The Journal of Physiology

Compensatory axon sprouting for very slow axonal die-back in a transgenic model of spinal muscular atrophy type III

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Key points

- $Smn^{+/-}$ transgenic mouse is a model of the mildest form of spinal muscular atrophy.
- Although there is a loss of spinal motoneurons in 11-month-old animals, muscular force is maintained.
- This maintained muscular force is mediated by reinnervation of the denervated fibres by surviving motoneurons.
- The spinal motoneurons in these animals do not show an increased susceptibility to death after nerve injury and they retain their regenerative capacity.
- We conclude that the hypothesized immaturity of the neuromuscular system in this model cannot explain the loss of motoneurons by systematic die-back.

Abstract Spinal muscular atrophy (SMA) is a common autosomal recessive disorder in humans and is the leading genetic cause of infantile death. Patients lack the *SMN1* gene with the severity of the disease depending on the number of copies of the highly homologous *SMN2* gene. Although motoneuron death in the $Smn^{+/-}$ transgenic mouse model of the mildest form of SMA, SMA type III, has been reported, we have used retrograde tracing of sciatic and femoral motoneurons in the hindlimb with recording of muscle and motor unit isometric forces to count the number of motoneurons with intact neuromuscular connections. Thereby, we investigated whether incomplete maturation of the neuromuscular system induced by survival motoneuron protein (SMN) defects is responsible for die-back of axons relative to survival of motoneurons. First, a reduction of ~30% of backlabelled motoneurons began relatively late, at 11 months of age, with a significant loss of 19% at 7 months. Motor axon die-back was affirmed by motor unit number estimation. Loss of functional motor units was fully compensated by axonal sprouting to retain normal contractile force in four hindlimb muscles (three fast-twitch and one slow-twitch) innervated by branches of the sciatic nerve. Second, our evaluation of whether axotomy of motoneurons in the adult $Smn^{+/-}$ transgenic mouse increases their susceptibility to cell death demonstrated that all the motoneurons survived and they sustained their capacity to regenerate their nerve fibres. It is concluded the systematic die-back of motoneurons that innervate both fast- and slow-twitch muscle fibres is not related to immaturity of the neuromuscular system in SMA.

Abbreviations ALS, amyotrophic lateral sclerosis; CP, common peroneal; EDL, extensor digitorum longus; FG, fluorogold; FR, fluororuby dextran tetramethylrhodamine; ITS-MUNE, incremental twitch subtraction motor unit number estimation; MG, medial gastrocnemius; MUNE, motor unit number estimation; SMA, spinal muscular atrophy; SMN protein, survival motoneuron protein; *SMN1*, survival motoneuron gene 1; SOL, soleus; TA, tibialis anterior; WT, wild type.

Introduction

Spinal muscular atrophy (SMA) is the second most common autosomal recessive inherited disease in humans and is caused by mutations of the survival motoneuron gene (*SMN1*) (Lefebvre *et al.* 1995). *SMN1* encodes for the ubiquitously expressed SMN protein. The protein is involved in the assembly of ribonucleoproteins that regulate pre-mRNA splicing and translation for protein synthesis and may regulate the expression/trafficking of a number of mRNAs that are locally translated in axons (Monani *et al.* 1999; Zhang *et al.* 2008; Liu-Yesucevitz *et al.* 2011; Fallini *et al.* 2012; *Kye et al.* 2014). It is duplicated alongside a highly homologous copy, *SMN2*, which may act as the phenotypic modifier of the disease but produces insufficient levels of SMN protein for normal motoneuron function and survival (Melki *et al.* 1994; Lefebvre *et al.* 1995; Burghes & Beattie, 2009). Based on age of onset and severity of the disease phenotype, SMA is classified into three types (Munsat & Davies, 1992). The most severe type I SMA (Wernig-Hoffmann) is characterized by generalized weakness and hypotonia shortly after birth, with death usually occurring within 6 months. Type II is the intermediate form; affected children are able to sit but they cannot stand unaided and survive for more than 2 years. Type III is the relatively mild form where patients begin to develop muscle weakness after 18 months of age but survive into the adult years (Roberts *et al.* 1970; Pearn, 1978).

SMA is a neuromuscular disorder which affects all components of the motor unit, the motoneuron and the muscle fibres that it supplies, but the most prominent feature is the loss of spinal motoneurons resulting in progressive weakness and muscle atrophy (Crawford & Pardo, 1996). Pathology includes visible loss of nerves in ventral roots whereas the brain and spinal cord appear normal, and muscles are small and pale (Batten, 1911). Chromatolytic changes in motoneurons that are typically seen after cutting the axons (axotomy) in several species, including *Aplysia*, rats and cats (Lieberman, 1971; Gordon, 1983; Berdan *et al.* 1990), occur in conjunction with progressive degenerative changes in the peripheral nerves. The latter include loss of large myelinated fibres (Chou & Nonaka, 1978; Crawford & Pardo, 1996), and reduced calibre of spinal nerve roots (Simic *et al.* 2000). Active apoptotic death in motoneurons within fetal anterior horn sections was demonstrated by Soler-Botija *et al.* (2002) who suggested that the cause of the selective neuro-muscular degeneration in SMA may be over-activation of

naturally occurring apoptotic 'pruning' processes during development. Nonetheless, it is clear that in all three types of human SMA, motoneuron death occurs *after* neuromuscular connections are made (Soler-Botija *et al.* 2002), indicating that the SMN deficiency negatively impacts these connections. Normally these connections are essential for the survival of immature motoneurons with extensive evidence of their demise by apoptosis in neonatal rats when the neuromuscular connections are disrupted in the first week of life but not thereafter (Lowrie *et al.* 1982; 1987; Greensmith & Vrbová, 1996; de Bilbao & Dubois-Dauphin, 1996; Rossiter *et al.* 1996; Kemp *et al.* 2015). Possibly, the SMA motoneurons remain immature in the sense that they are susceptible to cell death after separation from their target muscles: once the 'pruning' of excess contacts at the neuromuscular junction occurs, the 'immature' SMA motoneurons may lose sufficient contact with their muscle fibres for each nerve fibre to retract completely from contact with muscle fibres, undergoing axonal die-back (Greensmith & Vrbová, 1997, Hausmanowa-Petrusewicz & Vrbová, 2005). In light of reported defects in motor axon growth (Rossoll *et al.* 2003; McWhorter *et al.* 2008), these motoneurons may fail to regenerate effectively and the susceptible motoneurons will die. Evidence that restoring SMN protein to SMA animal models is therapeutic and, importantly, that it is most effective during a relatively brief window in time early in the disease (Le *et al.* 2011; Lutz *et al.* 2011) was recently substantiated by findings that SMN is critical for the maturation of the neuromuscular junction during postnatal life (Kariya *et al.* 2014).

Presently, data are limited to findings of reduced numbers of surviving motoneurons in the lumbosacral cord of transgenic SMA mice at one or more time points in the lifespan of the mice (Jablonka *et al.* 2000; Monani *et al.* 2000, 2003; Balabanian *et al.* 2007). In order to determine if motor nerves progressively die back prior to death of the motoneurons in the ventral horn of the spinal cord, it is necessary to enumerate motoneurons that have functional connections and to establish the time course of the loss of functional motor units. It is also essential to document die-back of motor axons by backlabelling motoneurons of the lumbosacral cord through intact axons located within the peripheral nerves that innervate hindlimb muscles. Thus the first objective of the present study was to use the backlabelling technique of applying a fluorescent dye to a hindlimb nerve to establish the time course of motor unit and motoneuron loss in the C57B6*Smn*$^{+/-}$ SMAIII transgenic mouse and to determine whether there is a

temporal progression from disconnection of functional neuromuscular junctions to death of the motoneurons.

Normally, mature adult motoneurons, unlike immature neonatal motoneurons, survive the injuries that disrupt their neuromuscular contacts, namely axotomy (Xu *et al.* 2010). They undergo a change in gene expression from a chemically transmitting to a growth state that, in turn, supports the outgrowth and regeneration of their lost axons (Fu & Gordon, 1997). If indeed, SMN deficiency sustains a susceptibility of the motoneurons to cell death, death of at least some of the neurons might be expected when their neuromuscular contacts are disrupted. A second objective of this study was to determine whether the adult motoneurons in the C57B6$Smn^{+/-}$ SMAIII transgenic mouse model of SMA display an abnormal susceptibility to cell death following axotomy.

Data from transgenic mice in which the *Smn* gene was deleted (SMAII) indicate persistent muscle immaturity as evidenced by the loss of motoneurons, enduring muscle weakness, deficient axonal growth, and the continued expression of a neonatal muscle fibre phenotype (Frugier *et al.* 2000; Cifuentes-Diaz *et al.* 2001; Fan & Simard, 2002; Arnold *et al.* 2004; Biondi *et al.* 2008). SMAII transgenic mice also display immature features in motor nerve terminals at the neuromuscular junction (Frugier *et al.* 2000; Cifuentes-Diaz *et al.* 2001; Fan & Simard, 2002; Arnold *et al.* 2004; Biondi *et al.* 2008) that pre-cede motoneuron death (Frugier *et al.* 2000; Monani *et al.* 2000; Soler-Botija *et al.* 2002). A particularly notable feature is the impaired clustering of acetylcholine receptors revealing a persistent developmental interaction of peripheral motor nerves with their target muscle fibres (Grondard *et al.* 2005; Biondi *et al.* 2008; Kariya *et al.* 2008; Murray *et al.* 2008). Thus the third and final objective of the present study was to investigate axonal sprouting at motor nerve terminals to determine whether axonal sprouting occurred in the muscles of the C57B6$Smn^{+/-}$ SMAIII transgenic mice concurrent with loss of motoneurons.

Methods

Ethical approval

All surgical procedures and perioperative care measures were performed with strict accordance with the National Institutes of Health guidelines, The Canadian Council on Animal Care (CCAC) and were approved by the University of Alberta Health Sciences Animal Welfare and Policy Committee.

Animals

Breeding pairs of C57BL/6-$Smn^{+/-}$ transgenic mice (Jablonka *et al.* 2000) and C57BL/6-wild type (WT) mice were obtained from Jackson Laboratories (Bar Harbor,

ME, USA) and housed in the Health Sciences Laboratory Animal Services (HSLAS) at the University of Alberta. Animals were housed in standard conditions, in soft bedding and artificial 12 h dark/light cycles, with food and water available *ad libitum.*

A total of 71 C57BL/6-$Smn^{+/-}$ transgenic and 69 C57BL/6-WT mice were used in the experiments. The mice were genotyped by PCR amplification of DNA extracted from ear biopsies according to protocols specified by Jackson Laboratories.

Anaesthetic protocols, monitoring and terminal procedures

All surgical procedures, application of retrograde dyes and *in vivo* recordings of muscle and motor unit isometric contractile force were made under deep anaesthesia and using a sterile surgical technique. The mice were anaesthetized with a ketamine/Atravet cocktail (100 mg ml^{-1} ketamine and 10 mg ml^{-1} acepromazine in sterile saline at a dose of 17.5 ml (kg body weight)$^{-1}$ by intraperitoneal injection). If a supplementary dose was needed, one-third of the dose was administered half an hour after the first dose. The depth of anaesthesia was determined by manually pinching the tail or the foot and the reflex response was monitored. The absence of a reflex had to be noted in order to proceed with the different procedures. After the surgery or the retrograde dye application, the wound was sutured and disinfected with iodine. Thereafter, the mice received a single dose of buprenorphine (0.05 mg (kg body weight)$^{-1}$, by subcutaneous injection) and they were kept in a warm environment until they recovered fully from the anaesthesia. All the mice that were subjected to nerve surgery were periodically examined for signs of distress or autotomy at the denervated hindlimb. No signs of distress or autotomy were detected in any of the mice during the over survival periods of up to 4 months.

In the terminal experiments mice were either anaesthetized for hindlimb muscle contractile force recordings (see below) or they were deeply anaesthetized and perfused with 50 ml saline followed by 100 ml 4% paraformaldehyde at pH 7.4 through the aorta. Following perfusion, the lumbosacral spinal cord was removed to count backlabelled femoral or sciatic motoneurons. After the experiments in which force recordings were made under deep surgical anaesthesia, the mice were killed by cervical dislocation.

Confirmation of compliance

All the authors state that they understand the ethical principles under which *The Journal of Physiology* operates and their work complies with the animal ethics procedures outlined in Grundy (2015).

Backlabelling of motoneurons

Femoral and sciatic motoneuron pools. In 4-month-old C57BL/6-$Smn^{+/-}$ ($n = 6$) and C57BL/6-WT ($n = 5$) mice, the right sciatic nerve was exposed and crushed at the mid-thigh. The left femoral nerve was exposed and crushed in the ventral part of the thigh. Five per cent fluororuby dextran tetramethylrhodamine (FR) and 4% fluorogold (FG; hydrostilbamidine bis(methane-sulphonate); Sigma-Aldrich no. 39286, St Louis. MO, USA) in distilled water (Molecular Probes, Eugene, OR, USA) were used to backlabel the motoneurons in the lumbosacral spinal cord, each of the two dyes identifying the motoneuron pool of the two nerves. About 100 μl of the dyes was injected at the crush site using a micropipette attached to a picospritzer (Intercel Picospritzer III, Parker Hennifin, Cleveland, OH, USA) as described previously (Brushart *et al.* 2002). The two dyes have been shown to yield similar counts of motoneurons (Al-Majed *et al.* 2000; Boyd & Gordon, 2001) but they were alternated between contralateral nerves within animals to avoid variability in the motoneuron counts. The data obtained are shown in Fig. 3.

Four-, 5-, 7- and 12-month-old C57BL/6-$Smn^{+/-}$ ($n = 30$) and C57BL/6-WT ($n = 30$) mice were anaesthetized for exposure and crush of the sciatic nerve in order to backlabel and count the sciatic motoneurons that survive as a function of age. Right sciatic nerves were transected at mid-thigh level just above the division of the sciatic into tibial and common peroneal (CP) nerves. The tip of the proximal sciatic nerve stump was dipped for 1 h in a solution of FG or FR that was contained in a Vaseline well (Al-Majed *et al.* 2000; Boyd & Gordon, 2001). The exposure time was 1 h, after which the well was removed and the nerve stump washed with saline. The data that were obtained are plotted in Fig. 2.

Sciatic motoneurons after axotomy. To evaluate the survival of sciatic motoneurons after 2 and 4 months of prolonged axotomy, the sciatic nerve in the right hindlimb of $Smn^{+/-}$ SMA ($n = 8$ and 8, respectively) and WT ($n = 7$ and 8, respectively) 4-month-old mice was transected and axon regeneration was prevented by suturing the proximal nerve stump into the adjacent innervated muscles (Fu & Gordon, 1995). Two and 4 months later, the intact sciatic nerve of the left side was cut at the mid-thigh level and the proximal nerve stump dipped for 1 h in a solution of FG or FR, contained in a Vaseline well. In the right operated hindlimb, either dye, depending on the dye applied on the left sciatic nerve, was applied to the right sciatic nerve proximal to the axotomy site. The dyes were alternated on the left and right sides in order to distinguish the neurons in the ventral horns of the spinal cord. The data obtained in these experiments are plotted in Fig. 8.

Sciatic motoneurons after transection and surgical coaptation (resuture). The capacity for axon regeneration was evaluated in the right hindlimbs of $Smn^{+/-}$ SMA ($n = 8$) and WT ($n = 7$) 4-month-old mice. The right sciatic nerve was exposed at the mid-thigh, transected (40 mm proximal to the tip of the third digit) and surgically coapted by direct suture of the proximal and distal nerve stumps with two 9-0 nylon sutures (Ethicon Inc., Somerville, NJ, USA). The wound was sutured closed and the mice allowed to recover from anaesthesia. Axonal regeneration was allowed for 3 weeks and afterwards the right CP nerve was cut prior to its entrance to the tibialis anterior (TA) muscle, distal to the CP nerve repair site. The tip of the proximal stump was dipped for 1 h in the 5% FR solution contained in a Vaseline well. The left CP nerve was also exposed for simultaneous backlabelling of the unoperated CP motoneurons. As described above, the dyes that were used, either FG or FR, were applied so that the neurons were backlabelled on left and right sides with separate dyes for identification. One week later, animals were killed and their spinal cords removed to visualize and count the motoneurons on both sides of the cord. The data are plotted in Fig. 9.

Motoneuron counts

Seven days after the application of fluorescent dyes to backlabel motoneuronal cell bodies, the mice were deeply anaesthetized for perfusion via the aorta with 50 ml saline followed by 100 ml 4% paraformaldehyde at pH 7.4. After the perfusion, the spinal cord was dissected and the segment T11–L2 (containing the femoral, the sciatic and the common peroneal nerve motoneuron pools) was removed and post-fixed with 30% sucrose in 4% paraformaldehyde solution overnight. The tissue was then frozen in liquid nitrogen after being embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA, USA).

Frozen samples were sectioned in a cryostat (Jung CM3000, Leica, Wetzlar, Germany). Longitudinal spinal cord sections were cut at 40 μm thickness. The fluorescent bodies of the retrogradely labelled motoneurons of the different nerve pools were visualized and counted at $\times 40$ under fluorescence at barrier filters of 580 nm for FR and an ultraviolet filter for FG. The number of motoneurons counted was corrected according to the thickness of the sections and the diameter of the bodies by the method of Abercrombie and Johnson (Abercrombie & Johnson, 1946).

Isometric contractile force recording

To evaluate the number of adult sciatic motoneurons that sustained their connections with skeletal muscles, the right hindlimb of 7-month-old ($n = 6$) and 11-month-old

($n = 6$) $Smn^{+/-}$ SMA transgenic mice and 7-month-old ($n = 4$) and 11-month-old ($n = 4$) WT mice were dissected under deep anaesthesia for *in vivo* isometric contractile force recording from each of four hindlimb muscles: the medial gastrocnemius (MG), soleus (SOL), extensor digitorum longus (EDL) and tibialis anterior (TA) muscles (Fig. 1*A*). All other hindlimb muscles were denervated by cutting their nerve supplies.

Each of the muscles was attached in random order to a force transducer for recording isometric contractile forces. Evoked isometric forces were amplified and visualized on an oscilloscope and digitized using Axoscope (Version 8.0, Axon Instruments, Union City, CA, USA). Muscle length was adjusted for maximal isometric twitch force in response to stimulation of the sciatic nerve. Whole muscle twitch and tetanic forces were recorded in response to single and repetitive suprathreshold (twice threshold voltage at 100 ms duration) stimulation of the sciatic nerve at 0.5 and 100 Hz, respectively. The tetanic contractions lasted for 210 ms and the maximum tetanic force was measured. An example of muscle and motor unit force recordings from a 60 day control mouse TA muscle is shown in Fig. 1*B*. To estimate the number of motor units in the hindlimb muscles of the mice, we used a modified version of the mechanical motor unit number estimation (MUNE) that was first described by McComas *et al.* (1971) for EMG measurements of

single motor units. The validity of this new method, the incremental twitch subtraction motor unit number estimation (ITS-MUNE) method of counting motor units, was described previously from our laboratory (Major *et al.* 2007). In this study, we used the ITS-MUNE method to overcome potential problems of alternation, namely that motor units can be recruited in numerous combinations by stimulating a motor nerve (Galea *et al.* 1991), which, in turn, may result in overestimation of the numbers of motor units (Stein & Yang, 1990). An interactive software program was used that randomly selects from a set of stimulus-evoked, incremental muscle twitch force waveforms (Major *et al.* 2007). The basic principle that underlies all MUNE techniques is the division of the total muscle response by the estimate of the average motor unit response. The difference between the different MUNE techniques is the method used to estimate the average single motor unit response. With the ITS-MUNE method, 8–20 isometric twitch responses were selected randomly from ~100 incremental force waveforms. The waveforms were recorded from each of the four muscles in response to a manually controlled incremental rise in the stimulus voltage of 100 μs duration to the sciatic nerve from 0 to 10 V at a frequency of 0.5 Hz until the recorded twitch force was ~66% of the whole muscle twitch force. This resulted in incremental increases in muscle twitch force that have been shown to reflect a random
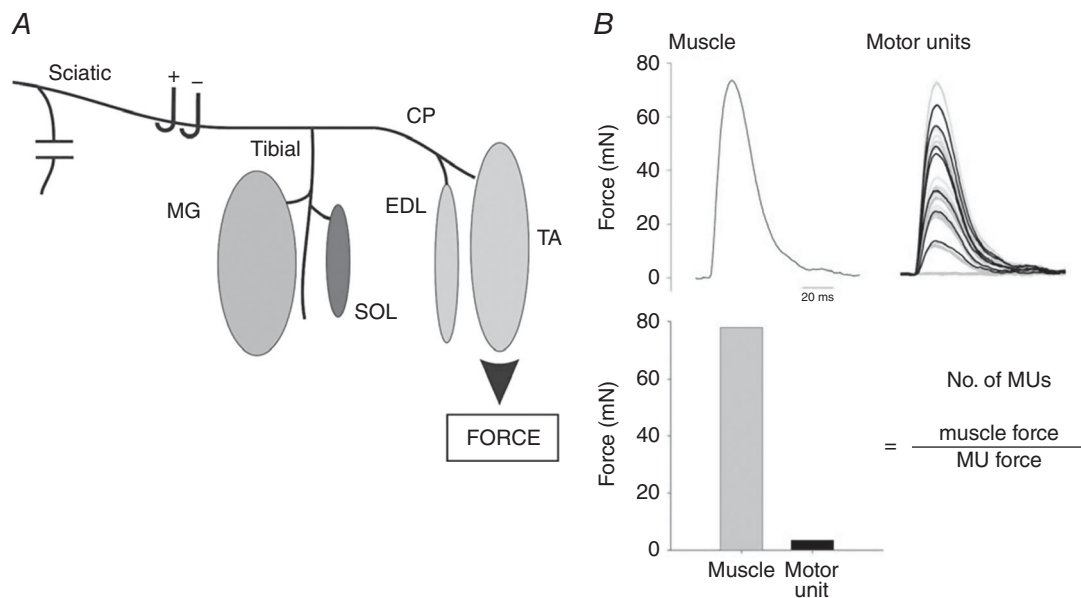

Figure 1. *In vivo* isolation of the sciatic nerve and fast-twitch medial gastrocnemius (MG), extensor digitorum longus (EDL) and tibialis anterior (TA) muscles and the slow-twitch soleus (SOL) muscle for isometric contractile force recording
A, all nerves except the sciatic and its tibial and CP nerve branches to the MG and SOL muscles and to the EDL and SOL muscles were denervated. Each of the muscles was attached to a force transducer for the recording of maximal muscle twitch contractile force and, in response to all-or-none increments in stimulus intensity on the sciatic nerve, the progressive recruitment of single motor units (*B*). The number of motor units (MUs) was obtained from the ratio of muscle and MU forces as described in the text.

distribution of axon thresholds (Major *et al.* 2007). The custom computer program – a graphical user interface based in MATLAB called ITS-MUNE – filtered and aligned incremental twitch force traces, randomly selected twitch responses, rejected non-physiological motor unit twitches with regions of negative force, subtracted two rank-ordered force traces to generate a candidate motor unit twitch response, and finally averaged motor unit twitch samples to estimate mean twitch force and to calculate ITS-MUNE (Major *et al.* 2007) (Fig. 1*B*). The data are plotted in Figs 4 and 6.

After completion of the muscle force and motor unit force recordings, the muscles were excised, blotted dry, and weighed. The data are plotted in Fig. 5.

Combined silver/cholinesterase histochemical staining

Under deep anaesthesia, 11-month-old $Smn^{+/-}$ SMA transgenic mice ($n = 6$) and WT mice ($n = 4$) were perfused with 50 ml saline followed by 100 ml 4% formalin at pH 7.4 through the aorta. The TA muscles from both hindlimbs were dissected, cryoprotected by subsequent overnight incubation in gum sucrose solution and then frozen in isopentane at $-74°C$. Cryostat longitudinal sections at 100 μm were cut and stained using combined silver/cholinesterase (Ag/AChE) histochemical staining to visualize the motor axons, sprouts and the muscle endplates. For AChE staining, 100 μm cryostat longitudinal sections were collected in distilled water and incubated for 25–30 min at room temperature in a mixture of 0.01 M Tris-HCl buffer, pH 7.2, bromoindoxyl acetate, 1.65% potassium ferricyanide, and 2.11% calcium chloride. For silver staining, sections from AChE staining were first incubated in 20% silver nitrate for 15 min, washed in distilled water, incubated in 3% sodium sulfite for 10 min and washed again, and finally, developed in a mixture of silver nitrate and physical developer. The number of axon sprouts (having visible axonal attachment to muscle endplates) and free endplates (having no visible axonal attachment) were subsequently counted from the longitudinal muscle sections. A sample of 500 endplates from the middle portion of each TA muscle was examined under light microscopy at a total magnification of $\times 160$ or $\times 400$ and the axonal sprouts categorized as intranodal, terminal and ultraterminal sprouts with intranodal sprouts originating from the last node of Ranvier (Fig. 7*A*), terminal sprouts originating from the axons after the last node of Ranvier (Fig. 7*B*), and the ultraterminal sprouts originating from the endplate area and contacting another endplate (Fig. 7*C*). The quantity of endplates that were reinnervated by axonal sprouts and those that were not contacted by an axon (free endplates) was determined and expressed as a percentage of all the endplates counted. The motor axons were stained brown and the motor

endplates were revealed by greenish-blue staining of the AChE enzyme at the synaptic lamina. Unlike acetylcholine receptors, AChE remains indefinitely after denervation.

Statistics

Data are reported as mean $\pm$ the standard error of the mean (SEM). Group means were compared using an independent samples Student's t test. Differences were considered significant at $P < 0.05$. P values are reported.

Results

Survival of motoneurons in $Smn^{+/-}$ transgenic mice

The numbers of labelled sciatic motoneurons in the ventral horn of the lumbosacral (L4–L5) spinal cord of the $Smn^{+/-}$ transgenic mouse model of SMAIII did not show any decline until 7 months when a significant reduction of 19% was detected between 5 and 7 months ($P < 0.05$), continuing to a 35% reduction ($P < 0.001$) at 12 months of age (Fig. 2). When direct comparisons are made at each age between the C57BL/6-wild type transgenic mice and the corresponding $Smn^{+/-}$ type III transgenic mice, it becomes clear that the number of lumbosacral motoneurons with intact axons in the sciatic nerve declined significantly only in the 12-month-old mice. The numbers of motoneurons in the wild type mice did not change with age.

Counts of the number of femoral motoneurons in 4-month-old $Smn^{+/-}$ and wild type control transgenic
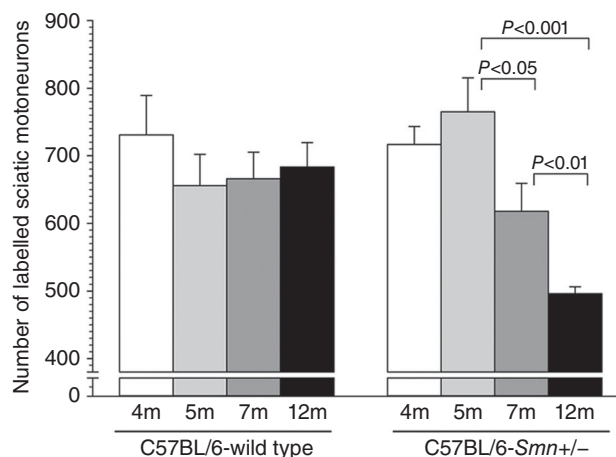

Figure 2. Number of sciatic motoneurons in $Smn^{+/-}$ transgenic mice as compared to wild type mice at 4, 5, 7 and 12 months (m) of age
Each bar represents the mean $\pm$ SEM of data obtained from 6–8 mice. The numbers of motoneurons was counted after application of either fluorogold or rubyred to the sciatic nerve mid-thigh. Note that the number of motoneurons in the $Smn^{+/-}$ transgenic mice began to decline significantly by 7 months.

mice were in agreement with the data for the sciatic motoneurons with there being no detectable difference between the smaller number of femoral motoneurons innervating more proximal muscles in the $Smn^{+/-}$ transgenic and wild type mice as was observed for the sciatic motoneurons at the same age (Fig. 3). That the numbers of surviving sciatic motoneurons at 4 months of age was the same whether the motoneurons were backlabelled with retrograde dyes applied to the nerve at a crush site by a picosprizer (Fig. 3) or by dipping the cut proximal stump into a pool of the dye, verifies the validity of the two methods.

Reduced numbers of intact motor units in $Smn^{+/-}$ transgenic mice

To address whether the number of lumbosacral motoneurons that were counted by backlabelling via the sciatic nerve have intact neuromuscular connections or whether the motor axons die-back, we recorded maximal contractions of representative hindlimb muscles innervated by the sciatic nerve and counted the number of intact motor units using isometric force recording as described in the Methods. These recordings were made at 7 and 11 months of age, the ages when we had noted significant declines in motoneuron numbers
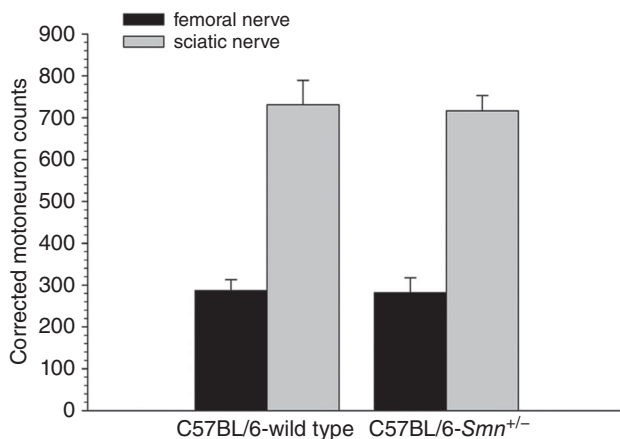

Figure 3. Number of femoral as compared to sciatic motoneurons in the lumbosacral (L2–L4) spinal cord in 4-month-old $Smn^{+/-}$ transgenic mice and wild type control mice

Motoneuron counts after backlabelling the femoral or sciatic nerves with either fluorogold or rubyred were completed on consecutive 40 μm thick longitudinal spinal cord sections between T11 and L2; motoneurons in all sections were counted. The data are corrected for thickness of the longitudinal sections. Each bar represents the mean ± SEM of data obtained from 5 (C57BL/6-wild type) and 6 (C57BL/6-$Smn^{+/-}$ transgenic) mice. Note that the number of motoneurons in the $Smn^{+/-}$ transgenic mouse is not different from the wild type control for either femoral ($P > 0.91$) or sciatic ($P > 0.83$) nerves at 4 months of age.

(Fig. 2). As shown in Fig. 4, the fast-twitch tibialis anterior (TA), medial gastrocnemius (MG) and extensor digitorum longus (EDL) and the slow-twitch soleus (SOL) hindlimb muscles in the $Smn^{+/-}$ transgenic mice developed the same maximal twitch and tetanic isometric forces at 7 and 11 months of age as the corresponding wild type control mice. The weights of the muscles for both the larger TA and MG muscles as well as the smaller EDL and SOL muscles were also the same, consistent with the sustained muscle forces (cf. Figs 4 and 5). Yet the average number of intact TA, MG and SOL motor units at both 7 and 11 months of age in the $Smn^{+/-}$ transgenic mice had declined by 30% ($P < 0.01$) compared with the corresponding muscles of the wild type control transgenic mice (Fig. 6). The findings that the maximal nerve stimulation resulted in the development of normal contractile forces indicated that the remaining intact motor units had enlarged by axon sprouting to reinnervate the muscle fibres denervated by the loss of axon connections.

Sprouting of axons in hindlimb skeletal muscles of $Smn^{+/-}$ transgenic mice

In order to evaluate sprouting directly, we visualized axons and the muscle endplates in the TA muscle of $Smn^{+/-}$ and wild type control transgenic mice. Nodal and terminal sprouting has been reported in the homozygous mouse model of SMA/III at 4 months of age when muscle fibres become denervated in association with a reduction of ∼30% in the numbers of viable motoneurons (Monani *et al.* 2003). In light of our findings of reductions of ∼19% ($P < 0.01$) in numbers of motoneurons with nerve fibres traversing the sciatic nerve between 7 and 12 months of age (Fig. 2), we visualized the nerves and their innervation of end-plates in longitudinal muscle sections with Ag/AChE histochemistry in 11-month-old mice. As described previously (Tam *et al.* 2001), ∼10% of endplates are free of silver stained axons as a result of the cryostat sectioning in both the $Smn^{+/-}$ and wild type control transgenic mice (Fig. 7D). In line with the findings of significant reductions in motoneuron numbers (Fig. 2), we observed sprouting in the muscles of the $Smn^{+/-}$ transgenic mice (Fig. 7). As shown in the typical examples of intramuscular nerves stained with silver chloride and their innervation of muscle endplates stained for cholinesterase in Fig. 7A–C, all types of sprouts – nodal, preterminal and ultraterminal – typical of partially denervated muscles (Tam *et al.* 2001) were seen in the muscles. The mean number of axon sprouts increased by 4-fold ($P < 0.01$), being sufficient to reinnervate all the denervated fibres in the partially denervated muscles (Fig. 7D) and for full reinnervation of the partially denervated muscles and recovery of their muscle contractile forces and muscle weights (Figs 5 and 6).

Motoneuron survival after axotomy in *Smn*^{+/−} transgenic mice

If mature motoneurons that make functional connections in SMA nevertheless fail to make the complete transition from a growth to a transmitting phenotype, we would expect that these motoneurons would be highly susceptible to cell death after axotomy, as has been demonstrated for immature motoneurons at postnatal ages of 1–5 days in rats (Lowrie *et al.* 1987). We cut the sciatic nerve in the right hindlimb of *Smn*^{+/−} SMA transgenic and wild-type control mice at 3 months of age (prior to any significant motoneuron death, see Fig. 3) and evaluated survival of axotomized sciatic motoneurons 2 and 4 months post-axotomy. We prevented nerve fibre regeneration in these experiments by suturing the proximal nerve stump into innervated muscle. We did not observe any difference between the *Smn*^{+/−} and wild type control mice in the survival of their motoneurons that were back-labelled from the sciatic nerve 2 months ($P > 0.60$) and 4 months ($P > 0.16$) post-axotomy (Fig. 8). The number of motoneurons in the lumbar spinal cord was the same whether the motor nerves were intact or sub-jected to prolonged axotomy. The slow age-dependent decline in motoneuron numbers (see Fig. 2) did not affect the survival after axotomy ($P > 0.83$), the percentage of motoneurons that survived the axotomy being ∼90% in both the *Smn*^{+/−} transgenic and the wild type control mice after both 2 and 4 months (Fig. 8).

Motoneuron capacity for nerve regeneration in *Smn*^{+/−} transgenic mice

We went on to address the possibility that a sustained 'growth' mode of motoneurons, in *Smn*^{+/−} mice, that were encouraged to regenerate their axons, may be sufficient to promote death of motoneurons. This possibility was again negated by our data. We found that the number of CP motoneurons that regenerated their axons was not reduced as compared to wild type controls 3 weeks after axotomy and resuture of the proximal and distal sciatic nerve stumps ($P > 0.47$) (Fig. 9). Hence the axotomized motoneurons that regenerated their axons survived as well as those axotomized motoneurons that were prevented from regenerating their axons. These findings indicate that the sustained 'growth' mode did not detrimentally affect the survival of the motoneurons in the SMAIII C57BL/6-*Smn*^{+/−} transgenic mice.
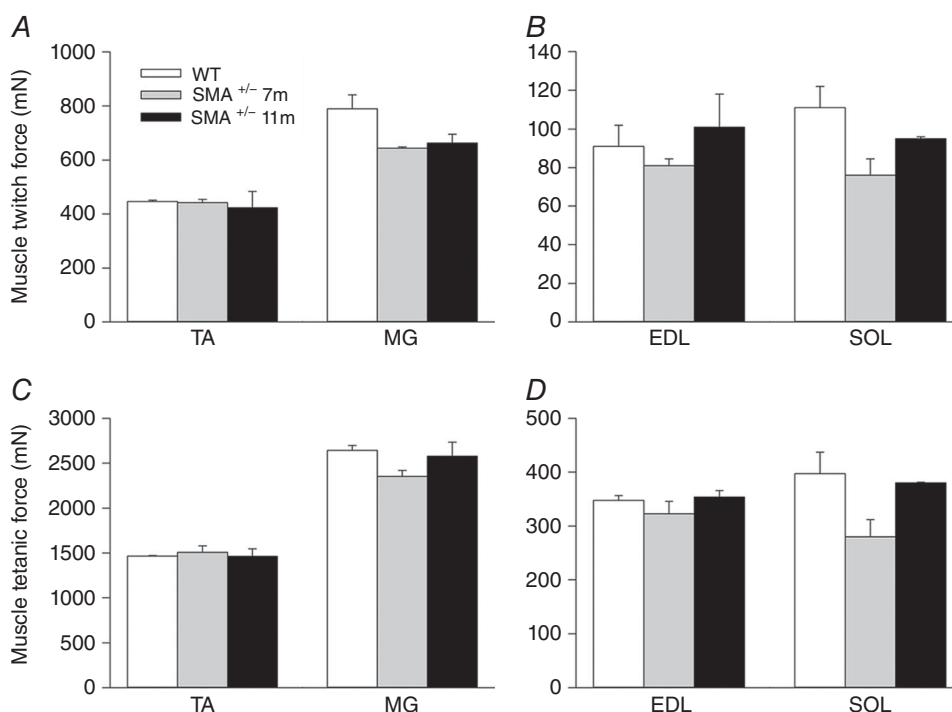

Figure 4. Mean (±SEM) isometric contractile forces developed by fast-twitch tibialis anterior (TA), medial gastrocnemius (MG) and extensor digitorum longus (EDL) muscles, and slow-twitch soleus (SOL) muscles
Maximal twitch forces in response to 2× threshold electrical stimulation of the sciatic nerve at 0.5 Hz. *A* and *B*, and, at 100 Hz, maximal tetanic forces (*C* and *D*) forces in C57BL/6-*Smn*^{+/−} transgenic and C57BL/6-wild type mice. The twitch forces of *Smn*^{+/−} transgenic mouse muscles were 89.6 ± 17.8% ($P > 0.16$) of those of the wild type controls; muscle tetanic forces of the *Smn*^{+/−} transgenic mice were 93.6 ± 3.3% ($P > 0.20$) of wild type controls.

Discussion

The major findings of the present study were threefold. (1) There were surprisingly small reductions in the number of motoneurons with peripheral axons that survived during 12 months in the $Smn^{+/-}$ SMAIII transgenic mouse. (2) The sprouting capacity of the remaining motor nerve fibres accounted for the maintained weights of both fast- and slow-twitch muscles and the capacity of the muscles to generate normal muscle contractile forces for up to 12 months in the normal lifespan of 28 months in the mice. (3) Motoneurons survived after axotomy in the adult mouse, demonstrating a normal capacity for nerve regeneration. These data indicate that, in the mild form of SMAIII disease, minimal motoneuron loss occurs in a die-back fashion. Moreover, the survival of all motoneurons after axotomy negates the hypothesis that incomplete maturation of the neuromuscular system induced by SMN defects is responsible for axon die-back and death of motoneurons.

Significant reductions in the numbers of surviving motoneurons were reported for the same mouse using a Kluver Barrera stain to identify the surviving motoneurons in cross-sections of the ventral horn of the lumbosacral spinal cord (L1–L6) (Balabanian et al. 2007). Importantly though, our counts of motoneurons are those motoneurons *with* nerve fibres that project as far as the sciatic nerve in the thigh. These counts of motoneurons with nerve fibres being substantially higher than the counts of Nissl- and Kluver Barrera-stained motoneurons suggest that the published reduced motoneuron counts probably overestimated the death of motoneurons in these type III SMA mouse models. Importantly, the die-back of motor nerve fibres shown by the small but significant decline in numbers of dye-labelled motoneurons at 7 and 12 months

of age in the motoneurons of $Smn^{+/-}$ SMAIII transgenic mice indicates that motoneuron death (Fig. 2), if it occurs at all in this strain of mice, is likely to be later than 7 months of age. Reductions in surviving motoneurons were reported as early as 5 weeks of age (Balabanian et al. 2007), but in the same mouse strain losses of ~40 and 54% were reported at 6 and 12 months, respectively (Jablonka et al. 2000). Whilst it could be argued that there is more severe loss of motoneurons that supply the more proximal musculature, our backlabelling of femoral motoneurons via the femoral nerve in the groin at 4 months of age did not reveal an early loss of these motoneurons (Fig. 3). Similarly, only a 23% reduction in facial motoneurons was described in these mice at 12 months (Jablonka et al. 2000).

Thus, reduction in SMN protein content in these mice does not impact severely on the survival of motoneurons. Hence the time course of motoneuron loss is clearly slow in this mouse, and considerably slower than in the $Smn^{-/-}$ type III transgenic mouse; the reduction in spinal motoneurons of 35% by 12 months in the $Smn^{+/-}$ type III mice (Fig. 2) compares with the equivalent reduction of ~29% reported already by 3.5 months of age in the $Smn^{-/-}$ type III mice (Monani et al. 2003). Indeed the rate of motoneuron loss correlates with the onset of disease symptoms, with symptoms being minor in the $Smn^{+/-}$ type III transgenic mouse in contrast to more severe symptoms of muscle weakness as early as 1 month of age in the $Smn^{-/-}$ type III transgenic mouse.

This mouse model of SMAIII with a normal lifespan (Jablonka et al. 2000) is consistent with the normal lifespan of patients with SMA type III (Lunn & Wang, 2008). However, the full reinnervation of hindlimb musculature in the mouse accounts for the findings of relatively normal
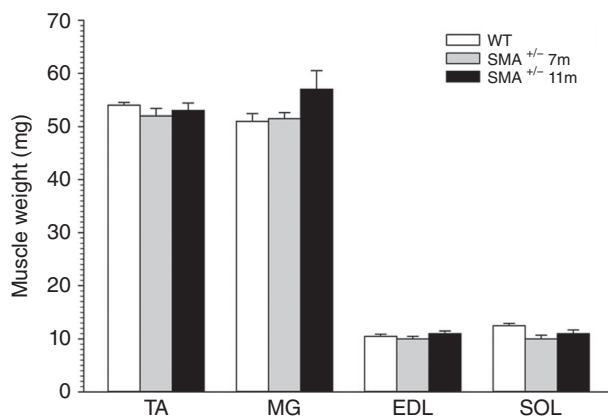

Figure 5. Mean ± SEM of the wet weights of fast-twitch TA, MG and EDL muscles and slow-twitch SOL muscles of C57BL/6-$Smn^{+/-}$ transgenic and C57BL/6-wild type mice
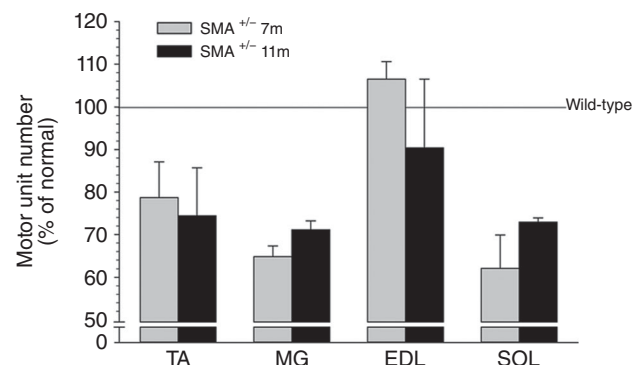Muscle weights of $Smn^{+/-}$ were 97 ± 2.7% (P > 0.44) of control wild type.

Figure 6. Mean ± SEM of the estimated numbers of intact motor units (MUNE) in hindlimb muscles of C57BL/6-$Smn^{+/-}$ transgenic mice expressed as a percentage of corresponding muscles in C57BL/6-wild type control mice
The mean number of motor units was 30% lower (P < 0.01) in TA, MG and SOL of the $Smn^{+/-}$ transgenic mice as compared with wild type control muscles.

movement in these mice in contrast to the patients, many of whom are wheelchair bound in and/or after teenage years (Lunn & Wang, 2008). The progressive weakness in the patients may be associated with continuing loss of functional motor units until the numbers decline below 15–20% of normal when the upper limit of fivefold increase in motor unit size by sprouting fails to reinnervate all the denervated muscle fibres, and muscle contractile force, in turn, declines (Gordon *et al.* 1993, 2004).

It is interesting that we did not observe any contractile force deficits in the partially denervated muscles at 7 and 11 months of age in the *Smn*$^{+/-}$ SMAIII transgenic mice relative to their wild type control muscles (Fig. 4). In addition, there was no observable muscle atrophy with muscle weights being the same in the *Smn*$^{+/-}$ SMAIII and the wild type control mice (Fig. 5). Impaired acetylcholine receptor clustering at the endplate region of the muscles in SMAII transgenic mice suggested that neuromuscular transmission might be compromised (Biondi *et al.* 2008). This was not the case in the *Smn*$^{+/-}$ transgenic mouse model of SMAIII where stimulation of the sciatic nerve effectively elicited twitch and tetanic contractions of the same force as the muscles from the corresponding

wild type control mice. This was so for both fast- and slow-twitch muscles (Fig. 4).

The ~30% reduction in numbers of motoneurons with intact neuromuscular contacts in representative hindlimb fast- and slow-twitch muscles (Fig. 6) was the same as for the surviving sciatic motoneurons that were enumerated after their backlabelling (Fig. 2), the equivalency attesting to the validity of the ITS-MUNE method of counting motor units in rodents (Major *et al.* 2007). Despite the motor axon die-back, the partially denervated muscles retained their normal contractile force capability in the *Smn*$^{+/-}$ III transgenic mouse (Fig. 4). Enlargement of motor units with increased motor unit contractile forces is the likely explanation with our histological evidence for substantial axon sprouting to reinnervate denervated muscle fibres (Fig. 7). This explanation is consistent with previous findings of significant sprouting in the triceps surae muscles of 4-month-old type III *Smn*$^{-/-}$ transgenic mice (Monani *et al.* 2003). In our study, the axonal sprouting was effective in reinnervating all denervated motor endplates because the muscle contractile forces were not reduced in line with reduced numbers of intact motor units, as in partially denervated rat and cat muscles
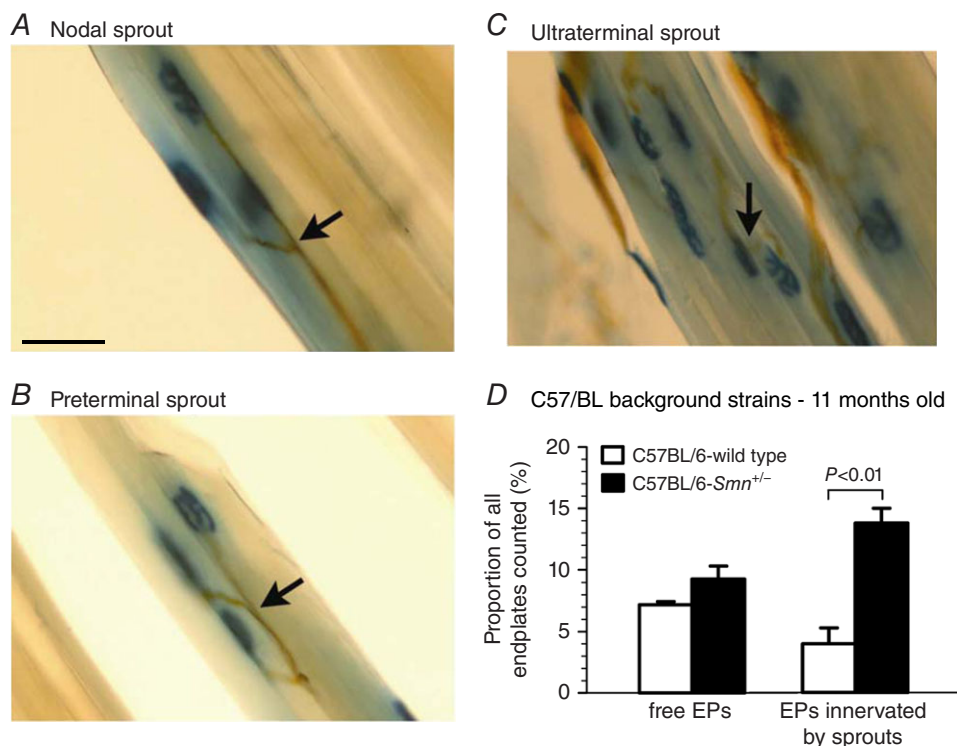

Figure 7. Photomicrographs of longitudinal sections of the TA muscle from an 11-month-old C57BL/6-*Smn*$^{+/-}$ transgenic mouse stained with the combined silver/cholinesterase stain to visualize axons and motor endplates (EPs), respectively
Examples of nodal (*A*), preterminal (*B*) and ultraterminal (*C*) sprouts observed in muscles are shown. *D*, mean (±SEM) proportions of endplates that were free of nerve endings (free EPs) or were innervated by axon sprouts (EPs innervated by sprouts) are plotted for C57BL/6-*Smn*$^{+/-}$ transgenic and C57BL/6-wild type control mice. Scale bar is 50 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

(Rafuse *et al.* 1992; Tam *et al.* 2001, Tam & Gordon, 2003). Hence, the sprouting effectively compensated for the loss of the innervation by a proportion of motoneurons in the $Smn^{+/-}$ III transgenic mice. It is likely that the sprouting resulted in some but not extensive fibre-type clumping in the partially denervated muscles because clumping becomes obvious only after extensive partial denervation (Gordon & Tyreman, 2010).

The capacity for sprouting in SMA differs fundamentally from findings in transgenic mouse models of amyotrophic lateral sclerosis (ALS). In the latter ALS model, the large motoneurons with intact neuromuscular connections fail to sprout and reinnervate the type IIB muscle fibres that are progressively denervated by selective die-back of their motor axons (Frey *et al.* 2000; Hegedus *et al.* 2007, 2008), in association with the expression of the inhibitory semaphorin 3A that is present at the endplates of these muscle fibres (Frey *et al.* 2000; Pun

et al. 2006; De Winter *et al.* 2006). The greater resilience of the small motoneurons and their sustained capacity to sprout and reinnervate denervated muscle fibres resulted in delayed susceptibility of these motoneurons to die-back (Hegedus *et al.* 2008). In association with this preferential die-back of axons from the type IIB fast glycolytic muscle fibres, a progressive conversion of the remaining reinnervated muscle fibres occurs probably associated with compensatory hyperactivity of the motor units (Hegedus *et al.* 2008). In the $Smn^{+/-}$ III transgenic mouse, where axon die-back occurs very slowly indeed and is not substantial, even at 12 months of age, there was no evidence of preferential die-back of axons from fast-twitch as opposed to slow-twitch muscles, the extent of motor unit loss being indistinguishable in the fast- and slow-twitch muscles (Fig. 6).

Whilst the basis for the motor axon die-back in both SMA and ALS is not understood, the gene defect in the
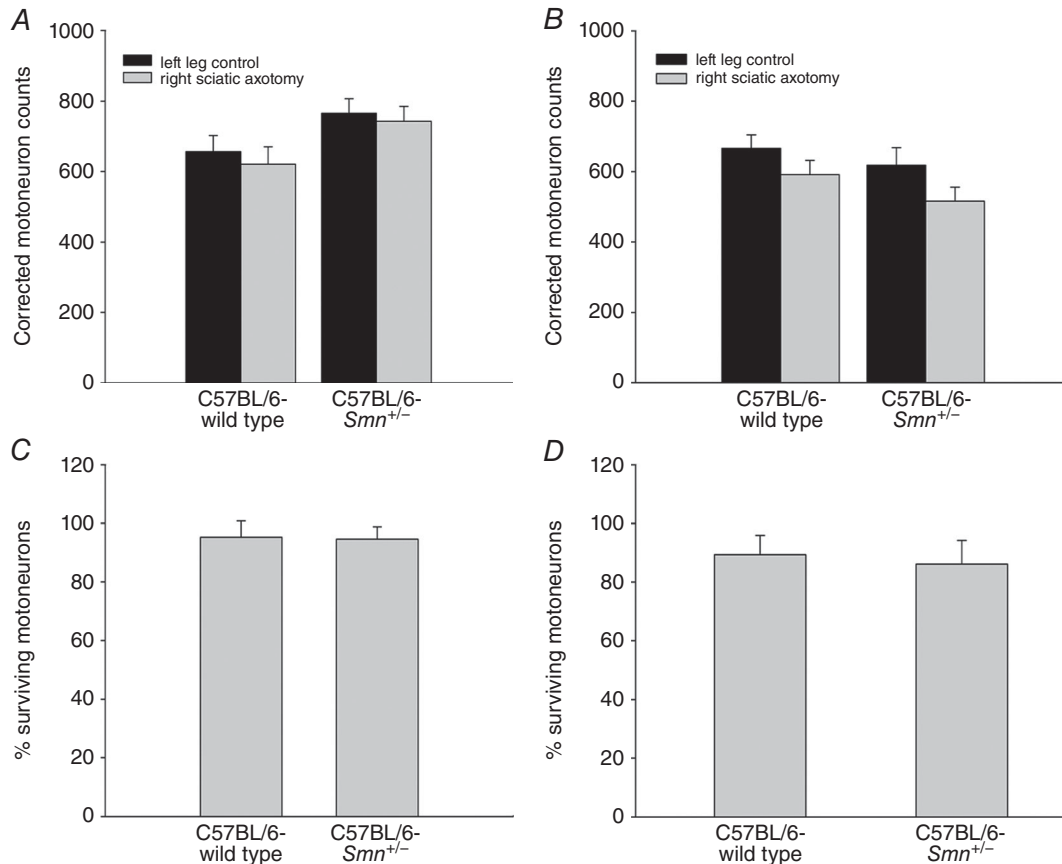

Figure 8. Mean (±SEM) numbers of motoneurons of C57BL/6-$Smn^{+/-}$ transgenic and C57BL/6-wild type control mice, back-labelled from the sciatic nerve 2 months (*A*) and 4 months (*B*) after left sciatic nerve section and suture of the proximal nerve stump to an innervated muscle to prevent regeneration
The corresponding proportions of surviving motoneurons after axotomy are shown in *C* and *D*. The left sciatic nerves served as intact internal controls. Two and 4 months after axotomy, the proximal stump of the sciatic nerve was cut and exposed to FR to back-label the motoneurons that survived the injury. Mice were 5 and 7 months old at the end of experiments in *A* and *B*. Motoneuron counts were completed on consecutive 40 μm thick longitudinal spinal cord sections between T11 and L2; all sections were counted. The data are corrected for thickness of longitudinal sections. ($n = 7$ for C57BL/6-wild type and $n = 8$ for C57BL/6-$Smn^{+/-}$ transgenic mice).

two motoneuron diseases is fundamentally different and the onset of the disease is early in life in SMA and later in life in ALS (Al Chalabi & Leigh, 2000; Nicole *et al.* 2002; Iannaccone *et al.* 2004; Monani, 2005; Russman, 2007). That motoneuron survival is prolonged in the mouse model of ALS by axotomy is intriguing with the capacity of axonal transport to sustain small rather than large axons being a possible explanation (Kong & Xu, 1999). In contrast, in SMA where axonal transport may also be compromised, we determined whether axotomy might increase the loss of motoneurons in the spinal cord. We hypothesized that, if indeed the motoneuron is in an immature state and thereby more susceptible to cell death, akin to the susceptibility of neonatal immature motoneurons in the rat being highly susceptible to cell death (Greensmith *et al.* 1996), we might observe increased loss of motoneurons after axotomy as has been observed with axotomy in neonatal rats at 1–3 days (Lowrie *et al.* 1987; de Bilbao & Dubois-Dauphin, 1996; Rossiter *et al.* 1996). The axon transection to axotomize sciatic motoneurons did *not* promote any motoneuron loss and hence our findings did not support the hypothesis (Fig. 8). We must conclude from these findings that there is a

very gradual loss of motoneurons that project their axons to the sciatic nerve, that the die-back of axons precedes motoneuron loss and that the immaturity of the neuro-muscular system is not, in of itself, an explanation for the progressive die-back in SMA. Moreover, the evidence that the axotomized common peroneal motoneurons regenerated their axons normally when regeneration was encouraged by sciatic nerve transection and surgical repair (Fig. 9), demonstrates the normal capacity of these SMA motoneurons to regenerate as well as to sprout under conditions of nerve injury and partial denervation, respectively.

It is important to note that in both SMA and ALS, there is evidence to support the possibility that increased neuromuscular activity may enhance the survival of intact motor units and/or the transgenic mice. In SMA where a deficiency in expression of the NR2A subunit of the NMDA receptor was reported in conjunction with defective clustering of acetylcholine receptors at the end-plate regions of skeletal muscle in SMA, exercise of the mice upregulated the gene for the subunit in conjunction with enhanced survival of the mice (Biondi *et al.* 2008). Similarly in the G93A transgenic mouse model of ALS, our findings demonstrated that all remaining intact motor units in the affected hindlimb muscles survived in conjunction with the compensatory hyperactivity of the remaining motor units (Gordon *et al.* 2010).
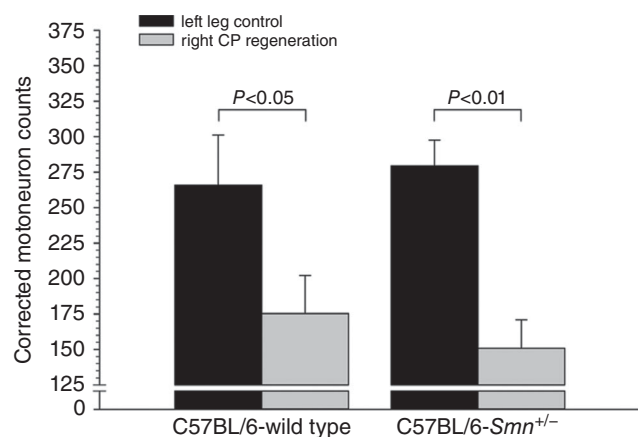

Figure 9. Numbers of backlabelled common peroneal (CP) motoneurons that regenerated their nerves after sciatic nerve transection and resuture in C57BL/6-*Smn*$^{+/-}$ transgenic and C57BL/6-wild type control mice

Left CP nerves served as intact internal controls. The right sciatic nerves of 4-month-old mice were cut and subsequently repaired (coapted) by direct suture of the proximal and distal stumps. Three weeks later, left control and the right axotomized CP motoneurons were backlabelled via the CP nerve at sites that were distal to the repair site of the right CP nerve. Motoneuron counts were completed on 20 μm thick longitudinal spinal cord sections between T11 and L2; every second section was counted through the entire spinal cord. Data are corrected for thickness of the longitudinal sections. Each bar represents the mean (±SEM) of data obtained from 8 C57BL/6-*Smn*$^{+/-}$ transgenic and 7 C57BL/6-wild type mice. The corrected motoneuron counts after right CP nerve regeneration were significantly different from counts from the left intact CP nerves but the counts were not different between the *Smn*$^{+/-}$ transgenic and the wild type mice.

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Additional information

Competing interests

None declared.

Author contributions

Experiments were performed in the laboratories of T.G. and C.T.P. E.U. and N.T. participated in the acquisition, analysis and interpretation of data and drafted and critically revised the work. L.R.H. and V.E.C. participated in the acquisition, analysis and interpretation of data and critically revised the work. T.G. and C.T.P. designed the work, participated in the analysis and interpretation of data, and drafted and critically revised the work. All authors approved the final version of the manuscript and ensure the accuracy and integrity of any part of the work presented, and all qualify for authorship. All those who qualify for authorship are listed.

Funding