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Review

Role of mitofusin 2 mutations in the physiopathology of Charcot–Marie–Tooth disease type 2A

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ABSTRACT

Charcot–Marie–Tooth disease (CMT) is the most common form of hereditary peripheral neuropathy. The main axonal form of CMT, CMT2A, preferentially affects peripheral neurons with the longest neurites. CMT2A has been recently linked to mutations in the mitofusin 2 (*Mfn2*) gene. Mfn2 participates in mitochondrial fusion a process that together with mitochondrial fission, contributes to mitochondrial morphology. Many hypotheses have been postulated to understand how mutations in Mfn2 lead to CMT2A. In this review, we will describe the physiological role of Mfn2, the pathophysiology of CMT2A and current hypotheses about the deleterious role of mutant *Mfn2* in neuronal function.

Contents

Charcot–Marie–Tooth disease type 2A

Charcot–Marie–Tooth (CMT) disease (also known as hereditary motor and sensory neuropathy) represents a group of clinically and genetically heterogeneous inherited neuropathies affecting motor and/or sensory neurons and associated Schwann cells. It has been first described independently in France and Great Britain at the end of the 19th century (Charcot and Marie, 1886; Tooth, 1886). The CMT disease is the most common inherited neuromuscular disorder with a prevalence estimated at 1/2500 (Skre, 1974). According to electrophysiological criteria, CMT is subdivided into two main categories: demyelinating neuropathies with motor nerve conduction velocity (MNCV) reduced under 38 m/s and axonal forms, which affect mostly axons and is characterized by normal MNCV (>38 m/s). The intermediate form of CMT, displays intermediate and/or heterogeneous electrophysiological (MNCV from 25 to 45 m/s) and clinical features. The availability of recently developed mouse models made possible the detailed analyses of molecular mechanisms underlying different forms of demyelinating peripheral neuropathies (for review see Houlden and Reilly, 2006, Meyer zu Horste et al., 2006). However, much less is known about the axonal forms of CMT.

Based on genetic studies, three main forms of axonal CMTs have been described: dominant (CMT2), recessive (CMT4C or AR-CMT2) and recessive X-linked (CMTX). CMT2A is the most prevalent type of axonal dominant forms with a frequency of up to 20% among all CMT2 patients (Verhoeven et al., 2006; Zuchner et al., 2005) and accounts for about 20 to 40% of all CMTs (Barisic et al., 2008; http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=gene&part=cmt). Typical clinical symptoms of CMT2A are progressive distal limb muscle weakness and/or atrophy, stepping gait, distal sensory loss, and mobility impairment, which can lead to wheelchair dependency. Puzzlingly, the disease onset seems to be very diverse from one case to another but seems to be tightly related to disease severity; the sooner symptoms will appear the more severe they will be (Chung et al., 2006). Accordingly, the number of peripheral axons in the sural nerve

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of early onset patients is dramatically reduced while it appears unchanged in late onset patients (Chung et al., 2006). This characteristic of late onset CMT2A suggests that neurodegeneration may not be the cause of the symptoms in this group of patients. Maybe the most intriguing hallmark of CMT2A is the fact that it affects a highly specific set of neurons; only the neurons with the longest axons are diseased (Young and Suter, 2003; Zuchner and Vance, 2006). Corollary, CNS perturbations as well as respiratory deficiencies are very rare. However, optic atrophy has been detected in some cases of CMT2A patients leading to classify them in the subtype of HMSN VI neuropathy (Sommer and Schroder, 1989). Curiously, some patients of this group experienced recovery of visual acuity several years following onset of the symptoms (Zuchner et al., 2006).

A major step in the comprehension of the disease has been reached when several studies identified mutations in the Mitofusin 2 (*Mfn2*) gene as responsible for CMT2A (Chung et al., 2006; Kijima et al., 2005; Verhoeven et al., 2006; Zuchner et al., 2004). In other, less frequent dominant axonal CMT2s, causative mutations have also been identified in several genes including RAS-associated GTP-binding protein gene (*CMT2B*), glycyl-tRNA synthetase gene (*CMT2D*), neurofilament light chain protein gene (*CMT2E*), myelin protein (CMT2I and CMT2J), heat shock proteins (CMT2F and CMT2L) (for review, see Barisic et al., 2008). This review will focus on CMT2A and the pathophysiological mechanisms by which *Mfn2* mutants affect peripheral axons.

Mitofusins and mitochondrial fusion

Mitochondria are not isolated organelles but rather form highly dynamic structures that continuously fuse and fragment. Together with Mfn1 and OPA1, Mfn2 plays a major role in the mitochondrial fusion process in mammalian cells (for review see Chan, 2006). The first mitochondrial fusion protein called Fzo has been described in *Drosophila* (Hales and Fuller, 1997). Mutants for the Fzo protein displayed sterility due to abnormal mitochondrial fusion in the spermatids. Instead of the classical perinuclear Nebenkern structure composed of fused mitochondria, spermatids of mutant flies had a fuzzy onion like structure made of unfused accumulated mitochondria. Whereas only one Fzo homolog has been described in yeast (Hermann et al., 1998), two mammalian homologs of Fzo have been characterized: mitofusin 1 and 2 (Santel and Fuller, 2001). The two mitofusins are large nuclear-encoded dynamin-like GTPase proteins. These proteins are anchored in the outer mitochondrial membrane thanks to two transmembrane domains situated close to the C-terminal end. Thus a large N-terminal and a smaller C-terminal fragment of the protein are exposed in the cytoplasm (Fig. 1). Three domains are of particular importance in the mitofusin proteins. First, as all the dynamin-like proteins, mitofusins have a GTPase domain, which is situated close to the N-terminus. GTP hydrolysis is required for mitochondrial fusion activity of mitofusins (Santel et al., 2003, Santel and Fuller, 2001). Interestingly, the GTPase activity of Mfn1 is higher than that of Mfn2, also explaining its higher mitochondrial fusion activity (Ishihara et al., 2004). Second, mitofusins 1 and 2 contain two hydrophobic heptad repeat domains (HR) localized at the basis of the two arms of the protein (Fig. 1). These motifs are the basis for most coiled-coil interactions. The C-terminal HR2 domain has been shown to be of particular importance for mitochondrial fusion activity. This domain forms antiparallel coiled-coil interactions with adjacent HR domains of Mfn1 or 2 on apposing mitochondria (Koshiba et al., 2004). Mutations which disrupt the HR2 domain of Mfn1, reduce the stability of the HR2 coiled-coil and cannot rescue the mitochondrial fragmentation in mitofusin-null cells. This HR2 structure is probably involved in the first step of mitochondrial fusion. Finally, a $p21^{RAS}$ signature has been identified in the rat Mfn2 sequence. Co-immunoprecipitation studies have confirmed the interaction of Mfn2 with Ras (Chen et al., 2004). Recently, in addition to be present in the

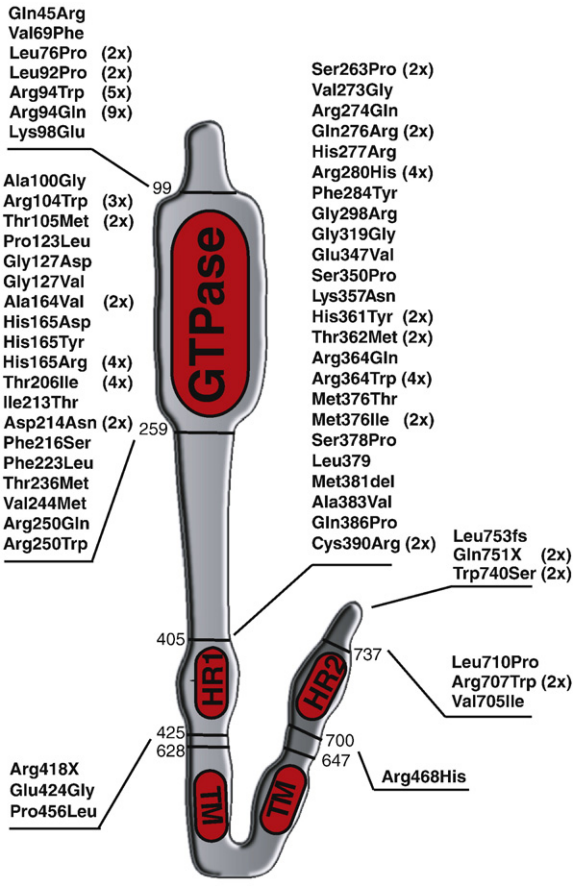

Fig. 1. Schematic representation of the human Mfn2 protein. Characterized domains are depicted in red. Mutations found in CMT2A and reported until the end of 2008 are indicated with their corresponding frequency. Abbreviation: HR; heptads repeated region, TM; transmembrane domain. For size issue, the distance between amino acid 425 and 628 is not on scale.

mitochondrial outer membrane, Mfn2 has also been shown to be associated with the endoplasmic reticulum (ER) and to tether mitochondrial and ER membranes (de Brito and Scorrano, 2008). In the absence of Mfn2, contacts between mitochondria and ER are lost, leading to calcium homeostasis perturbations and ER network fragmentation. The Ras domain has been found to be required to maintain a normal ER morphology in MEFs.

It has to be noted that the Mfn2 pool retrieved in the ER by de Brito and Scorrano (2008) corresponds to only 7% of the total Mfn2 proteins. Thus, a very small population of Mfn2 protein is able to control ER morphology and its contact to adjacent mitochondria. Studies on cells lacking Mfn1, Mfn2 or both, revealed that the function of the two mitofusins may not be fully redundant. Indeed, the mitochondrial network of MEFs is much more affected by the deletion of Mfn1 (Chen et al., 2003). Moreover, the pattern of tissue expression of Mfn1 and Mfn2 seems to be different. Whereas Mfn1 is expressed ubiquitously at a similar level, Mfn2 mRNA is detected in higher amounts in heart, skeletal muscle and brain (Santel et al., 2003). Finally as described above, the efficiency of GTP hydrolysis is also not identical between mitofusins (Ishihara et al., 2004).

The distribution of mutations found in CMT2A patients along the Mfn2 protein is depicted in Fig. 1. About 60 mutations on the *Mfn2* gene have already been reported in CMT2A patients (http://www.molgen.ua.ac.be/CMTMutations/Default.cfm). Almost all of these mutations are single point mutations in the coding sequence causing an amino acid substitution. Inexplicably, some hot spots appear to be more prone to be mutated than others. The amino acid residue at

position 94 of the Mfn2 protein displays a mutation rate much higher than any other site. Mutations in this codon have been reported 13 times in independent patients suffering from CMT2A (5 times R94W and 7 times R94Q) (Cho et al., 2007; Chung et al., 2006; Kijima et al., 2005; Neusch et al., 2007; Verhoeven et al., 2006; Zuchner et al., 2006; Zuchner et al., 2004). This position is not located in the GTPase domain but immediately upstream of it. Interestingly, it is a highly conserved amino acid from human to *C. elegans* (Zuchner et al., 2004). MEFs lacking Mfn2 were nicely rescued by the expression of *Mfn2* mutants associated with CMT2A whereas cells lacking Mfn1 were not rescued by such mutants (Detmer and Chan, 2007a). Thus, *Mfn2* mutants appear to be able to promote mitochondrial fusion through interaction with Mfn1 but not with wild type Mfn2. These data suggest that the cellular level of Mfn1 could be crucial to determine whether *Mfn2* mutants will be deleterious or not. Interestingly, whereas *Mfn2* R94Q mutant is able to rescue mitochondrial network of Mfn2 null cells, it fails to restore ER morphology (de Brito and Scorrano, 2008). This lack of effect could be due to the absence of Mfn1 on the ER membrane. Even though mutations in Mfn2 have been found to be associated with CMT2A, until recently it was not clear whether Mfn2 mutations were responsible for the pathology. This question has been addressed by Chan et al. who generated a transgenic mouse in which a GTPase mutant of Mfn2, *T105M*, was expressed under the control of the HB9 motoneuronal promoter (Detmer et al., 2008). Expression of Mfn2T105M was found to be sufficient to induce some of the clinical symptoms that are encountered in CMT2A, including severe muscle atrophy due to massive motor axon degeneration. However, the authors have not tested the possibility that over-expression of wild type Mfn2 could have a similar effect. The mechanisms by which *Mfn2* mutants disrupt the function of peripheral neurons in CMT2A and in the mouse model developed by Chan et al. are still unclear. Below, we discuss some mechanisms that have been proposed to explain the pathophysiology of CMT2A associated with Mfn2 dysfunctions.

CMT2A due to mitochondrial fusion impairment

Knock down of mitofusins 1, 2 and OPA1, three proteins involved in mitochondrial fusion, has a considerable impact on mouse development. Mitofusin1 and 2 deficient mice are unviable. They die at embryonic day 12.5 and 11.5, respectively. Mfn2 KO lethality is due to a fatal defect of the giant cell layer of the placenta, which is less abundant and contain smaller nuclei than normal (Chen et al., 2003). Notably, no defect in placental development was reported for the Mfn1 KO embryos. Recently, the same group published a conditional Mfn2 KO mouse that bypasses the development lethality caused by placental defects (Chen et al., 2007). They used mice in which Mfn2 alleles were floxed and the Meox-Cre mouse line that expresses the Cre recombinase throughout the embryo except in cell lineages giving rise to the placenta. Then, Mfn2 KO mice were born normally. Nevertheless, one third of the KO mice died on postnatal day 1. The surviving pups displayed severe defects in balance and movement, and all died by P17. Surprisingly, histological investigations revealed that the Purkinje cells of the cerebellum had degenerated (Chen et al., 2007). Mitochondria from cultured Purkinje cells isolated from these animals were improperly distributed in the dendritic branches. Moreover, dendrites devoid of mitochondria had less spines than normal. In contrast Mfn1 conditional KO mice did not show any particular phenotype up to 1 year of age. These important data reveal the crucial importance of Mfn2 in neurons of the cerebellum.

OPA1, another protein anchored in the inner membrane of mitochondria and required for mitochondrial fusion, has been shown to be mutated in autosomal dominant optic atrophy (ADOA), which leads to the degeneration of the optic nerve (Alexander et al., 2000; Delettre et al., 2000). Using ENU mutagenized DNA approach, a mouse model of ADOA carrying the Q258X has been established (Davies et al., 2007). This mutation leads to a protein truncated at the beginning of the GTPase domain. Although, the homozygous embryos died at approximately E12, heterozygous animals were viable and fertile. Skeletal muscle cell explants showed a fragmented mitochondrial network but no histological defects were reported in the main organs of mutant mice including brain and optic nerve. However, at 6 months of age, mutant mice displayed a slight reduction in the number of retinal ganglion cells and at 9 months the optic nerve showed myelin abnormalities. Interestingly, retinal ganglion cell degeneration and atrophy of the optic nerve are the hallmarks of ADOA (Johnston et al., 1979; Kjer et al., 1983). The fact that mutations in Mfn2 and OPA1, two distinct proteins that both participate in mitochondrial fusion, result in neuropathologies points to mitochondrial fusion alteration as the cause of neuronal dysfunction. It remains to understand why is mitochondrial fusion so important for neuronal function and why, when the process is dysfunctional, only a subset of neurons in the central or peripheral nervous system is affected.

The correct distribution of mitochondrial DNA (mtDNA) appears to rely upon mitochondrial fusion. This has been primarily observed in yeast, using a thermosensitive mutant of Mgm1, an OPA1 homolog (Jones and Fangman, 1992). When these yeast cells were cultured at 34 °C, a defect in mtDNA synthesis leading to a decrease in the number of mtDNA molecules per cell was observed. Such a relation between mitochondrial fusion and mtDNA maintenance has also been observed in mammalian cells. In HeLa cells and fibroblasts, mtDNA is distributed throughout the mitochondrial tubules. When cells are treated with carbonyl cyanide m-chlorophenylhydrazone (CCCP) to induce a dramatic mitochondrial fragmentation, around 25% of rounded isolated mitochondria are found to be without any mtDNA (Legros et al., 2004). Moreover, in MEFs lacking Mfn2, Mfn1 or both, a significant amount of mitochondria is devoid of mtDNA (Chen et al., 2007). Notably, the same phenotype is found in OPA1 depleted cells (Chen et al., 2007). Additionally, the number of mtDNA copies per cell has been shown to be reduced in leukocytes of ADOA patients (Kim et al., 2005). Therefore, it can be postulated that mitochondrial fusion could be a mechanism allowing mtDNA maintenance through exchange of DNA molecules between mitochondria. Such a function has been previously demonstrated by a very elegant experiment (Ono et al., 2001). The authors fused two populations of respiratory-deficient cells isolated from patients suffering from mitochondriopathy due to mutations in mitochondrial $tRNA^{Alle}$ or mitochondrial $tRNA^{Leu}$. Fourteen days after fusion of the two cell types, a normal respiratory function and a normal mitochondrial network were observed, indicating a role of mitochondrial fusion in mtDNA exchange between organelles. mtDNA encodes 13 proteins of the respiratory chain and a depletion of mtDNA is expected to lead to oxidative phosphorylation impairment. In the Mfn2 conditional KO mouse a decrease in cytochrome *c* oxidase activity has been measured in Purkinje cells. It is therefore possible that a dysfunction of Purkinje cells is the result of mtDNA loss due to impaired mitochondrial fusion (Chen et al., 2007). Surprisingly, only Purkinje cells have been found to be affected in these animals. Although the reason for this selectivity is unknown, one explanation could be the low level of Mfn1 expressed in these cells, that would be insufficient to compensate the Mfn2 deficit (Chen et al., 2007).

Together, these data suggest that a defect in mitochondrial fusion, leading to a loss of mtDNA and an impairment in oxidative phosphorylation could participate in the pathology of CMT2A.

Mfn2 and cell bioenergetics

The implication of Mfn2 in cell bioenergetics was first proposed by Zorzano et al. They reported that a down-regulation of Mfn2 in L6E9 myotubes induced a reduction in glucose oxidation and mitochondrial membrane potential (Bach et al., 2003). They further showed a down-regulation of Mfn2 mRNA and protein in skeletal muscles of obese Zucker rats and patients. Still in human skeletal muscles, Mfn2

expression has been shown to be up-regulated after acute exercise (Cartoni et al., 2005). Additionally, its expression is regulated by PGC-1 α and β, two key players in cell metabolism (Cartoni et al., 2005; Liesa et al., 2008). Studies with fibroblasts from CMT2A patients carrying a Mfn2 mutation have shown a mitochondrial-coupling defect leading to reduced OXPHOS (Loiseau et al., 2007). Of course, these bioenergetic alterations could be largely indirect and be, for example, the result of mitochondrial fusion impairment. However, some studies argue for a mitochondrial fusion independent role of Mfn2 in bioenergetics. Pich et al., (2005) reported that overexpression of a truncated form of Mfn2 that lacks the transmembrane domain was unable to affect mitochondrial morphology although the membrane potential of mitochondria was substantially increased. How this mitochondrial fusion-independent activity of Mfn2 is achieved is unclear. One possibility is that it may involve RAS since, as mentioned above, a Ras signature motif has been identified in rat Mfn2 protein and Mfn2 has been shown to interact with RAS through this domain (Chen et al., 2004).

Mfn2-induced CMT2A: a mitochondrial transport disorder

Mitochondrial transport and distribution are particularly crucial for neurons. Because of their polarity with long axons and dendrites, high energy levels are often required far from the soma, for example at synapse levels. This is possible thanks to mitochondria. Current models propose that a mitochondrial transport defect could be the cause of CMT2A. Zhao et al. (2001) were the first to link axonal cargo transport dysfunction to CMT2A. They characterized KIF1Bβ as a new isoform of the mitochondrial transporter motor protein KIF1B (Nangaku et al., 1994). Interestingly, they discovered a Japanese family affected by CMT2A carrying a KIF1Bβ mutation. They further showed that heterozygous knockout (KO) mice for the *KIF1Bβ* gene recapitulate symptoms of axonal peripheral neuropathy. However, no other CMT2A patient has been found to display a KIF1Bβ mutation. These results raise the possibility that mutations in Mfn2 could trigger CMT2A by disrupting mitochondrial movement. Furthermore, they raise the question as to whether there could be a direct link between Mfn2 function and the mitochondrial transport machinery.

Mouse embryonic fibroblasts isolated from Mfn2 KO embryos do not appear to display major mitochondrial transport defects. In these cells, short tubular as well as round mitochondria are present. Round mitochondria lost directed movement but were still able to merge with tubular mitochondria, resulting in mitochondria with undirected movement. Round mitochondria were also able to fragment and detach from tubular mitochondria that were then able to move correctly (Chen et al., 2003). The authors concluded that the defect in mitochondrial movement is not due to the lack of Mfn2 *per se*, but rather to incorrect mitochondrial shaping induced by the depletion of Mfn2. This study shows that the consequence of Mfn2 loss in MEFs results in relatively minor transport impairments mostly attributed to mitochondria morphological abnormalities. It also underlines the importance of Mfn2 in the maintenance of the tubular shape of mitochondria. In contrast, over-expression of CMT2A-related *Mfn2* mutants in neurons, in vitro or in vivo, was found to lead to severe mitochondrial transport defects. Over-expression of several *Mfn2* mutants in cultured rat DRG neurons induced mitochondrial aggregation around the nucleus (Baloh et al., 2007). As a consequence, neurites were almost completely lacking mitochondria. In addition, the few mitochondria that were present in axons appeared to be mostly static. On the other hand, in the transgenic mice expressing Mfn2T105M in motoneurons, mitochondria also appeared to collapse around the nucleus and only few of them reached distal parts of axons (Detmer et al., 2008). These transgenic animals displayed severe motoneuron degeneration with massive muscular atrophy. Interestingly and unlike in human disease where a single copy of *Mfn2* mutants is sufficient to induce CMT2A, this severe phenotype was not found in the heterozygous which remained asymptomatic. As previously demonstrated in *Drosophila* (Guo et al., 2005; Stowers et al., 2002; Verstreken et al., 2005; and see below), these sets of data clearly show that mitochondria need to be properly distributed in peripheral axons to allow neurons to function properly. How relevant are these observations for the pathophysiology of CMT2A? Perinuclear mitochondrial aggregation could be the consequence of a transgene dosage and irrelevant for the human disease. By manipulating Mfn2 expression levels in MEFs, Chan's laboratory showed that the majority of *Mfn2* mutants induced a perinuclear collapse of mitochondria when the retrovirus multiplicity of infection (MOI) used to over-express the mutants was high. In contrast, when the MOI was low, ensuring a physiological level of the mutant, the mitochondria did not collapse (Detmer and Chan, 2007a). Importantly, Eura et al. (2003) reported the same mitochondrial collapse when both *Mfn2* WT or GTPase mutants were over-expressed in HeLa cells. Thus, it seems that the collapse of mitochondria around the nucleus could well be the result of protein over-expression (Detmer and Chan, 2007a, 2007b). Nevertheless, the interest of these studies is that they point to a mitochondrial transport defect that should be considered as a possible cause of CMT2A associated with Mfn2 mutations. This possibility was reinforced by the observation of mitochondrial distribution abnormalities in Purkinje cells of Mfn2 deficient mice (Chen et al., 2007). Based on all these studies, it is tempting to speculate that Mfn2 could be part of a motor complex involved in anterograde movement of mitochondria. We can also speculate that by positioning mitofusin 2 at the interface between mitochondrial fusion and mitochondrial transport, a kind of quality control would be ensured since only mitochondria that are able to fuse – and that would be functional – would be transported. The other mitochondria would remain in the soma where they could be degraded by autophagy.

The kinesin and dynein motor proteins are in charge of displacing mitochondria along axonal microtubules in anterograde and retrograde direction, respectively (for reviews see Hollenbeck and Saxton, 2005). In the recent years, adaptor/receptor proteins that connect mitochondria to the motor proteins have been described. In *Drosophila*, the milton/miro complex has been characterized as a key player of mitochondrial distribution. Milton has been identified *via* a *Drosophila* mutagenesis genetic screen for mutants with photoreceptor defects (Stowers et al., 2002). Mitochondria are absent in photoreceptor axon terminals of milton mutant flies but are concentrated in cell bodies, leading to abnormal synaptic transmission. Using a similar screen (Guo et al., 2005), another protein, miro, was found to be required for correct distribution of mitochondria in neurons. Miro is a transmembrane GTPase, which is bound to the outer membrane of mitochondria. Glater et al., using an *in vitro* system, could demonstrate that milton can recruit the kinesin heavy chain (KHC). They also showed that miro not only associates with milton but that a dominant negative mutant of miro lacking its transmembrane domain displaces milton from mitochondria (Glater et al., 2006). Under physiological conditions, miro would bind milton-KHC to mitochondria. Based on the observation of mitochondrial aggregation in the cell body in Mfn2 over-expressing cells, it is tempting to speculate that Mfn2 may form heterocomplexes with miro or Milton to promote mitochondrial transport. High levels of Mfn2 could disrupt these heterocomplexes and impair mitochondrial transport.

In CMT2A patients, mitochondria accumulation in the soma of motoneurons or DRG neurons has not been reported. Rather, in two anatomo-clinical reports, mitochondria have been found to accumulate in the distal part of sural nerve axons (Vallat et al., 2008; Verhoeven et al., 2006) (Fig. 2). The possibility of a mitochondrial transport defect, either anterograde or retrograde as suggested by the observation so far made in patients, should therefore be considered as

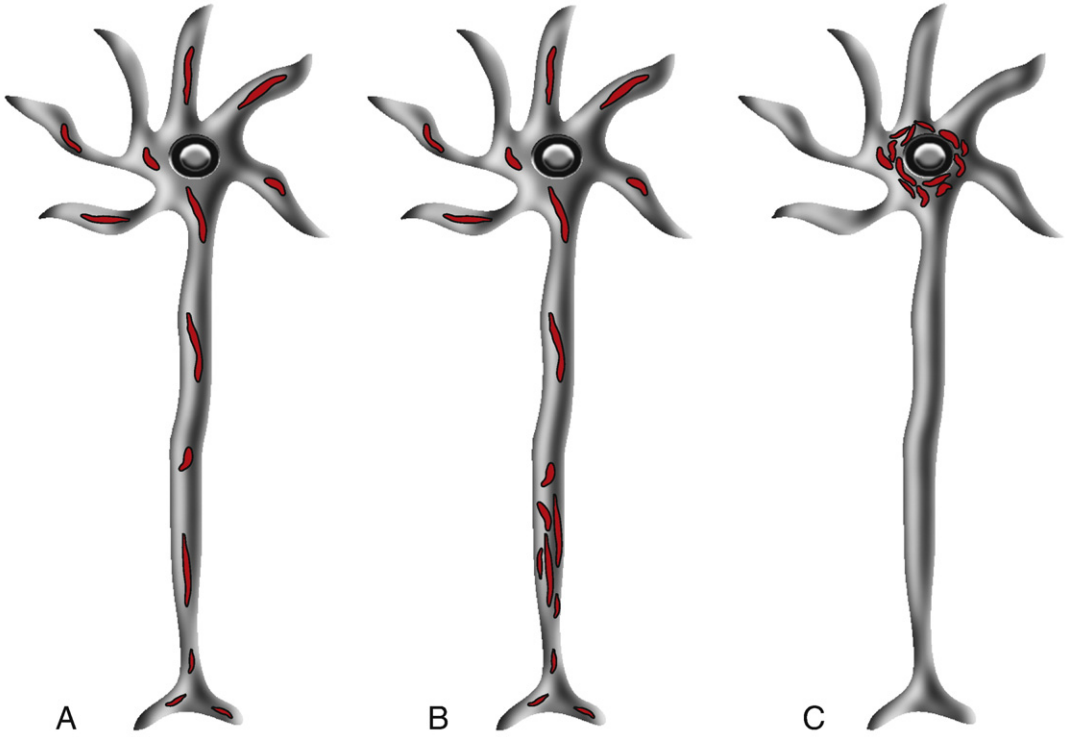

Fig. 2. Mitochondrial transport impairments. (A) Normal situation: mitochondria are distributed throughout the soma and neurites. (B) Mitochondria accumulate in distal axons as shown in two patients with CMT2A. (C) Mitochondria accumulate in the soma of neurons when Mfn2 mutant is over-expressed (or when Mfn2 is depleted as in Purkinje cells).

a possible mechanism responsible for the pathophysiology of the disease.

Perspectives and conclusions

CMT2A peripheral neuropathy is an invalidating disease affecting approximately 40,000 people in the US. Major advancement in our understanding of this disease has been made with the identification of Mfn2 mutations in a substantial set of CMT2A patients. So far there is no treatment available for this disease. Over the last recent years we have learnt a lot about the mechanism of action of Mfn2 and its implication in the disease. The generation of an animal model of the disease should be useful to further understand the pathology of the disease and also to test therapeutic strategies against this disease.

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