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Nerve-dependent changes in skeletal muscle myosin heavy chain after experimental denervation, cross-reinnervation and in a demyelinating mouse model of Charcot-Marie-Tooth disease type 1A

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Abstract

Innervation regulates the contractile properties of vertebrate muscle fibers, in part through the effect of electrical activity on expression of distinct myosins. Here we analyse the role of innervation in regulating the accumulation of the general, maturational and adult forms of rodent slow myosin heavy chain (MyHC) that are defined by the presence of distinct antigenic epitopes. Denervation increases the number of fibers that express general slow MyHC, but it decreases the adult slow MyHC epitope. Cross-reinnervation of slow muscle by a fast nerve leads to an increase in the number of fibers that express fast MyHC. In both cases, there is an increase in fibers that express slow and fast IIA MyHCs but without the adult slow MyHC epitope. The data suggest that innervation is required for maturation and maintenance of diversity of both slow and fast fibers. The sequence of slow MyHC epitope transitions is a useful biomarker, and it may play a significant role during nerve-dependent changes in muscle fiber function. We applied this detailed muscle analysis to a transgenic mouse model of Human Motor and Sensory Neuropathy IA, also known as Charcot-Marie-Tooth disease Type 1A (CMT1A), in which electrical conduction in some motor neurons is poor due to demyelination. The mice display atrophy of some muscle fibers and changes in slow and fast MyHC epitope expression suggestive of a progressive increase in innervation of muscle fibers by fast motor neurons, even at early stages. The potential role of these early changes in disease pathogenesis is discussed.

Keywords

Muscle; myosin; human motor and sensory neuropathy IA; denervation; innervation; fast; slow; type I; type II; fiber type; antibody; post-translational modification; demyelination

INTRODUCTION

Each adult mouse muscle contains a characteristic mixture of fast and slow fiber types⁵⁸. Patterning of vertebrate muscle fiber types in development is initially nerve-independent^{6,12}. Later, innervation by inappropriate motor neurons can override other regulation of fiber type, in both the embryo^{69,26} and the adult animal⁵. Thus, development of mature muscle fiber type pattern requires matching between innervating motor neurons and their muscle

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targets so that slow motor neurons connect to slow fibers and fast motor neurons to fast fibers (reviewed in ref.³⁶).

Changes in fiber type are a common correlate of neuropathic disease, and of changes of use. Amyotrophic lateral sclerosis, human motor and sensory neuropathies (HMSN) and human motor neuropathies are all accompanied by gradual changes in both innervation and muscle fiber types¹⁵. In none of these conditions is the contribution of fiber type alteration, as opposed to altered neuronal drive, to the functional deficit in patient mobility clear. Aging is also paralleled by increases in slow fiber proportion and decreases in the fastest classes of fibers, both in humans and other mammals^{42,43}, which may contribute to physical impairment and dependency. Endurance or strength training also lead to fiber type changes (reviewed in refs^{11,20,33}). In most cases, the extent to which fiber type changes in humans are brought about by altered innervation, by change of firing patterns of nerves without alteration of innervation, or by other causes (such as hormonal changes) is unclear.

HMSN type 1A, also known as Charcot-Marie-Tooth disease type 1A, is caused by mutation of the *PMP22* gene that leads to over-expression of the PMP22 protein in Schwann cells. This causes motor neurons to become focally demyelinated leading to conduction block, followed by re-myelination and consequent slow nerve conduction. For reasons that are not clear, but may involve reduction in electrical stimulation of muscle fibers, this leads to debilitating muscle wasting, particularly in the lower leg. Over the long term, the disease involves progressive axonal degeneration and loss of motor neurons, and remaining neurons have increased motor unit size. Fiber type grouping is observed within muscle, as a consequence of collateral innervation by remaining motor neurons that is a hallmark of peripheral neuropathy¹⁷. Thus, in the late stage disease, it is likely that the primary cause of disability is loss of motor neurons. However, the cause of motor neuron changes in early stages of the disease is unclear, but it could relate to direct effects of demyelination, altered sensory feedback or changes in neurotrophic support for motor neurons from their muscle targets as a result of altered stimulation of the muscle.

An effect of altered electrical stimulation on adult muscle fibers is to change their 'fiber type'. This involves changes in a wide range of contractile, regulatory, metabolic and other proteins. Myosin heavy chain (MyHC), which is responsible, together with actin, for the contraction of muscle fibers, is now frequently used to classify fibers, because it correlates with their contractile properties^{52,41}. The electrical activity imposed on muscle fibers by their motor neurons regulates fiber contractility in large part through regulation of distinct MyHC genes^{56,1} (reviewed in ref²⁸). As well as diverse genes encoding MyHC, there is additional MyHC diversity which cannot simply be explained by expression of distinct MyHC genes. For example, a maturational series of three forms of slow MyHC have been described in rat, human and mouse skeletal muscle yet there is only a single known mammalian slow β -cardiac MyHC gene expressed^{34,44}. Post-translational modifications leading to distinct epitopes on the slow MyHC protein backbone may explain this maturation series^{44,50}.

The maturational series of slow MyHC forms is characterised by a series of five monoclonal antibodies that recognize distinct epitopes on the product of the mammalian β -cardiac slow MyHC gene^{34,44}. Two 'general slow' MyHC epitopes, recognized by antibodies A4.840 and BA-D5, parallel the reported expression pattern of the mRNA for the β -cardiac *myh7* gene⁵³. Two additional epitopes, which we have termed 'maturational slow' epitopes, are recognized by antibodies BA-F8 and N2.261. They appear a little later than the general slow epitopes in developing slow muscle. Another epitope, we have termed the 'adult slow' epitope, is recognized by antibody A4.951. The adult slow MyHC epitope appears in developing slow fibers even later than the maturational slow MyHC epitope. The N2.261-

reactive maturational slow antibody also detects the fast IIA MyHC, which can be uniquely identified by antibody A4.74^{34,44}. These data are summarized in Table 1. Single muscle fibers in adult animals occasionally express the general slow MyHC epitopes, without the adult slow MyHC epitope. These fibers appear to be IIC fibers, in transition between mature slow and fast IIA character^{34,44}. It is generally believed that such natural fiber type transitions in freely-moving animals are the result of alterations in nerve-induced electrical activity. The mechanism for generation of the distinct slow MyHC epitopes at a molecular level remains unclear, and how they are regulated by electrical activity has not been investigated.

In addition to regulating adult fiber type and MyHC isoform/epitope accumulation, electrical activity also influences the MyHC transitions that occur during development. First expression of the adult fast IIA, IIX and IIB MyHC isoforms does not show an absolute requirement for innervation^{7,49}. However, manipulations that prevent correct formation or maturation of innervation inhibit fast MyHC maturation, suggesting that at least some muscle fiber maturation is nerve-dependent^{51,56,54,55}. Moreover, in the chicken and rat, expression of slow MyHC in myotubes grown in cell culture can be influenced by electrical activity or neural signals^{18,70,47,30}. Therefore, we examined the role innervation plays in the accumulation of distinct slow MyHC epitopes.

We report that denervation and cross-reinnervation cause reciprocal changes in a set of slow and fast MyHC epitopes. Distinct slow MyHC epitopes are differentially neurally-regulated in mammalian muscle. Applying this data to transgenic mice which over-express human peripheral myelin protein 22 (PMP-22), a mouse model of CMT1A disease, we suggest underlying causes for the complex muscle pathology observed in this mouse model.

MATERIALS AND METHODS

Muscle tissue

Mouse muscle tissue was obtained from T0 and other strains bred at King's College London and killed by CO₂ inhalation. Transgenic mice³⁷ containing copies of the human *PMP22* gene on a mixed C57BL/6J and CBA/Ca background were bred at Imperial College School of Medicine and were analysed at 4 to 5 months old. The C22 line has seven copies of the *PMP22* gene, C61 has four copies and C16 has a single copy containing a deletion and is not expressed.

Monoclonal Antibodies

Hybridomas of all antibodies used in this paper are available from the American Type Culture Collection and/or the German Hybridoma Bank at Braunschweig, Germany. Antibodies A4.840 (IgM), N2.261 (IgG1), A4.951 (IgG1) N3.36 (IgM) and A4.74 (IgG1) have been characterised in detail previously^{14,34,10}, and most can be purchased from Alexis, Switzerland. Antibodies BA-F8 (IgG2b), BF-F3 (IgM), BA-D5 (IgG2b) were produced in the laboratory of Dr Stefano Schiaffino⁵⁷.

Immunohistochemistry

Mouse lower hindlimb muscle was sectioned and stained as described previously³⁴. Briefly, sections were pre-incubated with 0.1 mg/ml goat anti-mouse IgG (heavy and light) Fab (Cappel), 5% HS in PBS for 30 min to prevent non-specific binding. Sections were then incubated with undiluted tissue culture supernatant containing the anti-MyHC monoclonal antibody for 1 hour. Sections were incubated in PBS, 5% HS for 1 hour before replacement with either 1:100 biotin-conjugated goat anti-mouse IgM (μ -chain specific; Vector) or 1:400 biotin-conjugated horse anti-mouse IgG (heavy and light chain specific; Vector), to detect

IgM and IgG respectively, diluted in PBS, 5% HS. Slides were washed and endogenous peroxidase was blocked with 5% H₂O₂ in methanol. Slides were then rewashed, and avidin-biotin complex (Vectastain ABC Elite kit, Vector) was applied to the sections for 1 hour. Slides were again washed, and horseradish peroxidase reactivity was developed for approximately one minute with 0.6 mg/ml diaminobenzidine, 50 mM Tris HCl pH 7.2, 0.03% CoCl₂ and 0.05% H₂O₂ and mounted in polyvinyl alcohol.

Denervation and Cross-reinnervation

Limbs of TO outbred mice were denervated at 4-5 weeks of age by right sciatic nerve section at the upper thigh level. Although some fibrillation of the denervated muscle is possible, no movement was observed after the operation or when animals were tested for lack of sensory and motor function in the lower hindlimb at the time of sample collection. A visible nerve was not present within the thigh on the operated side. The superficial peroneal nerve of adult Wistar rats was cut and implanted onto the surface of the soleus muscle. Two weeks later the normal innervation to the soleus was removed by nerve resection²¹. Reinnervation was permitted for 60 days, by which time almost all fibers on the nerve-implant side of the soleus were reinnervated. Cross re-innervated limbs were the generous gift of Dr. Wesley Thompson.

RESULTS

Denervation causes differential slow MyHC induction and loss

To examine the role of innervation in slow and fast MyHC isoform/epitope expression, mouse lower hindlimbs were denervated by sciatic nerve section. Ipsi- and contralateral limb slow soleus muscles were compared for changes in slow MyHCs. Atrophy caused by denervation of ipsilateral muscle fibers was detected after one week, but it was successively more marked after three or seven weeks of denervation (Fig. 1). Contralateral control soleus showed no significant difference from un-operated control animals of similar age and sex (Fig. 1, 2). Most fibers in contralateral control soleus were either classic type I fibers with A4.840⁺BA-D5⁺BA-F8⁺A4.951⁺A4.74⁻ phenotype (about 60% of fibers, marked i in Fig. 1) or type IIA fast fibers with A4.840⁻BA-D5⁻BA-F8⁻A4.951⁻A4.74⁺ phenotype (about 30%, marked ii in Fig. 1). About 8% of control soleus fibers had a IIC character, reacting with a subset of slow MyHC antibodies, generally A4.840 and BA-D5, and these fibers also reacted for some fast MyHC (Fig. 2). In denervated atrophying limbs, there was a widespread induction of general slow MyHC in the soleus, but relatively little detectable increase in maturational or adult slow MyHCs. The number of fibers that express general slow MyHC rose to 88% (from 68%) within three weeks (Fig. 1, 2), and to almost 100% by seven weeks after denervation (Fig. 1G). However, there was relatively little increase in maturational or adult slow MyHC. On the contrary, although few if any fibers entirely lose adult slow MyHC, there is a marked decline in the intensity of adult slow-reactivity in the denervated muscles, both compared to contralateral control muscle (Fig. 1B,E,H,K), and compared to general slow-reactivity in adjacent sections of the denervated muscle (Fig. 1A,B,G,H). In addition, shortly after denervation there is a striking increased heterogeneity in intensity of reactivity between fibers and a difference in reactivity of individual identified fibers with the two general slow antibodies, A4.840 and BA-D5. As the A4.840 epitope depends on phosphorylation status⁵⁰, this may reflect post-translational changes in slow MyHC. Such variation is not present in innervated muscle of either developing or mature mice (Fig. 1). The increase in general slow MyHC is not accompanied by a detectable reduction in fast MyHC expression, giving numerous type IIC fibers with an A4.840⁺A4.951⁻A4.74⁺ phenotype (marked iii in Fig. 1). There are also a few fibers with a A4.840⁺A4.951⁺A4.74⁺ phenotype (marked iv in Fig. 1).

Overall, the response of slow muscle to denervation is up-regulation of the general slow MyHC epitopes in fast IIA fibers, without concomitant increase in adult or maturational slow MyHC epitopes. This change was not accompanied by a significant change of fibers expressing fast IIA MyHC, although a slight trend towards increased IIA MyHC-expressing fibers could not be ruled out (Fig. 1, 2). This finding suggests that denervation of soleus muscle leads to a loss of mechanisms that normally repress slow MyHC when innervation is intact.

In fast peroneus muscle, on the other hand, denervation leads to more mild atrophy and up-regulation of general slow MyHC, significant ectopic appearance of adult fast IIA MyHC and a marked loss of fast IIB MyHC (Fig. 1M-O and data not shown). There was also a striking regional difference in these fast muscle changes. In general, in deep regions of fast muscles that already contained some slow and IIA fibers, changes like those in Fig. 1M-O occurred. In contrast, in some superficial fast muscle regions more marked atrophy was observed, but there was little change in MyHC. Taken together, the distinct responses of these regions to denervation suggests that nerve-independent differences in the regulation of MyHC expression exist between a) soleus fibers and most fast muscle fibers and, b) deep fast muscle fibers and certain superficial fast muscle regions.

Cross-reinnervation leads to stable loss of adult slow MyHC

To further test the role of electrical activity, we examined the effects of distinct patterns of electrical activity upon slow MyHC in muscle to which electrical stimulation had been altered, rather than removed. For this purpose mouse limbs are too small, so we turned to the rat, an animal that is large enough to permit manipulation of innervation and which responds to changes in electrical activity and innervation by a well-characterized alteration of the balance of fast and slow MyHCs³⁹. We have previously shown that rat soleus contains around 90% slow fibers³⁴. So we examined the effect of cross-reinnervation of the slow soleus muscle by the peroneal nerve, which normally innervates fast muscles that have both fast and slow motor units. This manipulation has the advantage of also denervating the antagonist peroneal muscles and thereby unloading the soleus. As unloading also occurs in hindlimb denervation, the unloading in cross-reinnervated muscle makes comparison between cross-innervation and denervation effects on the soleus simpler, as unloading is present in both cases.

Un-operated, mock-operated and contralateral rat muscles had a similar appearance. Most soleus slow fibers expressed the three epitopes of general (A4.840), maturational (N2.261) and adult (A4.951) slow MyHC and no fast MyHC (type I, $A4.840^+N2.261^+A4.951^+N3.36^-A4.74^-$, arrows I in Fig. 3). A few fibers were missing the adult slow reactivity ($A4.840^+N2.261^+A4.951^-$), and somewhat less than 10% of the fibers were of a fast IIA type ($A4.840^-N2.261^+A4.951^-N3.36^+A4.74^+$, arrowheads IIA in Fig. 3). In contrast, the cross-reinnervated soleus showed a striking loss of slow MyHC reactivities in many fibers and the classic fiber type grouping typical of re-innervated muscle (Fig. 3). Adult slow MyHC showed the largest decrease; no fibers in some regions of the muscle contained detectable $A4.951^+$ MyHC. However, the same fibers displayed heterogeneous reactivity with the general slow MyHC antibody (Fig. 3B,D). Fibers that lost adult but retained general slow MyHC also expressed fast MyHCs (compare Fig. 3B,D,F,H). The fibers that also showed decreased or absent general slow MyHC had converted to a fast IIX/IIB phenotype that is not found in control soleus (compare Fig. 3B,D,J). Some of these fibers may have derived from the fewer than 10% of fast IIA fibers present in control rat soleus. However, the frequency and distribution of IIX/IIB fibers in these areas was too high to be accounted for solely by IIA fiber conversion. $A4.840^+A4.951^-$ fibers retained $N2.261^+$ MyHC(s), but, due to the cross-reaction of N2.261 with both maturational slow and fast IIA

MyHC (Table 1), the latter of which is also expressed in these fibers, we could not determine whether maturational slow MyHC was altered (compare Fig. 3I,J).

Overall, these long term cross-re-innervations led to fiber type grouping with four major types of fibers being detected: residual fully slow and fully fast IIA fibers, fast fibers that have entirely lost slow MyHC and converted to fast IIX/IIB, and an abundant mixed IIC fiber type in which adult slow MyHC has been lost but general slow MyHC remains in the presence of IIA MyHC. Thus, denervation of antagonists combined with altered electrical activity in slow fibers re-innervated by a fast nerve leads to a population of fibers that have a mixed fast-plus-general-slow MyHC profile similar to that observed in denervated animals. This change is due to a down-regulation of adult slow MyHC and up-regulation of adult fast IIA MyHC, rather than up-regulation of general slow MyHC.

Transgenic PMP22 over-expressing mice lose adult slow MyHC and gain fast MyHC

Having shown that slow MyHC markers change when fibers experience altered electrical activity, we employed these markers in a pathological situation, a mouse model of CMT1A. *PMP22* transgenic mice over-express in the Schwann cells that myelinate motor neurons, a gene encoding the human peripheral myelin protein-22 (*PMP22*) driven by its own regulatory elements^{37,38}. These mice have altered electrical conduction in the sciatic nerve throughout life accompanied by ongoing demyelination and remyelination. A control transgenic line, C16, had no extra *PMP22* expression and normal motor nerve conduction velocity (30-50 ms⁻¹, control used in Fig. 4F-J). Line C61 has four extra copies of the *PMP22* transgene but showed no significant changes in MyHC profile from control mice (C61 in Fig. 5). This correlates with the lack of visible phenotype in the movement of these mice, even though they have quite thin myelin and reduced motor nerve conduction velocities (about 25 ms⁻¹). The C22 line has seven extra copies of the *PMP22* transgene and severe reduction in motor nerve conduction velocity (<10 ms⁻¹) with clear muscle wasting and weakness. These mice showed a profile of MyHC expression different from that seen in short-term denervated mouse muscle, but it was similar in some respects to that of cross-reinnervated rat muscle (Figs 4,5). The proportion of fibers that express some form of slow MyHC shows no significant change, being 68% in controls and 60% in the C22 transgenic mice. However, there was a significant decrease in A4.951⁺ adult slow MyHC-reactive fibers from 61% to 46% as a proportion of total fibers in the soleus, and from 89% to 77% as a proportion of fibers expressing any slow MyHC epitope (Figs 4,5). This change in slow MyHC epitope expression was paralleled by an increase in the proportion of fibers that express A4.74-reactive fast IIA MyHC from 41% to 71% of total fibers. The increase in fast MyHC-expressing fibers is not a full fiber type conversion from slow to fast. The proportion of fibers that express both general slow and IIA fast MyHCs rises from 10% in control animals to 31% in C22 *PMP22*-transgenic mice, whereas the proportion of fibers that express fast IIA MyHC without any slow MyHC epitope shows no significant increase (Fig. 5).

PMP22-transgenic mouse soleus muscle fibers are variably affected, as demonstrated by the presence of both atrophic and relatively normal-sized fibers (Fig. 4). Further insight into the changes occurring in C22 transgenic animals was gained by using fiber atrophy to distinguish between two kinds of fibers: 1) fibers that have recently had a serious disruption of innervation and are consequently small, and 2) fibers that are within the normal size range and are presumably better innervated (Fig. 5). Most normal-sized fibers in *PMP22*-transgenic mouse soleus muscle were either exclusively slow (31%), most reacting with all four anti-slow MyHC antibodies, or exclusively fast (47%), expressing A4.74-reactive IIA MyHC without slow MyHC reactivity, as in control muscle. Only 22% of normal-sized fibers expressed both fast and slow MyHCs, compared to 10% in control animals. On the other hand, the small 'denervated' fibers were much more likely to have an A4.840⁺A4.951⁻

pattern of reactivity characteristic of the short term denervated fibers (Fig. 4); 44% of atrophic fibers had both slow and fast MyHC expression. Thus, the small atrophic fibers in *PMP22* mice showed changes more similar to those induced by acute denervation than did the non-atrophic fibers.

Despite showing an increased mixed fast/slow MyHC phenotype the atrophic fibers of *PMP22* mice were not identical to the atrophic fibers of denervated soleus. The mixed fibers appeared to arise from an increase in the number of fibers that express fast MyHC, rather than an increase in the number of fibers that express slow MyHC as seen after short term denervation (compare Figs 2 and 5). The tendency towards fast IIA MyHC induction was also detectable in the normal-sized fibers of *PMP22*-transgenic mice. These fibers showed a marked loss of the typical type I slow MyHC profile and an increase in fast MyHC without a significant drop in the proportion of fast fibers that were pure fast IIA. Strikingly, as fast IIA MyHC is induced the adult A4.951 epitope and the maturational BA-F8 epitope decline in slow fibers. Interestingly, analysis of two female C22 *PMP22*-transgenic mice that survived to 16 months of age revealed no significant fiber type differences, grouping or atrophy (Chris Mann, CH and SMH, data not shown). We suspect these animals may have been less severely affected (one showed no gate defect).

DISCUSSION

This report contains two major findings. First, distinct modifications of electrical activity can induce various fiber types to take on an intermediate fast/slow phenotype that involves the presence of general-type slow MyHC in the absence of adult slow MyHC. Second, we used this new insight to analyse fast and slow MyHC epitopes in a murine model of CMT1A. The data reveals a CMT1A phenotype in which slow fibers convert to a faster character and suggests a contribution of this process to pathogenesis.

Electrical activity and slow MyHCs

Individual muscle fibers differ in their response to denervation of the whole leg, a manipulation which also removes much passive stretching of fibers. After three weeks of denervation, half of the type IIA fibers in the soleus and some type IIA fibers in fast muscles have accumulated detectable slow MyHC, but the vast majority of fast IIB fibers (the majority fiber type in fast muscles) have not done so. This suggests that, underlying the pool of fast fibers in adult muscle, there are nerve-independent differences that regulate the rate or extent of slow MyHC modulation in response to denervation. This is consistent with the differential response of neonatal MyHC expression to denervation⁵⁵ and observations in the rat⁴⁸. Similarly, although fast IIA MyHC is not accumulated in many slow fibers in the soleus upon denervation, this appears to be the case in some fibers in deep regions of fast muscles. Together with other evidence from the divergent response of different muscles to electrical stimulation and the expression of reporter transgenes^{25,71,35,72}, these data support the view that adult muscle fibers are not entirely dependent on their innervation to maintain distinct fiber types. Instead, other local factors are able to retain fiber type differences over periods of many weeks. Clearly, the nerve, through electrical activity, has a dominant role in determining adult fiber type, but it appears to act on fibers with underlying intrinsic differences that have been called ‘adaptive range’⁷¹.

We observe induction of general slow MyHC epitopes in previously fast fibers, and no increase in fibers expressing fast MyHC, upon acute denervation of the soleus muscle. Induction of both neonatal fast and fast IIA MyHC at both protein and mRNA levels is the reported effect of denervation of adult rat soleus muscle^{56,39,46,55,40}, although enhanced slow character is seen in denervated slow rabbit muscles^{13,2}. An accentuation of slow traits has been reported in rat, cat and guinea pig^{65,29,3,27}. Denervation of other muscles with

substantial slow fiber content, such as the diaphragm, gives variable results^{8,22,61,73,23}. However, it is clear that denervation represents a complex process that involves reduced electrical activity, loss of tone, fibrillation and, in particular, serious fiber atrophy. Thus, comparisons of whole muscle behavior or myosin content with single fiber data can be misleading, because they will not reflect differential changes in the several fiber populations found within muscles. Slow muscles undergo more severe atrophy than fast muscles in the early stages of denervation²⁴, and protein and mRNA levels do not always change in parallel²³. We suggest that, were this also true at the single fiber level, it could prevent an induction of slow MyHC mRNA and protein from being detected when relative levels of MyHC molecules are assayed in muscle homogenates. In previous studies, the overall atrophy and mRNA down-regulation may have masked the increase in proportion of fibers that express slow MyHC molecules. Moreover, depending on the time course of atrophy and MyHC induction, distinct results may have been obtained.

Our data show that, at least in mouse muscle, slow MyHC is induced in addition to other MyHC isoforms by denervation, consistent with the prevailing view that denervated muscles adopt an earlier developmental character. As the slowest myosin in a mixture is thought to have a disproportionate effect on contraction rate, the induction of slow MyHC in fast IIA-expressing fibers may explain the slowing of denervated muscles. The induction of slow MyHC occurred both in soleus and, to a lesser extent, in some, but not all, fast muscle regions. The induced slow myosin does not contain the adult slow MyHC epitope. The increase reflects expression of general-type slow MyHC in fibers that previously did not express it, leading to the striking rise in fibers with a mixed slow/fast IIA phenotype. Yet, due to denervation-induced atrophy, the content of both proteins within the muscle has probably declined sharply. Although the proportion of slow MyHC-containing fibers increases, the content of A4.951-reactive adult slow fibers declines so that, as in innervated muscle, very few, if any, fibers contain both fast IIA MyHC and the adult slow epitope. These data support the view that, at least in rodent soleus, a function of innervation is to repress generation of inappropriate slow MyHC forms (e.g. the immature forms lacking the adult epitope) and maintain diversification of fibers into the mature end-stages of differentiation, as has been suggested in developing muscles^{9,68}.

The reciprocity between the adult slow MyHC and fast IIA MyHC accumulation is not only observed in denervated muscle fibers: stably cross-reinnervated rat soleus shows the same phenomenon in a large proportion of fibers. It has previously been observed that electrical stimulation of denervated rat soleus muscle with a fast stimulation regime leads to co-expression of fast and slow MyHC isoforms in the vast majority of fibers²⁵. Our results with cross-reinnervated rat soleus muscle are consistent with this finding but further show that the slow MyHC expressed in these mixed fibers is subtly different from that present in the unmanipulated soleus. It does not contain the adult slow MyHC epitope. Unlike the situation in the denervated soleus, the presence of mixed general-slow/fast IIA fibers is caused by up-regulation of fast IIA MyHC in slow fibers, accompanied by down-regulation of adult slow MyHC epitope, rather than by up-regulation of general slow MyHC in fast fibers. This difference between denervation and cross-reinnervation is likely to be due to electrical activity imposed on the muscle fibers by the nerve, rather than changes in passive stretch, as the antagonistic peroneal muscles were also denervated in our cross-reinnervation experiment. It is quite possible that, with longer periods of cross-reinnervation, slow MyHC would disappear completely. Nevertheless, we cannot determine whether induction of fast IIA MyHC is due to sarcolemmal electrical activity itself, the resultant active contraction or, less likely, some other effect of the new nerve.

In rat denervated fast extensor digitorum longus (EDL) muscle, slow soleus-type electrical activity alone can trigger a transition to pure slow MyHC expression⁷². As no fast MyHC

was observed in these fibers, we predict that the adult slow MyHC epitope is present. However, the converted EDL slow fibers have a twitch time to peak typical of EDL slow fibers in un-manipulated animals, as opposed to the even slower rate observed in soleus slow fibers⁷². So it is possible that differences in other slow MyHC epitopes, different from those examined here, or changes in other parameters that control twitch time to peak, are more refractory to alteration by electrical activity than is MyHC isoform expression per se.

Maturational slow MyHC epitopes and myosin stability

We have previously shown that the adult slow MyHC epitope is reciprocal to fast MyHC expression in mouse, rat and human muscle^{34,44}. In soleus, the reciprocity between adult slow MyHC and fast IIA MyHC accumulation is also observed in denervated muscle fibers and stably cross-reinnervated rat soleus fibers, with rare exceptions. It has been suggested that conformational change of slow MyHC, possibly regulated by phosphorylation or glycosylation, controls the appearance of the adult slow epitope^{44,50}. Fibers undergoing transition normally lose the adult slow epitope, indicating that this epitope correlates with stabilisation of slow MyHC in the filament lattice. Interestingly, MyHC protein and mRNA levels are differentially regulated in denervated slow muscle^{73,23}. Perhaps altered post-translational modification is one means by which protein turnover is controlled independent of translation rate.

In PMP22 muscle, the reciprocity of adult MyHC isoforms/epitopes dramatically breaks down in the atrophic fibers, as many small fibers react for both fast IIA and adult slow epitopes. Large soleus fibers in PMP22 mice, appear to retain/recover the reciprocity despite showing a shift in fiber type. We suggest that this reciprocity reflects properly innervated (and therefore not atrophied) fibers re-establishing normal MyHC metabolism. Why do so many atrophic fibers have both slow and fast adult MyHC forms? We speculate that this is due to the precise pattern of electrical activity the fibers receive. Different aspects of electrical activity pattern may control slow and fast IIA MyHC mRNA expression and protein stability, such that both can accumulate in these innervated small fibers. At least two reasons for altered firing can be envisioned. Residual conduction block might alter pattern irrespective of central drive. Alternatively, the functional failure of the postural soleus muscle might lead to compensatory changes in central drive and recruitment of fast motor units that poise the overall pattern of activity at a value few, if any, muscle fibers would receive in a healthy animal.

Fiber type change in PMP22 mice

PMP22-transgenic mice have numerous attributes that faithfully mimic human CMT1A^{37,45,38}, which is caused by excess *PMP22* expression in the Schwann cells of peripheral nerves and a consequent demyelination^{64,63,32,59,67,31}. The transgenic mice show progressive hindlimb weakness accompanied by nerve conduction rate decreases and demyelination within the nerve^{37,45,38}. However, the mice have relatively few fibers in the acute stage of denervation, as we do not detect a significant rise in slow MyHC, even in atrophied fibers. A mild early muscle defect becomes more pronounced with age: fiber type grouping is followed by focal fiber atrophy⁴⁵. Fiber type grouping is taken to indicate collateral reinnervation of denervated muscle fibers by relatively healthy nearby motor neuron terminals. In our animals, which were analysed at 2-4 months of age, fiber type grouping was not yet apparent. However, we found changes in these mice analogous to those in CMT1A, including an increase in the number of atrophied type II fibers compared to type I fibers, and an increase in the number of fast myosin-expressing fibers that might explain the changes in fiber type predominance that can be observed in the human disease^{15,19}. It would appear that an increase in motor unit sizes involving fiber type grouping,

similar to that which may occur in CMT1A through collateral branching (⁶² and Björn Falck, personal communication), is a later event in these mice.

Motor neuron death would lead to complete fiber denervation, yet the increase in slow MyHC typical of denervation is absent in PMP22 fibers. Therefore, we suspect that the number of completely denervated fibers in our PMP22 mice is limited. Focal demyelination of neurons will lead to lack of electrical signals to target muscles through a block of conduction; blockade might be predicted to be most marked for fast firing trains typical of fast motor neurons. One possible interpretation of our findings is that reduced activity may trigger fiber type changes similar to those observed after spinal cord transection, in which fibers atrophy and change to faster phenotypes ^{16, 66}. In other words, the presence of atrophied fibers with increased proportions of fast MyHC suggest reduced activity in slow motor units leading to simultaneous atrophy and conversion to a faster, but intermediate, fiber type in which both fast and slow MyHC is expressed. However, other data argue against this view. Fast fiber numbers are increased even among large normal-sized fibers: changes similar to those in cross-reinnervated rat soleus. So an alternative interpretation, which would explain the fiber type grouping seen later in the disease, is that fibers are denervated from an early stage, but they are rapidly re-innervated and that fast motor neurons are better than slow motor neurons at re-innervating denervated fibers in *PMP22* mice. In this scenario, atrophic fibers are re-innervated, re-growing and in the process of conversion to a fast type. After re-growth, conversion to the fast phenotype is complete. This explains the increased proportion of large purely fast fibers. Whichever of these views is correct, our data suggest that the preferential type II fiber atrophy observed in CMT1A might, in fact, be atrophy of slow fibers that are simultaneously converting to a faster phenotype. In the *PMP22* mice, such fibers also lose the adult slow MyHC profile. Presumably, fiber type grouping in late stage human disease reflects collateral innervation of muscle fibers by relatively healthy motor neurons. It is, therefore, important to know whether the changes toward faster fiber types in *PMP22* transgenic mouse MyHC expression might also occur in other aspects of fiber function. It seems likely that slow muscle fibers, which receive more electrical activity from their motor neurons, may be the first to be affected by demyelination. If the trophic support supplied to slow motor neurons by their muscle targets is reduced as a result of decreased activity, it would not be surprising that they fail to maintain neuromuscular connectivity. Alternatively, poorly stimulated slow fibers might be the first to emit signals to recruit further innervation. Continual expansion of the large fast motor units could lead to death of out-competed slower motor neurons and hasten the eventual functional decline in patients. If methods could be found to encourage slow muscle fibers to reject re-innervation by fast motor neurons, as happens during development but declines during maturation ⁶⁰, it might be possible to modify the disease course by preventing the rapid growth of some motor units at the expense of others. Our observation that some genetic carriers of the severe PMP22-transgene allele survive to middle age without obvious muscle changes is reminiscent of sporadic clinical reports of nerve defects with limited muscle pathology ⁴. It suggests that variables currently beyond our control can mitigate disease progression.

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Abbreviations

MyHC	myosin heavy chain
CMT	Charcot Marie-Tooth
HMSN	Human Motor and Sensory Neuropathy

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Figure 1. Denervation causes general-type slow MyHC induction in fibers that express fast IIA MyHC

Muscles of denervated or contralateral control mouse legs were analysed for general slow MyHC (A4.840⁺ A,D,G,J,M,P), adult slow MyHC (A4.951⁺ B,E,H,K,N,Q) and adult fast IIA MyHC (A4.74⁺ C,F,I,L,O,R). Soleus fibers were atrophied after 22 days denervation (A-C), compared to control (D-F), and general slow MyHC increased without a parallel induction of adult slow MyHC in fibers containing IIA MyHC. Whereas most fibers in the contralateral muscle were either classic type I slow fibers with A4.840⁺A4.951⁺A4.74⁻ phenotype (i), or type IIA fast fibers with A4.840⁻A4.951⁻A4.74⁺ phenotype (ii), numerous fibers in the denervated muscle were mixed type IIC with A4.840⁺A4.951⁻A4.74⁺ (iii, a group of three such fibers and one normal slow fiber are magnified in the inset). A few fibers had a A4.840⁺A4.951⁺A4.74⁺ phenotype (iv). By seven weeks of denervation, soleus was strikingly atrophied (G-I), compared to contralateral control (J-L). Most fibers had either a slow A4.840⁺A4.951^{weak}A4.74⁻ or mixed A4.840⁺A4.951^{-/weak}A4.74⁺ phenotype (insets show three fibers magnified). The fast peroneus longus muscle showed substantially

less atrophy (M-R), accompanied by an increase in both general slow A4.840⁺ MyHC in some fast fibers (arrow), and A4.74⁺ MyHC in more numerous fibers. Note that most peroneal fibers in both conditions react for N3.36 (data not shown).

Figure 2. Induction of specific isoform/epitopes of both slow and fast MyHC on denervation
Muscle fibers from soleus of seven control mice (top panel), two 7-day (middle panel) and two 22-day (lower panel) denervated mice were analysed for MyHC content. Individual fibers in serial cryosections reacted with each anti-MyHC antibody (A4.840, BA-D5, BA-F8, A4.951, A4.74) were identified and scored for MyHC from photomicrographs. Results are presented as bar charts with the upper row representing the proportion of fibers that express all four slow MyHC epitopes ($\pm$ fast, dark grey), some subset of slow epitopes ($\pm$ fast, light grey) or no slow MyHC (white). The lower row represents fibers that express fast IIA MyHC only (dark cross-hatching), fast IIA MyHC in combination with any slow epitope (light cross-hatching) or no fast IIA MyHC (white). The black box represents the proportion of total fibers that co-express slow and fast IIA MyHC. In each animal, 90-300 fibers were scored per muscle (124 ± 49 , mean $\pm$ s.d.). Controls, which consisted of one wild type muscle, the contralateral muscles from the four denervated animals and a single soleus muscle from each of two mice from the transgenic control line (PMP22 C16, that had a single mutant copy of the transgene that did not express), showed no significant differences. * $p < 0.01$ compared to control.

Figure 3. Cross-reinnervation of rat soleus muscle causes graded changes in slow MyHCs
 MyHC epitopes were detected immunohistochemically with A4.840 (general slow; A,B), A4.951 (adult slow; C,D), N3.36 (all fast; E,F), A4.74 (fast IIA; G,H) and N2.261 (slow and fast IIA; I,J) in cryosections of contralateral control (A,C,E,G,I) and cross-reinnervated (B,D,F,H,J) soleus muscles. Control muscle contained a majority of slow type I fibers ($A4.951^+A4.840^+N2.261^+N3.36^-A4.74^-$; arrows) and a minority of fast type IIA fibers ($A4.951^-A4.840^-N2.261^-N3.36^+A4.74^+$; arrowheads). The proportions of these two fiber types was reduced after cross-reinnervation, and three new fiber types became abundant: a fiber containing fast IIA and a non-adult slow MyHC ($A4.951^-A4.840^+N2.261^+N3.36^+A4.74^+$; arrow i), a mixed fiber containing general-type slow and a fast MyHC but neither maturational slow, adult slow nor fast IIA ($A4.951^-A4.840^+N2.261^-N3.36^+A4.74^-$; arrow ii) and a fast fiber containing only non-type IIA fast MyHC ($A4.951^-A4.840^-N2.261^-N3.36^+A4.74^-$; arrow iii).

Figure 4. Demyelination-induced activity reduction causes differential transitions in individual slow MyHCs

A-J. Slow soleus muscles of transgenic mice containing seven additional copies of the *PMP22* gene (PMP22 C22, A-E) or control mice carrying a single non-functional transgene on similar genetic background (PMP22 C16, F-J) were serially cryo-sectioned and reacted with four slow MyHC-specific and one fast IIA MyHC-specific antibodies. In control C16 mice, fibers were generally of two classes: large slow fibers in which all four slow MyHC epitopes were detected without IIA MyHC (arrow iv), and slightly smaller fast IIA fibers that contain only fast IIA MyHC (arrow v). In contrast, the transgenic mice had heterogeneous fiber size. Normal-sized fibers fell mainly into two distinct classes similar to those in wild type animals. However, some normal-sized fibers contained general slow and fast IIA MyHC with little or no maturational or adult slow (arrow i). The small, atrophied fibers in transgenic animals frequently contained general slow MyHC and fast IIA MyHC with little or no maturational slow MyHC (arrow ii), whereas intermediate-sized fibers showed a variety of combinations of general slow and fast IIA MyHCs without adult slow MyHC (e.g. arrow iii).

Figure 5. Chronic activity reduction induces fast MyHC in *PMP22*-transgenic mice

Muscle fibers from soleus of two C22 transgenic mice aged 4 and 5 months (PMP22 C22 7 copies), two C61 transgenic mice aged 4 months (PMP22 C61 4 copies) and seven control mice of similar age (Control) were analysed for MyHC content as in Fig. 4. Fibers in the C22 mice were also divided into two pools by visual inspection, based on their cross-sectional area within the transverse section, and each pool is shown separately. Approximately half of all fibers showed significant reduction in size (PMP22 C22 small fibers) compared to the normal range of areas in control animals of the same age and sex. The remainder were classified as being within the normal range (PMP22 C22 normal fibers). Note that the small fibers of the two C22 animals had a similar MyHC profile (low s.d.), whereas the larger fibers in the older animal showed a more dramatic change than those in the younger animals (large s.d.). * $p < 0.01$, † $p < 0.05$.

Table 1

Summary of antigenic characteristics of murine slow MyHC isoforms/epitopes

	Monoclonal Antibody					
	A4.840	BA-D5	BA-F8	N2.261	A4.951	A4.74
General slow	+	+	-	-	-	-
Maturation slow	+	+	+	+	-	-
Adult slow	+	+	?+	?+	+	-
Fast IIA	-	-	-	+	-	+

Data from ^{34,44}, ?+ indicates that reactivity of this protein form with the antibody is likely, based on the relative intensities of bands on Western blots or immunohistochemistry, but formal proof of cross-reaction is lacking. No conclusions in the current paper depend on whether or not these reactions actually occur. Reactivity with rat is the same, although BA-F8 has not been tested (ref. ³⁴ and data not shown).