Histol Histopathol (2010) 25: 1191-1206

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Molecular mechanisms of gap junction mutations in myelinating cells

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Summary. There is an emerging group of neurological disorders that result from genetic mutations affecting gap junction proteins in myelinating cells. The X-linked form of Charcot Marie Tooth disease (CMT1X) is caused by numerous mutations in the *GJB1* gene encoding the gap junction protein connexin32 (Cx32), which is expressed in both Schwann cells in the PNS and oligodendrocytes in the CNS. Patients with CMT1X present mainly with a progressive peripheral neuropathy, showing mixed axonal and demyelinating features. In many cases there is also clinical or subclinical involvement of the CNS with acute or chronic phenotypes of encephalopathy. Furthermore, mutations in the *GJA12/GJC2* gene encoding the gap junction protein Cx47, which is expressed in oligodendrocytes, have been identified in families with progressive leukodystrophy, known as Pelizaeus-Merzbacher-like disease, as well as in patients with hereditary spastic paraplegia. Recent studies have provided insights into the pattern of gap junction protein expression and function in CNS and PNS myelinating cells. Furthermore, *in vitro* and *in vivo* disease models have clarified some of the molecular and cellular mechanisms underlying these disorders. Here we provide an overview of the clinical, genetic, and neurobiological aspects of gap junction disorders affecting the nervous system.

Key words: Connexin32, Connexin47, Schwann cells, Oligodendrocytes, X-linked Charcot-Marie-Tooth Disease, Pelizaeus-Merzbacher-like disease

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X-linked Charcot-Marie-Tooth disease caused by *GJB1* mutations

The X-linked Charcot-Marie-Tooth disease (CMT1X) is the second most common form of demyelinating CMT. Following the initial discovery of the genetic etiology of the disease (Bergoffen et al., 1993) more than 300 different mutations in the *GJB1* gene encoding the gap junction (GJ) protein connexin32 (Cx32) have been reported to date in patients with CMT1X. They affect the non-coding region, as well as the open reading frame (ORF), including missense (amino acid substitutions) and nonsense (premature stop codons) mutations, deletions, insertions, and frameshifts (listed in (http://www.molgen.ua.ac.be/CMTMutations/default.cfm), predicted to affect all regions of the Cx32 protein (Fig. 1).

CMT1X is an X-linked dominant disease and therefore affects males more severely, with onset between 5 and 20 years of age (Hahn et al., 1990, 2000; Nicholson and Nash, 1993; Birouk et al., 1998). Patients present initially with difficulty running and frequently sprained ankles. In later stages they develop foot drop and distal sensory loss. As the disease progresses, the distal weakness may progress to involve the leg muscles and assistive devices may be required for ambulation. Weakness, atrophy, and sensory loss also develop in the hands, particularly in thenar muscles. Pes cavus, varus deformities, and "hammer toes" are frequently present. Affected women may be asymptomatic or they may have a later onset than men, after the end of second decade, and a milder version of the same phenotype at every age. This is likely due to random X-chromosome inactivation; only a fraction of myelinating Schwann cells express the mutant *GJB1* allele (Scherer et al., 1998).

Electrophysiological studies in CMT1X patients

show characteristically intermediate slowing of nerve conduction velocities (NCV) in the range of 30-40 m/s in affected males, and 30-50 m/s in affected females (Nicholson and Nash, 1993; Rouger et al., 1997; Birouk et al., 1998; Hahn et al., 1999; Senderek et al., 1999). These are faster than in most patients with demyelinating CMT (CMT1) and slower than in most patients with axonal CMT (CMT2). Compared to other CMT1 forms, conduction slowing in CMT1X is less uniform among different nerves and dispersion is more pronounced (Tabaraud et al., 1999; Gutierrez et al., 2000). Needle electromyography confirms the length-dependent loss of motor units as a result of axonal degeneration, which progresses with age (Rozear et al., 1987; Hahn et al., 1990, 1999; Nicholson and Nash, 1993; Rouger et al., 1997; Birouk et al., 1998; Senderek et al., 1999).

Typical pathological features in biopsied nerves from CMT1X patients include age-related loss of myelinated fibers, and in parallel an increasing number of regenerated axon clusters (Rozear et al., 1987; Hahn et al., 1990, 1999; Nicholson and Nash, 1993; Birouk et al., 1998; Sander et al., 1998; Senderek et al., 1998; Senderek et al., 1999; Tabaraud et al., 1999; Gutierrez et al., 2000; Vital et al., 2001; Kleopa et al., 2006). Many myelin sheaths are inappropriately thin for the axonal diameter, suggesting chronic segmental demyelination and remyelination, or remyelination after axonal regeneration (Sander et al., 1998; Hahn et al., 2001; Vital et al., 2001; Hattori et al., 2003; Kleopa et al., 2006). Ultrastructural studies have shown enlargement

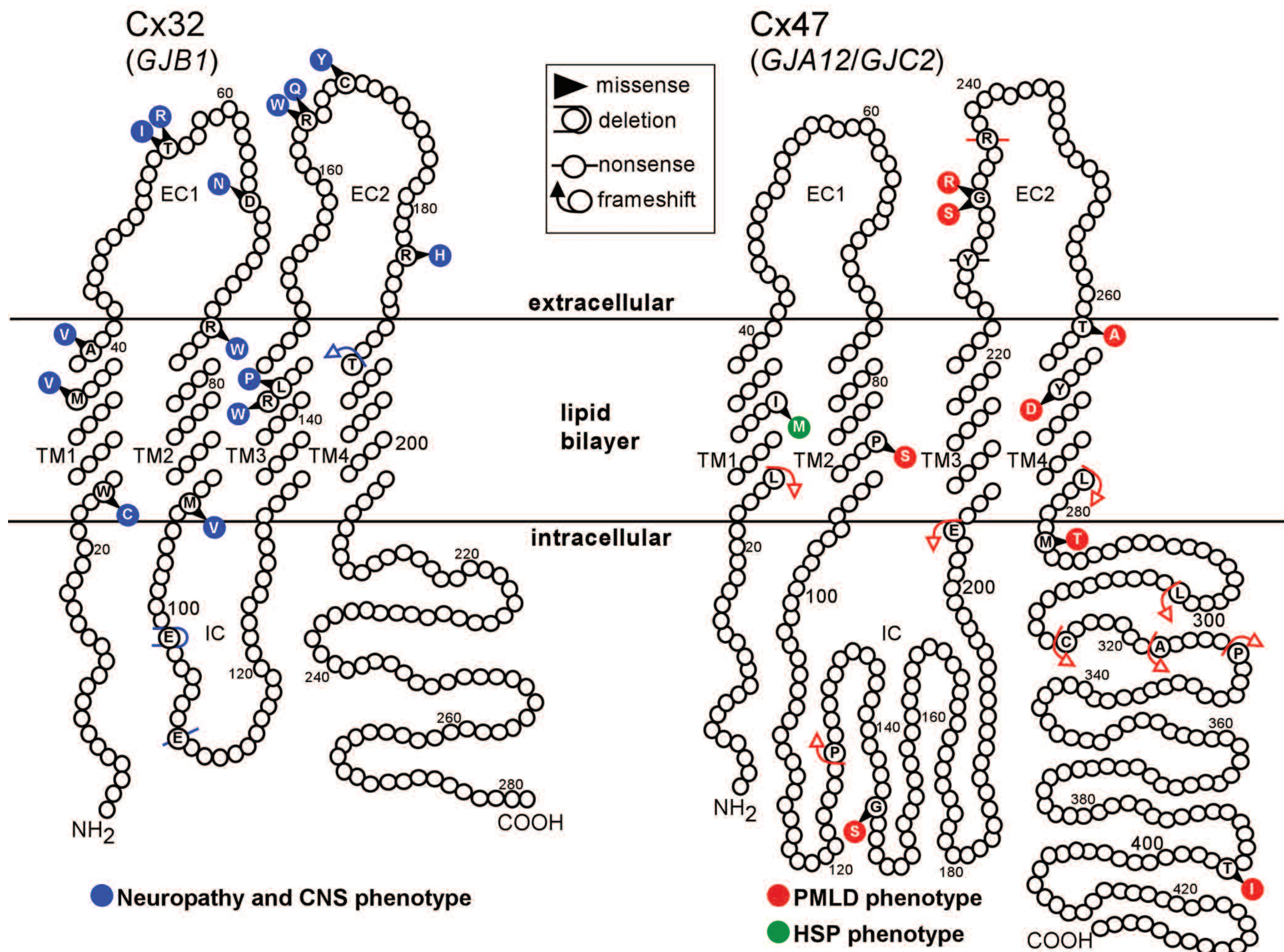

Fig. 1. Mutations in Cx32 and Cx47 and associated phenotypes. The structure of the connexin proteins (Yeager and Nicholson, 1996) is shown schematically including four transmembrane domains (TM1-4) linked by two extracellular loops (EC1-2) and one intracellular loop (IC). The diagram shows only a subset of the Cx32 mutations reported to date in CMT1X patients, which are associated with acute or chronic CNS phenotypes in addition to peripheral neuropathy. Over 300 other *GJB1* mutations not shown here occurring in all domains of the protein have been reported to cause only peripheral neuropathy, and are listed in http://www.molgen.ua.ac.be/CMTMutations/default.cfm. Cx47 (*GJA12/GJC2*) mutations associated with PMLD and one mutation associated with complicated HSP (I33M) are also shown.

and widening of the adaxonal Schwann cell cytoplasm, myelin discompaction and vesicle formation between degenerating innermost myelin layers (Senderek et al., 1999; Hahn et al., 2001; Kuntzer et al., 2003; Kleopa et al., 2006) (Fig. 2), as well as increased packing density of axonal neurofilaments (Hahn et al., 2001).

In addition to peripheral neuropathy, a high proportion of CMT1X patients have subclinical evidence of CNS involvement, such as abnormal brainstem auditory evoked potentials (Nicholson and Corbett, 1996; Nicholson et al., 1998). Central visual and motor pathways may also reveal abnormal responses (Bähr et al., 1999). Moreover, CMT1X mutations have been increasingly associated with clinical CNS phenotypes (Kleopa et al., 2002; Paulson et al., 2002; Taylor et al., 2003). These include signs of chronic corticospinal tract dysfunction, such as spasticity, extensor plantar responses and hyperactive reflexes (Bell et al., 1996; Bort et al., 1997; Panas et al., 1998; Marques et al., 1999; Lee et al., 2002; Isoardo et al., 2005; Kleopa et al., 2006), as well as progressive cerebellar ataxia, dysarthria, and delayed central somatosensory responses (Kawakami et al., 2002).

Furthermore, acute transient encephalopathy syndromes associated with MRI changes consistent with myelin dysfunction have been described in CMT1X patients with different *GJB1* mutations (Panas et al., 2001; Paulson et al., 2002; Schelhaas et al., 2002; Hanemann et al., 2003; Taylor et al., 2003). In most patients encephalopathy developed under conditions of metabolic stress caused by travel to high altitudes (Paulson et al., 2002), febrile illness (Schelhaas et al., 2002; Hanemann et al., 2003), hyperventilation (Srinivasan et al., 2008), or concussion (Halbrich et al., 2008). CNS involvement in CMT1X may be more common than initially appreciated, as the CNS manifestations in the individuals reported to date are usually subtle, and are likely masked by the typically more severe manifestations of the peripheral neuropathy. However, in some cases the CNS manifestations were the first or most prominent clinical presentation that led to the diagnosis of CMT1X (Panas et al., 2001; Taylor et al., 2003).

Pelizaeus-Merzbacher like disease and hereditary spastic paraplegia caused by GJA12/GJC2 mutations

Pelizaeus-Merzbacher disease (PMD) is an X-linked disorder that affects boys and is characterized by nystagmus and impaired psychomotor development within the first year of life, followed by progressive spasticity, ataxia, choreoathetosis and diffuse white matter changes on MRI (Nave and Boespflug-Tanguy, 1996; Hudson et al., 2004; Inoue, 2005). PMD is caused by mutations in *PLP1*, the gene encoding the main protein in CNS myelin, proteolipid protein (PLP). Pelizaeus-Merzbacher-like disease (PMLD) is clinically and neuroradiologically similar to classic PMD, but is not associated with *PLP1* mutations. Different homozygous and compound heterozygous *GJA12/GJC2*

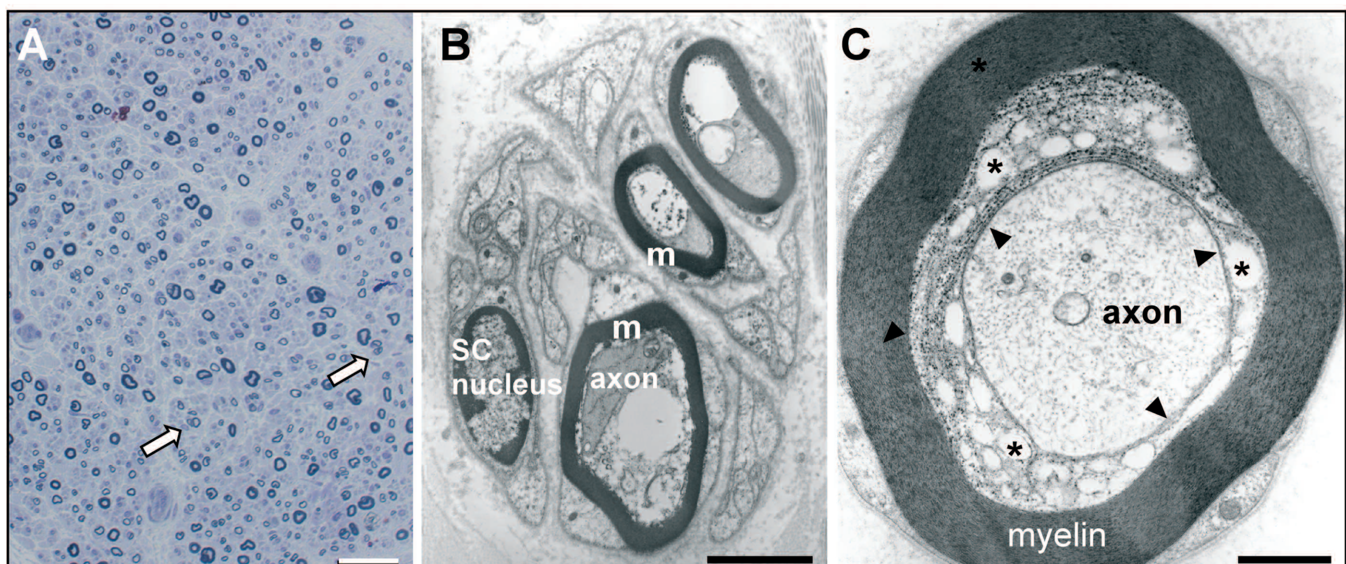

Fig. 2. Pathological features of CMT1X neuropathy caused by Cx32 mutations. Images of a semithin section **(A)** or electron micrographs **(B-C)** of a sural nerve biopsy sample from patient with a *GJB1* mutation causing the V140E substitution in Cx32. Panel A (toluidine blue) shows marked loss of large myelinated fibers and clusters of thinly myelinated fibers (arrows). Scale bar: 50 μm. **B.** Regenerative sprouting of thinly myelinated fibers. Axons appear retracted and separated from the degenerating innermost myelin (m) layers (SC: Schwann cell). Scale bar: 2 μm. **C.** Large myelinated fiber with typical abnormalities of the innermost myelin layers, including discompaction (black arrowheads) and vesicle formation between degenerating layers (asterisks). Scale bar: 1 μm.

mutations affecting the GJ protein Cx47 were identified in consanguineous and non-consanguineous PMLD families with autosomal recessive inheritance (Uhlenberg et al., 2004; Bugiani et al., 2006) (Fig. 1). Homozygous deletions in the *GJA12/GJC2* gene were also reported causing a similar phenotype (Salviati et al., 2007; Wolf et al., 2007) suggesting loss-of-function effect. Overall, *GJA12/GJC2* mutations do not appear to be a common cause of this phenotype, as they were detected in only 7.7% of a large series of PMLD families (Henneke et al., 2008).

Compared to "classical" PMD, PMLD patients initially have a milder phenotype reflected in higher cognitive levels and speech capacity achieved. However, neurologic deterioration starts earlier and progresses faster in patients with *GJA12/GJC2* mutations compared to patients with *PLP1* mutations, with shorter interval to loss of speech capacity, as well as loss of ambulation and wheelchair dependency (Henneke et al., 2008). One possible explanation for this clinical difference is that loss of Cx47-containing GJs leads to more rapid axonal degeneration than does loss of PLP (Cailloux et al., 2000). Axonal degeneration likely correlates best with the severity of the phenotype, as in CMT peripheral neuropathy (Krajewski et al., 2000) and in other CNS primary demyelinating disorders including PMD (Inoue, 2005; Garbern, 2007) and multiple sclerosis (Bjartmar et al., 2000).

A novel *GJA12/GJC2* mutation resulting in the I33M amino acid substitution in Cx47 has been recently identified in a family with complicated hereditary spastic paraplegia (HSP), expanding the phenotypic spectrum of Cx47 related disorders. Affected patients had a late-onset, slowly progressive, complicated spastic paraplegia, with normal or near-normal psychomotor development, preserved walking capability through adulthood, and no nystagmus. MRI and MR spectroscopy imaging were consistent with a hypomyelinating leukoencephalopathy (Orthmann-Murphy et al., 2009). Thus, *GJA12/GJC2* mutations, like *PLP1* mutations (Garbern et al., 1999; Inoue, 2005), can result in a milder phenotype than PMLD.

Connexins and gap junctions

GJs are found in most tissues, usually connecting adjacent cells, but in myelinating cells they also create a pathway through the myelin layers connecting different compartments of the same cell (Bruzzone et al., 1996; White and Paul, 1999). GJs may be involved in electrical connectivity, metabolic cooperation, growth control, cellular differentiation, and pattern formation during development (Goodenough et al., 1996). A GJ channel spans two adjacent plasma membranes and is formed by end-to-end docking of two hemichannels, also known as connexons. Each connexon is composed of six connexin molecules arranged around a central pore. Individual hemichannels can be composed of one (homomeric) or more (heteromeric) types of connexins (White and Bruzzone, 1996). Similarly, GJ channels may contain hemichannels with the same (homotypic) or different (heterotypic) connexins (Bruzzone et al., 1996; Kumar and Gilula, 1996). All connexins can form homomeric hemichannels, but some may preferentially form heteromeric channels. Different connexins can form heterotypic channels which may confer distinct channel functions (Bukauskas et al., 1995; Dahl et al., 1996).

GJ plaques are specialized regions in cellular membranes and contain tens to hundreds of GJ channels. The channel diameter is about 1.2 nm and allows only molecules smaller than 1000 Da, including ions, second messengers, metabolites, nucleotides and small peptides to pass between cells (Kumar and Gilula, 1996). The molecular size restriction at the channel entrance is also determined by a positively charged cytoplasmic entrance, a funnel, a negatively charged transmembrane pathway, and an extracellular cavity. The pore is narrowed at the funnel, which is formed by the six amino-terminal helices lining the wall of the channel. This structure also has implications for gating and transjunctional voltage (Maeda et al., 2009). However, this data is based on the analysis of a few GJ proteins and is only assumed to apply to all isoforms.

Connexins belong to a multigene family of over 20 integral membrane proteins that form GJs (Willecke et al., 2002). They are highly homologous, indicating that their structure and function were conserved as they evolved from a common ancestral gene. Each connexin protein is named according to its predicted molecular mass (in kDa). Their tertiary structure includes a cytoplasmic amino terminus, four transmembrane domains with alpha helix structure, one intracellular loop and two extracellular loops, and a cytoplasmic carboxy terminus (Bruzzone et al., 1996; Unger et al., 1999; White and Paul, 1999) (Fig. 1). All of these regions are highly conserved except for the intracellular loop and C-terminus (Yeager and Nicholson, 1996). These are the most divergent parts of the connexins, and differences in their sizes account for the different molecular masses of the connexins (Willecke et al., 2002). The first transmembrane domain probably forms the central pore, with polar residues lining the wall of the pore (Maeda et al., 2009). The two extracellular loops regulate the connexon-connexon interactions; three highly conserved cysteine residues are found in each loop connecting apposing loops via disulfide bonds.

Similar to other intrinsic membrane proteins, connexins follow the secretory pathway to form GJs. They are synthesized in the endoplasmic reticulum (ER) and transported to the Golgi apparatus before they are inserted as hexamers into the cell membrane (Yeager and Nicholson, 1996). Unlike most oligomeric trans-membrane proteins, multi-unit assembly of connexins may 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). Cx32 oligomerizes in the ER when expressed *in vitro* (Kumar et al., 1995; Das

Sarma et al., 2002; Maza et al., 2005), but also undergoes prenylation (Huang et al., 2005), a posttranslational modification that is accomplished on the cytoplasmic surface of the ER and Golgi (Silvius, 2002; Wright and Philips, 2006). Where other homomeric and heteromeric hemichannels oligomerize is largely unknown.

Expression and function of gap junction proteins in Schwann cells and oligodendrocytes

Connexins in Schwann cells

Cx32 was the first connexin to be cloned and is highly conserved across mammalian species: human, mouse and rat Cx32 proteins have 98% identical amino acid sequence. Cx32 is most abundant in liver, but it is also expressed by many other cell types, including oligodendrocytes and perhaps some neurons, as well as by myelinating Schwann cells (Scherer et al., 1995; Chandross et al., 1996; Söhl et al., 1996; Ressot and Bruzzone, 2000) (Fig. 3A). Despite this broad expression pattern, peripheral neuropathy and mild CNS phenotypes are usually the sole clinical manifestations of *GJB1* mutations in patients with CMT1X. Other tissues may be protected against the loss of Cx32 by the co-expression of one or more other connexins, which could have overlapping functions.

GJ-like structures were first observed by freeze-fracture electron microscopy at the Schmidt-Lantermann incisures and paranodes of myelin (Schnapp and Mugnaini, 1978; Sandri et al., 1982; Tetzlaff, 1982). The localization of Cx32 in the same areas by immunohistochemistry (Bergoffen et al., 1993; Scherer et al., 1995) suggested that Cx32 forms these GJs between the layers of the Schwann cell myelin sheath. This localization was recently confirmed by freeze-fracture replica immunogold labeling (Meier et al., 2004). Diffusion of low molecular mass fluorescent dyes across the myelin sheath has been documented by fluorescence microscopy following injection in the perinuclear region of living myelinating Schwann cells (Balice-Gordon et al., 1998). This radial pathway formed by GJs at these locations would be up to 1000-fold

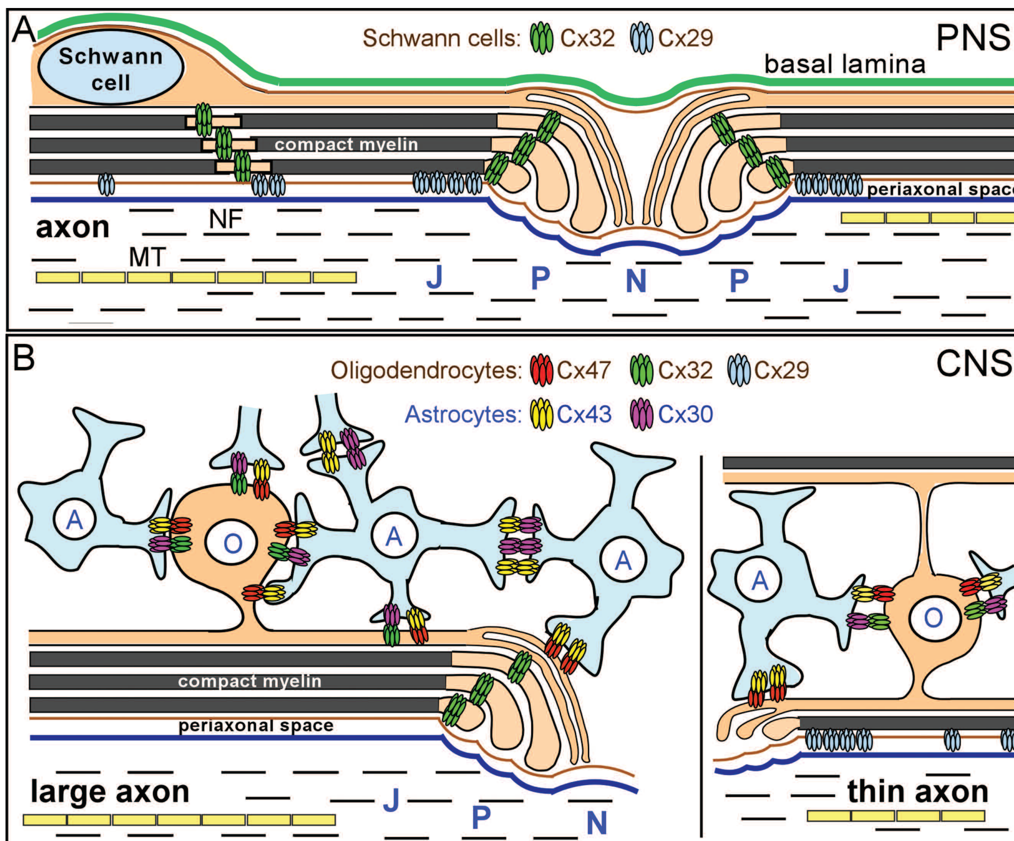

Fig. 3: Gap junction proteins in PNS and CNS myelinating cells. **A.** Diagram of a peripheral myelinated fiber showing the localization of Cx32 forming GJ channels through the layers of non-compact myelin at paranodes (P) surrounding the nodes (N) and Schmidt-Lantermann incisures interrupting the compact myelin, as well as the localization of Cx29 forming hemichannels in the Schwann cell membrane apposing the juxtaparanodes and paranodes, as well as at the innermost aspects of incisures. Both connexins may be directly relevant, not only for the myelinating Schwann cell, but also for the axon, as they form a pathway of direct communication from the abaxonal to the adaxonal Schwann cell cytoplasm and to the periaxonal space. NF: neurofilaments; MT: microtubules. **B.** Schematic drawing of GJ connectivity within oligodendrocytes (O) and between oligodendrocytes and astrocytes (A) showing the localization of Cx29, Cx32, and Cx47. Oligodendrocytes myelinating large axons (left) have numerous perikaryal GJ plaques, mainly comprised of Cx47. Cx32 forms GJ channels mainly on the outer aspects of the myelin sheath and in non-compact myelin areas, and Cx29 is absent. The astrocytic partner of Cx47 is Cx43, whereas Cx32 forms heterotypic channels with astrocytic Cx30, as well as most intracellular channels within the myelin sheath. Oligodendrocytes myelinating more than one small axons (right) have perikaryal GJ plaques comprised of Cx47 and Cx32, while Cx29 is localized to the juxtaparanodal and internodal regions of the myelin sheath forming hemichannels. Interastrocytic GJs are formed by homotypic connexons as indicated.

shorter than the circumferential pathway within the Schwann cell cytoplasm (Scherer et al., 1995).

Myelinating Schwann cells in rodents express a second connexin, Cx29 (Söhl et al., 2001; Altevogt et al., 2002), which is distinctly localized in the innermost part of the incisures, the juxtamesaxon and the innermost layer of myelin apposing axonal juxtaparanodes (Fig. 3A). Cx29 does not appear to form functional GJs, at least *in vitro* (Altevogt et al., 2002), and may instead form hemichannels with a different function (Ahn et al., 2008; Sargiannidou et al., 2008). The proximity of Cx29 hemichannels on the inner myelin membrane apposing the *Shaker*-type Kv1.1 and Kv1.2 channels on the axonal membrane suggests that Cx29 hemichannels could comprise a pathway for the removal of K^+ accumulating in periaxonal space during neural activity (Konishi, 1990; Chiu, 1991). In support of this notion, freeze-fracture studies reveal rosettes of particles in juxtaparanode, inner mesaxon, and incisures (Stolinski et al., 1985), which could correspond to Cx29 hemichannels. Thus, Kv1.1/1.2 channels in the axonal membrane, Cx29 hemichannels in the Schwann cell membrane, and Cx32 channels through the non-compact myelin sheath could function together to provide a mechanism for shuttling K^+.

Connexins in oligodendrocytes

Glial cells in the CNS show extensive gap junctional connectivity (Nagy and Rash, 2000; Rash et al., 2001). Rodent oligodendrocytes express at least 3 different GJ proteins, Cx32, Cx47, and Cx29 (Altevogt et al., 2002; Kleopa et al., 2004) and form intercellular GJ, mostly with astrocytes (Rash et al., 2001; Altevogt and Paul, 2004), in addition to intracellular GJs within and along the myelin sheath. The subcellular distribution of oligodendrocytic GJ proteins is complex (Fig. 3B): Cx47 is prominent in oligodendrocyte somata and proximal processes throughout the CNS (Menichella et al., 2003; Odermatt et al., 2003; Kleopa et al., 2004) forming GJs with astrocytic processes; its astrocytic partner is mainly Cx43 (Altevogt and Paul, 2004; Kamasawa et al., 2005; Orthmann-Murphy et al., 2007b). Cx32 is mainly expressed along the large myelinated fibers of the white matter (Altevogt et al., 2002; Kleopa et al., 2004) in Schmidt-Lantermann incisures and paranodes bordering nodes of Ranvier, forming most intracellular GJs within the myelin sheath, but also intercellular ones with astrocytic Cx30 (Rash et al., 2001; Nagy et al., 2003a,b; Altevogt and Paul, 2004; Kamasawa et al., 2005). Cx29 (Altevogt et al., 2002; Kleopa et al., 2004) and its human ortholog Cx31.3 (Sargiannidou et al., 2008) appear to form hemichannels within the myelin sheath of small fibers in some white matter tracts of the spinal cord, optic nerve and corpus callosum, as well as in the cortex. They rarely colocalize with any of the other CNS glial connexins (Altevogt and Paul, 2004), suggesting that as in the PNS they form hemichannels but no intercellular channels (Ahn et al., 2008).

Astrocytes are functionally coupled by GJs (Kettenmann et al., 1983; Ransom and Kettenmann, 1990; Giaume et al., 1991). They form an extensive gap junctional network that plays an important role in distributing the excess K^+ ions and glutamate during neuronal activity (Rouach et al., 2000), as well as in mediating Ca^{2+} waves (Cornell-Bell et al., 1990). Astrocytes express at least two different GJ proteins: Cx30 and Cx43, and possibly Cx26. Immunostaining and functional studies indicate that inter-astrocytic GJs are composed of Cx43/Cx43 and Cx30/Cx30 channels in the gray matter, and only Cx43/Cx43 in the white matter (Dermietzel et al., 1989; Yamamoto et al., 1990; Ochalski et al., 1997; Kunzelmann et al., 1999; Nagy et al., 1999; Altevogt and Paul, 2004; Orthmann-Murphy et al., 2007b). The distinct GJ channels coupling oligodendrocytes to astrocytes (Cx32/Cx30 and Cx47/Cx43) and adjacent astrocytes (Cx43/Cx43 and Cx30/Cx30) may serve distinct functions, as they show differences in voltage-gating properties and in their permeability to different molecules, such as cAMP, glucose, glutamate, glutathione, ATP, and IP3 (Niessen et al., 2000; Goldberg et al., 2002; Bedner et al., 2006; Orthmann-Murphy et al., 2007b).

The functional importance of astrocyte-oligodendrocyte connectivity throughout the CNS, especially for oligodendrocytes and the myelin sheath they form, is highlighted by the phenotype of CNS demyelination in patients with occulodentodigital dysplasia syndrome caused by mutations affecting the astrocytic Cx43 (Paznekas et al., 2003) and in animal models with deficient astrocytic GJs (Lutz et al., 2009). Thus, oligodendrocytes depend on gap junctional connectivity with astrocytes. Astrocytes are thought to serve as "intermediates" between successive oligodendrocytes permitting otherwise isolated oligodendrocytes to participate in the GJ-mediated "pan-glial syncytium" that regulates cell differentiation and tissue homeostasis in the CNS (Fig. 3B) (Rash et al., 1997; Hansson et al., 2000; Rash et al., 2001; Kamasawa et al., 2005). GJs formed by Cx32 and Cx47 within the myelin sheath, as well as between myelin and astrocytes, appear to form a structural pathway required for the transport of K^+ ions generated during neuronal activity, restoring concentration and osmotic gradients (Kofuji and Newman, 2004; Kamasawa et al., 2005).

Molecular and cellular mechanisms of GJB1 and GJA12/GJC2 mutations

Models of CMT1X and the molecular mechanisms of Cx32 mutants

A large number of Cx32 mutants that cause CMT1X have been studied in heterologous cells, as well as in mammalian cell lines. When expressed in *Xenopus* oocytes many mutants fail to form functional channels; some of them also exert dominant-negative effects on the coexpressed wild type Cx32 (Bruzzone et al., 1994).

Other mutants form functional channels with altered biophysical characteristics, such as reduced pore diameter that may prevent the diffusion of second messengers like IP3, cAMP, and Ca^{2+} (Oh et al., 1997). The position of the Cx32 mutation alone does not necessarily predict the molecular and functional

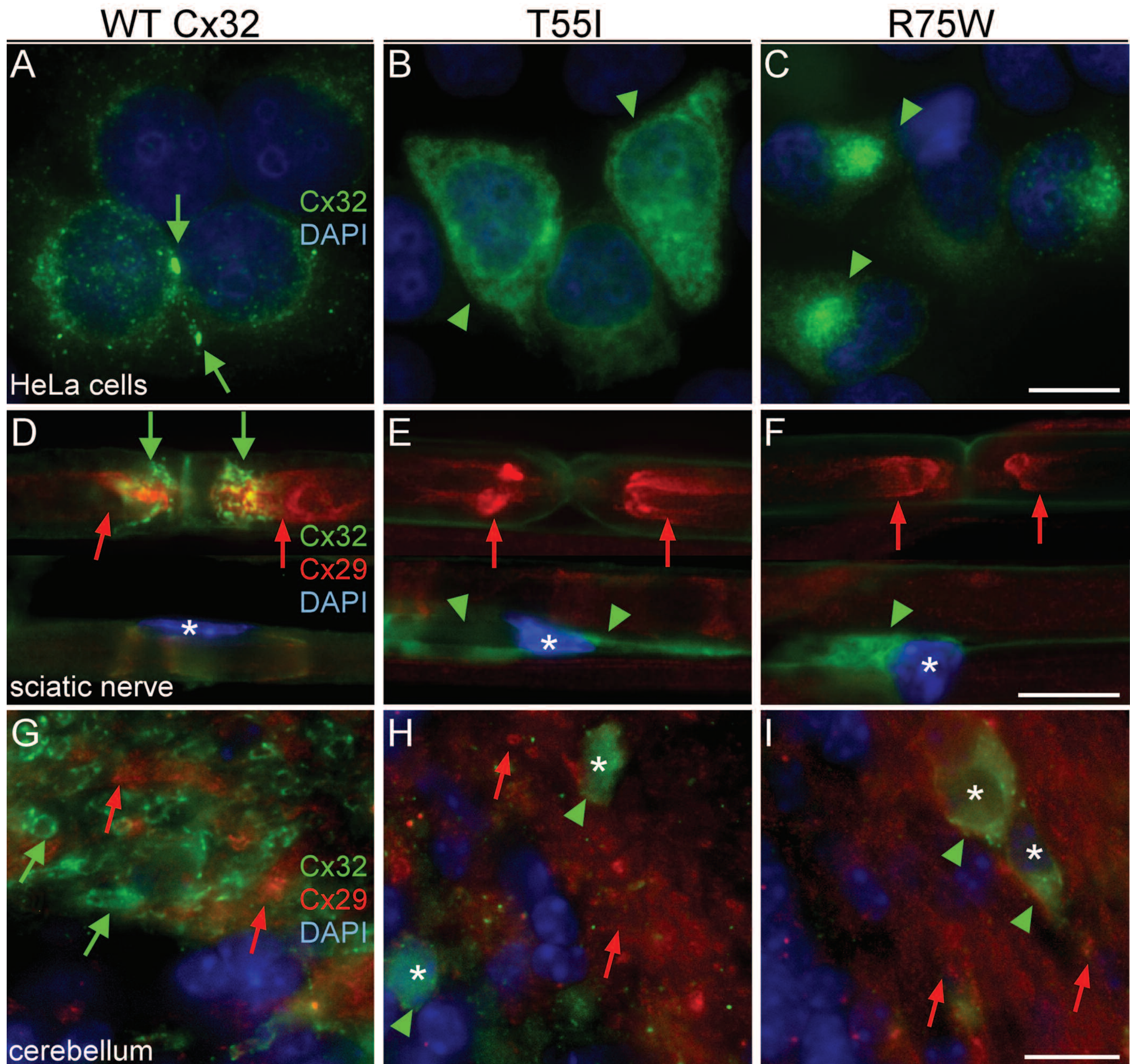

Fig. 4. Expression of Cx32 mutants *in vitro* and in myelinating cells. **A-C.** Images of HeLa cells transfected to express the wild type (WT) Cx32 **(A)**, the T55I **(B)** or the R75W **(C)** mutants show that only the WT protein forms gap junction plaques between apposing cells (green arrows in **A**). Both mutants fail to form gap junction plaques and are localized in the cytoplasm (arrowheads in **B-C**): the T55I is found around the nucleus **(B),** suggestive of endoplasmic reticulum retention, while the R75W mutant is concentrated on one side of the nucleus **(C)**, suggesting Golgi retention. **D-I.** Sciatic nerve teased fibers **(D-F)** or cerebellar sections **(G-I)** from WT **(D, G)** or Cx32 mutant mice expressing the T55I **(E, H)** or the R75W mutation **(F, I)** on a Gjb1-null background, double stained for Cx32 (green) and Cx29 (red). The WT Cx32 is normally localized at paranodal myelin areas of Schwann cells (green arrows in **D**) and along large myelinated fibers of the cerebellar white matter (green arrows in **G**). In contrast, the T55I and R75W mutants fail to reach the paranodal myelin of peripheral fibers **(E-F)**, or the myelin sheath of CNS fibers **(H-I)** and are instead localized in the perinuclear cytoplasm of Schwann cells **(E-F)** and oligodendrocytes **(H-I)** (green arrowheads). Cx29 is normally localized at the juxtaparanodal areas of Schwann cells (red arrows in **D-F**) and along small fibers in the cerebellum **(G-I)**. Nuclei of myelinating cells are marked with an asterisk. Scale bars: 10 μm.

consequences, as the R15Q and H94Q mutants form normal functional channels, whereas R15W and H94Y do not (Abrams et al., 2001).

Expression of Cx32 mutants in mammalian cells with more stringent protein trafficking requirements revealed that they are often abnormally localized (Omori et al., 1996; Yoshimura et al., 1998; Yum et al., 2002); either no Cx32 is detected, even though its mRNA is expressed (Deschênes et al., 1996), or Cx32 appears to be retained in the ER and/or Golgi (Fig. 4A-C). Co-localization studies with ER or Golgi markers confirmed that Cx32 mutants are retained predominately in the ER or Golgi, even if they form rare GJ-like plaques (Deschênes et al., 1997; Oh et al., 1997; Martin et al., 2000; Matsuyama et al., 2001; Kleopa et al., 2002; Yum et al., 2002; Kleopa et al., 2006), and are degraded via endosomal and proteasomal pathways (VanSlyke et al., 2000; Kleopa et al., 2002). Several mutants, the majority of which occur in the C-terminal domain were mainly localized on the cell membrane and show no significant difference to wild type protein (Kleopa et al., 2002; Yum et al., 2002), but they may be less stable or have abnormal biophysical properties (Rabadan-Diehl et al., 1994; Castro et al., 1999; Abrams et al., 2000). In one case they form leaky hemichannels with severe phenotype (Liang et al., 2005).

The cellular effects of Cx32 mutants *in vitro* highlight some structure-function correlations for Cx32 (Abrams et al., 2000). N-terminal mutations result in altered biophysical properties and may cause reversal of gating polarity by negative charge substitutions. This is in keeping with the role of this domain in the insertion of the nascent polypeptide chain into the ER, and along with the first transmembrane domain in the regulation of voltage gating (Maeda et al., 2009). Shifted voltage gating and abnormally increased opening has been shown for several mutants affecting the first and second transmembrane domain, which cause conformational changes (Abrams et al., 2002). Mutations affecting the cysteines residues in the two extracellular loops, which mediate the interactions between apposed connexons, lead to a loss of functional channels. Mutations of the intracellular loop and C-terminal domain may affect pH gating (Castro et al., 1999). Two mutations that affect a consensus prenylation motif of Cx32 (C280G and S281X) abolish prenylation, a lipid modification (Huang et al., 2005).

Several animal models have provided further insights into CMT1X pathogenesis. Mice with targeted deletion of the *Gjb1/cx32* gene develop a progressive, predominantly motor demyelinating peripheral neuropathy beginning at about three months of age (Anzini et al., 1997; Scherer et al., 1998). Heterozygous females have fewer demyelinated and remyelinated axons than age-matched *Gjb1/cx32*-null females or males (Scherer et al., 1998), in keeping with the clinical phenotype of affected women who are obligate carriers of CMT1X. Expression of wild type human Cx32 protein prevents demyelination in Gjb1/cx32-null mice (Scherer et al., 2005), confirming that the loss of Schwann cell autonomous expression of Cx32 is sufficient to account for CMT1X pathology. In addition, Cx32 knockout (KO) mice show subtle CNS myelin defects, including diminished myelinated fiber and myelin volume density, particularly in white matter tracts with prominent Cx32 expression, such as the ventral and dorsal funiculus of the spinal cord (Sargiannidou et al., 2009) but also in the neorcortex (Sutor et al., 2000). Optic nerves, which do not significantly express Cx32 (Kleopa et al., 2004), are free of pathology (Scherer et al., 1998).

Transgenic mice expressing the 175fs, T55I, R75W, R142W, C280G, and S281X Cx32 mutations have also been generated. No Cx32 protein could be detected in 175fs transgenic mice despite expression of the transgenic mRNA (Abel et al., 1999). In contrast, R142W transgenic mice showed retention of the mutant protein in the perinuclear region and developed a mild demyelinating neuropathy (Scherer et al., 1999). Moreover, the presence of the mutant Cx32 reduced the level of the endogenous mouse Cx32, indicating that R142W may have dominant-negative interactions with the wild type protein. However, this is not clinically relevant in patients with CMT1X as only one *GJB1* allele is expressed in each cell. The R142W mutant did not affect the coexpressed Cx29 in Schwann cells (Jeng et al., 2006). The C280G and S281X mutants were properly localized to incisures and paranodes of myelinating Schwann cells and appeared to prevent demyelination in *Gjb1/cx32*-null mice, indicating that they may form functional channels in the myelin sheath (Huang et al., 2005). How they cause neuropathy in humans remains unclear.

More recently, transgenic mice were generated that express two further Cx32 mutations, T55I and R75W in Schwann cells. Driven by the CNP promoter, they also express the mutants in oligodendrocytes, in order to explore the possibility that they have gain-of-function effects explaining the associated CNS phenotypes of CMT1X patients (Kleopa et al., 2002). In myelinating cells, as in cultured cells, the T55I mutant is localized in the ER and the R75W mostly in the Golgi (Fig. 4D-I). On a *cx32*-null background they cause a progressive demyelinating neuropathy (Fig. 5) as well as mild CNS myelination defects. However, they have no significant gain-of-function effects in a *Gjb1*-null background and did not appear to affect the expression and localization of coexpressed Cx29 or Cx47 in oligodendrocytes (Fig. 6). Thus, the loss of Cx32 function appears to be the main effect of the T55I and R75W mutants, in both the PNS and the CNS (Sargiannidou et al., 2009).

All Cx32 mutants expressed *in vivo* have a comparable localization in myelinating cells and in transfected cell lines (Deschênes et al., 1997; Kleopa et al., 2002; Yum et al., 2002) (Fig. 4) indicating that altered synthesis or trafficking and loss of GJ function is the fundamental mechanism in most CMT1X mutants. These results are in keeping with a large clinical study of

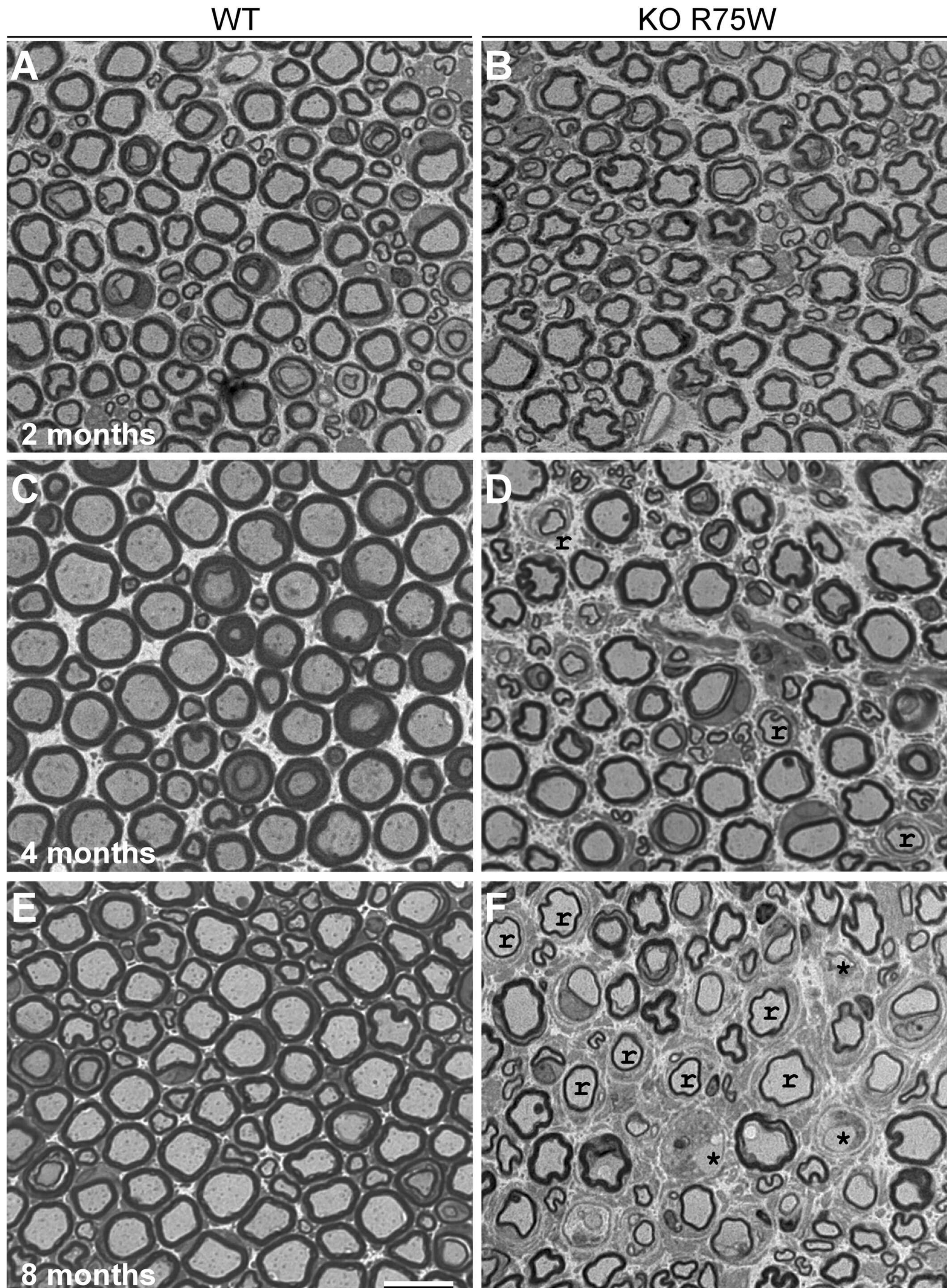

Fig. 5. Progressive demyelinating neuropathy in transgenic mice expressing the R75W mutant on a *Gjb1*-null background. These are semithin sections of femoral motor nerves from wild type (WT) **(A, C, E)** or mutant mice expressing the R75W mutant on a knockout background (KO R75W) **(B, D, F)** at the ages of 2 **(A-B)**, 4 **(C-D)** and 8 months **(E-F)**. There is progressive demyelination starting after 2 months of age, with increased numbers of abnormally myelinated fibers that are either remyelinated (r) or completely demyelinated (asterisk). Scale bar: 10 μm.

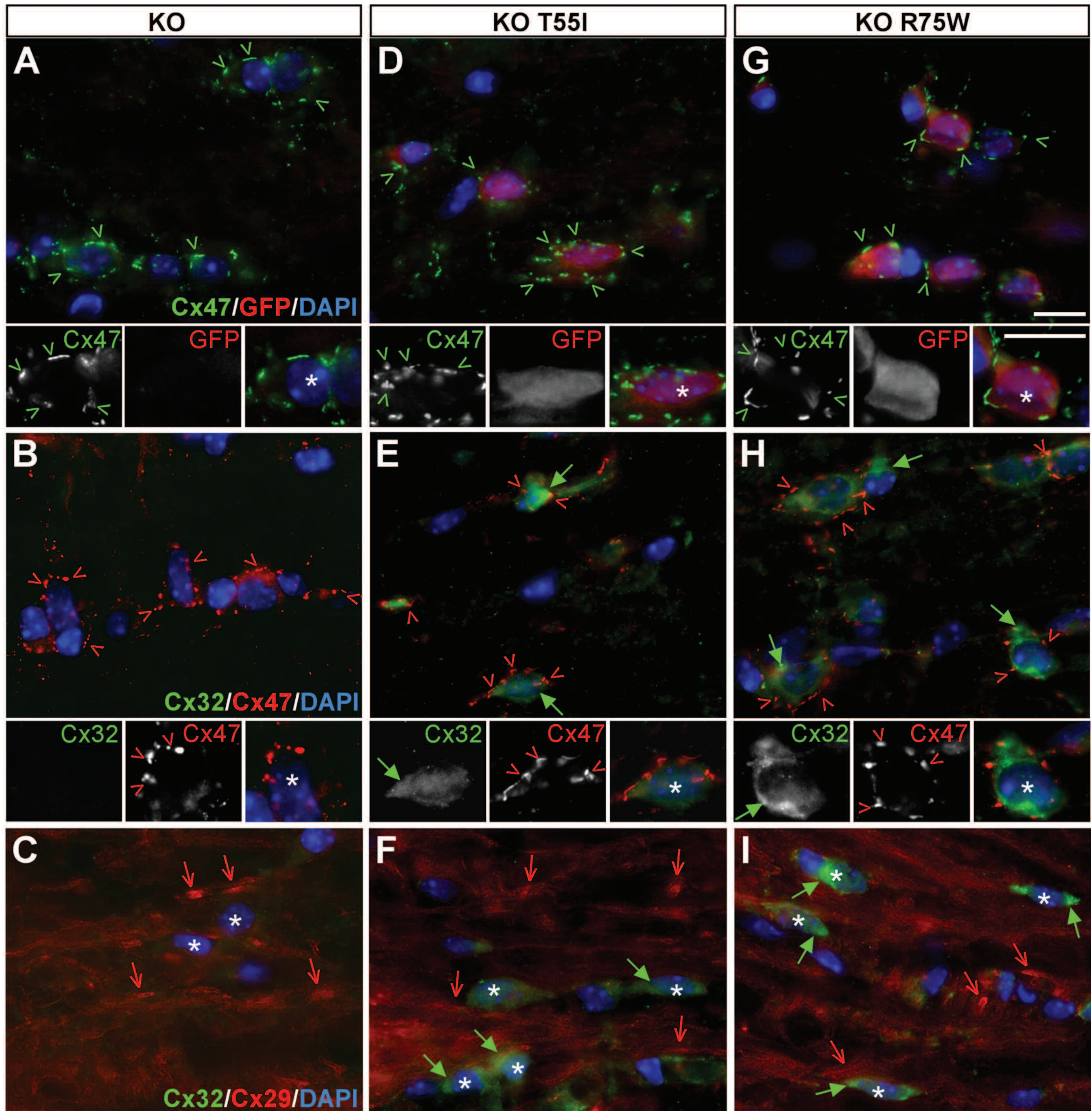

Fig. 6. Mutant Cx32 does not alter the localization of other gap junction proteins in oligodendrocytes. These are images of longitudinal sections through the white matter of spinal cords from Cx32 KO mice **(A-C)** as well as T55I **(D-F)** and R75W **(G-I)** mutant mice in a KO background as indicated. Sections are double labeled with mouse monoclonal antibodies (green) against Cx47 **(A, D, G)** or Cx32 **(B&C, E&F, H&I)** and rabbit antisera (red) against GFP **(A, D, G)**, Cx47 **(B, E, H)** or Cx29 **(C, F, I)**. Nuclei are labeled with DAPI (blue). In mutant mice **(D, G)**, EGFP-positive oligodendrocytes (asterisks) express Cx47, which forms numerous GJ plaques at the perikaryon and proximal processes (green arrowheads), as in Cx32 KO mice **(A)**. Cx32 is absent from Cx32 KO mice **(B-C)**, and mutant Cx32 is localized in the cytoplasm of oligodendrocytes in both KO T55I and KO R75W lines (green arrows in **E-F** and **H-I**); in the same cells Cx47 again appears to be normally localized, forming GJ plaques (red arrowheads in **B, E, H**). Cx29 is also normally localized along thin myelinated fibers (red arrows) in both mutant lines despite the presence of the Cx32 mutants **(F, I)**, as it does in Cx32 KO mice **(C)**. Scale bars (including insets): 10 μm. (With permission from: Sargiannidou et al., 2009; Copyright Society for Neuroscience).

CMT1X patients with various mutations, including deletion of the entire *GJB1* gene, who had a similar phenotype (Shy et al., 2007), as well as with studies showing that the severity of pathological findings in CMT1X nerve biopsies are not associated with particular *GJB1* mutations (Hahn et al., 2000; Nakagawa et al., 2001; Hattori et al., 2003). Thus, with notable exceptions (Liang et al., 2005), most *GJB1* mutations appear to cause CMT1X through loss of normal Cx32 function.

Molecular mechanisms of Cx47 mutants

Some PMLD missense mutations affecting Cx47 that have been expressed in cultured mammalian cells, including P87S, Y269D, and M283T, result in loss-of-function of the protein (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, suggesting that 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).

The I33M Cx47 mutation associated with the milder HSP phenotype forms GJ plaques similar to wild type Cx47 in transfected cells, but fails to form functional homotypic channels in scrape-loading and dual whole-cell patch clamp assays. Furthermore, I33M/Cx43 channels open only when a large voltage difference is applied to paired cells and probably do not function under physiological conditions, suggesting that although mutant Cx47 reaches the cell membrane, Cx47/Cx43 channels between astrocytes and oligodendrocytes are disrupted, similar to the PMLD mutants (Orthmann-Murphy et al., 2009). It remains unclear whether the HSP mutant in contrast to PMLD mutants, retains a function of Cx47 not directly related to forming functional GJ channels.

Cx47 KO mice, similar to Cx32 KO mice, develop a mild pathology consisting of vacuolation of nerve fibers, especially in the proximal optic nerve, where myelination starts, and have a normal lifespan. Vacuoles are present in the periaxonal space, as well as within the myelin itself (Menichella et al., 2003; Odermatt et al., 2003). In contrast to the mild phenotypes of single KO mice, Cx32/Cx47 double KO mice develop severe CNS demyelination. They present with a progressive coarse action tremor during the third postnatal week and tonic seizures begin during the fourth to fifth postnatal week. The seizures increase in frequency and severity until the animals die, typically during the sixth postnatal week (Menichella et al., 2003; Odermatt et al., 2003). At one month of age they exhibit severe CNS demyelination, axonal degeneration and oligodendrocyte apoptosis in the spinal cord funiculi and in the optic nerve. They have thinner myelin sheaths than age-matched littermates and edematous extracellular spaces separating degenerating axons from their myelin sheath (Menichella et al., 2003; Odermatt et al., 2003). Thus, Cx32 and Cx47 may have partially overlapping functions in oligodendrocytes, so that each of the two can partially compensate for the loss of the other, at least in mice. Why *Gja12/cx47*-null mice have a milder phenotype than humans with presumably recessive, loss-of-function mutations causing PMLD is unknown. It is possible that the hCx47 mutants have a dominant effect or that for unclear reasons the loss of Cx47 function has more severe consequences in the human than in the mouse CNS.

Further studies in Cx32 and Cx47 KO mice highlight the role of GJs in oligodendrocyte function, in relation to neuronal activity. Increasing retinal ganglion cell activity exacerbated vacuole formation in optic nerves of Cx32/Cx47 double KO mice, whereas inhibiting activity reduced vacuole formation (Menichella et al., 2006). Kir4.1, which is highly enriched at the perivascular endfeet of astrocytes, is an important contributor to K^+ homeostasis in the CNS (Neusch et al., 2003) and appears to share this role with GJs formed by Cx32 and Cx47: Neither Cx47 nor Kir4.1 heterozygotes display any significant pathology in a *Gjb1*-null background, whereas Kir4.1/Cx47 compound heterozygotes on a *Gjb1*-null background develop myelin vacuoles, similar to the Cx32/Cx47 double KOs (Menichella et al., 2006). Furthermore, astrocytic GJs accelerate extracellular K^+ clearance attenuating the propagation of spreading depression (Amzica et al., 2002), limit K^+ accumulation during synchronized neuronal firing and after spinal cord injury (De Pina-Benabou et al., 2001), and aid in radial K^+ relocation in the hippocampus, although GJ-independent processes also account for K^+ buffering (Wallraff et al., 2006). These findings indicate that GJs connecting oligodendrocytes to astrocytes may serve the spatial buffering of K^+ elaborated during the propagation of action potentials (Kamasawa et al., 2005; Menichella et al., 2006). Loss of Cx32 and Cx47 GJs could cause the build-up of axonally-derived potassium and osmotic water within myelin and may explain the periaxonal edema in double KO mice (Menichella et al., 2003; Odermatt et al., 2003).

Conclusion

The discovery of the genetic etiology of CMT1X and PMLD has highlighted the functional importance of GJ proteins in myelinating cells. Disease models that have been recently generated have provided insights not only into the pathogenesis of these neurological diseases, but also into the complexity and functional role of GJs in Schwann cells and oligodendrocytes. Mostly loss of function mutations affecting these proteins impair the intracellular and intercellular gap junctional connectivity of myelinating cells and cause phenotypes of peripheral and central demyelination and axonal degeneration. Understanding the cellular and molecular mechanisms of GJ dysfunction in myelinating cells will aid the development of therapeutic approaches for these and similar genetic and acquired disorders.

Acknowledgments. This work was supported by the Cyprus Research Promotion Foundation (Grants HEALTH/0506/04 and CY-GR/0406/11 to KAK), the National Multiple Sclerosis Society (USA) (Grant RG3457A2/1 to KAK), and the Cyprus Telethon. We thank Steven Scherer and Jennifer Orthmann-Murphy for helpful discussions and antibodies and Natalie Vavlitou for technical assistance.

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Accepted March 15, 2010