S1P$_3$ receptor influences key physiological properties of fast-twitch extensor digitorum longus muscle

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Running head: S1P$_3$ receptor and muscle fatigue and atrophy

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To examine the role of sphingosine 1-phosphate (S1P) receptor 3 (S1P3) in modulating muscle properties, we utilized transgenic mice depleted of the receptor. Morphological analyses of extensor digitorum longus (EDL) muscle did not show evident differences between wild type and S1P3-null mice. The body weight of three-month old S1P3-null mice and the mean cross sectional area of transgenic EDL muscle fibers were similar to those of wild type. S1P3 deficiency enhanced the expression level of S1P₁ and S1P₂ receptors mRNA in S1P3-null EDL muscle. The contractile properties of S1P3-null EDL diverge from those of wild type, being largely more fatigable and less able to recover. The absence of S1P₃ appears responsible for a lower availability of calcium during fatigue. S1P supplementation, expected to stimulate residual S1P receptors and signaling, reduced fatigue development of S1P₃-null muscle. Moreover, in the absence of S1P₃, denervated EDL atrophies less than wild type. The analysis of atrophy-related proteins in S1P₃-null EDL evidences high levels of the endogenous regulator of mitochondria biogenesis peroxisome proliferative activated receptor-γ coactivator 1α (PGC-1α), that preserving mitochondria could protect the muscle from disuse atrophy. In conclusion, the absence of S1P₃ makes the muscle more sensitive to fatigue and slows down atrophy development after denervation, indicating that S1P₃ is involved in the modulation of key physiological properties of the fast-twitch EDL muscle.

**Key words:** contractile properties, fatigue, atrophy, fast muscle, sphingosine 1-phosphate, S1P₃ receptor

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**INTRODUCTION**
The bioactive phospholipid, sphingosine 1-phosphate (S1P), is an important regulator of skeletal muscle properties (14). S1P is generated intracellularly as a relevant metabolite of cell membrane sphingomyelin degradation pathway, by the action of two distinct sphingosine kinases (SphK) that catalyze the phosphorylation of sphingosine. Catabolism of S1P includes its conversion back to sphingosine catalyzed by specific S1P phosphatases (SPP1 and SPP2) and its irreversible cleavage exerted by S1P lyase (SPL). Substantial amounts of S1P are also present in the blood, within erythrocytes and platelets, and in serum, mainly associated to apolipoprotein M in the HDL fraction (39, 61). Skeletal muscle possesses several sphingomyelin metabolism enzymes, including the isoform 1 and 2 of SphK, the phosphatase SPP1 and SPL (14, 53).

The mechanism of action of S1P is quite complex, being this bioactive lipid an intracellular mediator as well as a receptor ligand (14). Indeed, intracellular S1P regulates gene transcription by directly affecting HDAC activity (25) or NF-kB signaling (62). S1P is released in the extracellular environment through various transporters (42) where it acts by autocrine/paracrine mechanisms as ligand of five distinct S1P receptors, S1P1-5. S1P receptors are coupled to specific GTP-binding proteins that activate distinct signaling pathways (38, 39, 52, 61).

Three S1P receptor subtypes (S1P1-3) are expressed in adult skeletal muscle. S1P1 and S1P3 receptors are localized both in the cell and nuclear membrane of adult fibers and in the neuromuscular junction (69). S1P1 and S1P3 are expressed by quiescent satellite cells of adult muscle, whereas S1P2 is only transiently expressed in activated satellite cells (13, 19, 26). The molecular mechanisms for the action of S1P and the role of individual receptors in skeletal muscle are still not well understood. Our previous results, using agonist and antagonist of S1P receptors, indicate that S1P1 and S1P3 display opposite actions in controlling the growth of regenerating fibers in rat soleus muscle (13). Moreover, S1P2 plays a critical role during the early phases of muscle regeneration: the lack of S1P2 resulted in a delay of regenerating muscle fiber growth (24, 36).

Recently, it has been demonstrated in vivo that in the absence of S1P3, acute regeneration is enhanced both after a single and repeated cycles of muscle injury (19). Finally, in vitro experiments...
demonstrated that supplementation of S1P attenuates the development of EDL muscle fatigue, induced by low-frequency repeated short tetani (11), indicating that S1P signaling is involved in this important property of skeletal muscle, even though the putative role of S1P receptors has not yet investigated in detail.

A striking characteristic of skeletal muscle is plasticity, i.e. the ability to adapt its mass, fiber type composition, metabolism and performance to different functional requests. Activity plays a fundamental role in controlling skeletal muscle characteristics. Increased activity, such as strength exercise and functional overload, produces the enlargement of muscle fiber (hypertrophy), while muscle disuse, such as bed rest, spinal cord injury, denervation, immobilization and microgravity, induces atrophy, characterized by the reduction of muscle mass (58). Muscle atrophy occurs when protein degradation surpasses protein synthesis. Two major pathways regulate protein degradation, the ubiquitin - proteasome system and the autophagy - lysosome pathway (55). The IGF-1/Akt/mTOR/FoxOs signaling pathway positively regulates muscle growth (59), whereas the myostatin-SMAD2/3 pathway, acts as a negative regulator (32). The IGF-1-dependent phosphorylation of Akt, also known as PKB, stimulates mTOR and protein synthesis and prevents the nuclear localization and transcriptional activity of FoxO, which is critical for the atrophy process (33). The reduced activity of the Akt pathway, observed in various models of muscle atrophy, leads to decreased protein synthesis and a marked increase in nuclear FoxO, where it stimulates the expression of diverse atrophy genes (57). However, it is worth reporting that recent evidence indicates that denervation may be Akt-independent (37). In any case, nuclear FoxO mainly stimulates the expression of two atrophy-specific E3 ubiquitin ligases, atrogin-1 and MuRF1, which direct the ubiquitination and degradation of muscle proteins (4). Moreover, FoxO proteins induce the expression of many autophagy (such as, LC3-II, p62, and Beclin1) and lysosomal genes (70). In this respect, an autophagy-mediated removal of mitochondria (mitophagy) as well as a mitochondrial-mediated apoptosis has been observed during denervation (46, 60). Additional key players are active during muscle atrophy, such as the NF-kB pathway, myogenin, and the
peroxisome proliferative activated receptor-γ coactivator 1α (PGC-1α) (17, 63). In particular, PGC-1α, master regulator of mitochondria biogenesis (34), is indicated to be involved in the atrophy-related mitochondrial dysfunction and to affect the development of muscle atrophy both in soleus and EDL muscles (5, 6).

Injury of motor nerve causes the progressive atrophy of muscle fibers, fiber-type specific MyHC transformation and impairment of functional properties (41). We demonstrated that exogenous application of S1P counteracts the denervation atrophy of rat soleus, a slow-twitch muscle, while the depletion of the extracellular lipid with a specific anti-S1P monoclonal antibody accelerated the atrophy caused by denervation of mouse soleus. In addition, infusion with S1P attenuated the slow-to-fast transformation due to denervation in soleus muscle of rat (69).

In the present work, by using S1P3-null mice, we explored the functional role of S1P3 on skeletal muscle. We investigated the morphological and contractile properties of the fast-twitch skeletal muscle EDL and analyzed the effects of denervation on atrophy development of the transgenic muscle. Overall, the study demonstrates that S1P3 ablation affects the physiological properties of EDL muscle, its fiber-type composition, and the expression of residual S1P receptors. The absence of S1P3 makes EDL muscle more sensitive to fatigue and slows down atrophy development in the denervated muscle. Moreover, exogenous S1P supplementation affects fatigue development of S1P3-null EDL.
METHODS

The Italian Health Ministry approved all animal experimental protocols. C57BL/6J (wild type) and S1P3-null mice, generated by Jerold Chun, Scripps Institute, La Jolla, USA (27) and kindly provided by Dr. Bodo Levkau, University of Essen, Germany (the genotype of S1P3-null mouse was confirmed by PCR) were fed ad libitum with standard chow/water diet and kept under a 12-h light-dark cycle in an air-conditioned room. Three-month-old male mice were used.

EDL muscle contractile properties

The experiments were performed in vitro in a vertical muscle apparatus (300B, Aurora Scientific Inc, Canada) containing a Ringer solution of the following composition: 120 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 3.15 mM MgCl2, 1.3 mM NaH2PO4, 25 mM NaHCO3, 11 mM glucose, 30 µM d-tubocurarine, pH 7.2-7.4, 30°C, bubbled with 95% O2 - 5% CO2. Muscles were stretched to the optimal length (i.e. the length that allowed maximal tension development in response to a single pulse) and electrically stimulated, by two parallel electrodes, with supramaximal pulses (0.5 ms duration) delivered by a Grass S44 electronic stimulator through a stimulus isolation unit (Grass SIU5). Muscle response was recorded through an isometric force transducer (Grass-FT03) connected to an AT-MIO 16AD acquisition card (National Instruments). Data were analyzed by the specific module of the National Instruments LabView software (23). Contraction and half relaxation times of the twitch were measured. Twitch and tetanic tensions were normalized to the muscle wet weight (specific tension, Nxg\(^{-1}\)). Force-frequency curve was determined by stimulating EDL muscle at 1, 30, 45, 60, 75, 90, 110, 130 and 150 Hz. Muscles were weighed at the end of each experiment.

Muscle fatigue

Small intact bundles of about 10-20 fibers from the EDL or 5-10 fibers from flexor digitorum brevis
(FDB) were mechanically dissected in Tyrode solution, of the following composition: 121 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 0.4 mM NaH₂PO₄, 24 mM NaHCO₃, 5.5 mM glucose, 0.1 mM EDTA, and 0.05% DMSO (8, 45). The solution was bubbled with 5% CO₂ - 95% O₂ which gave a pH of 7.4. The experiments were performed at 24°C in control solution supplemented or not with 1 μM S1P. S1P was prepared by dilution of 2 mM stock solution in DMSO that was used as vehicle control of S1P. The use of small bundles of fibers (called fibers in this paper, for simplicity) offers ideal mechanical conditions for fast stiffness measurements. Small aluminum clips were attached to tendons as close as possible to the fiber ends (50-100 μm) to mount horizontally the fibers in an experimental chamber between the lever arms of an home-made capacitance force transducer (resonance frequency 15-30 kHz) and of an electromagnetic motor (minimum stretch time 150 μs) used to apply fast length changes to the fibers. Fibers were electrically stimulated with bipolar stimuli (0.5 ms duration and 1.5 times threshold strength) applied by means of two parallel platinum-plate electrodes. The fiber length was adjusted to that giving maximum tetanic force ($P₀$) corresponding to a mean sarcomere length of about 2.5-2.6 μm.

Mean clip to clip fiber length ($l_i$) was 1131 ± 27 μm ($n = 16$) in FDB wild type and 1217 ± 41 μm ($n = 20$) in FDB S1P₃ null; 6317 ± 160 μm ($n = 10$) and 6386 ± 237 μm ($n = 8$) in EDL wild type and S1P₃-null, respectively. Resting sarcomere and fiber length were measured under ordinary light illumination using a microscope and on digital images acquired by a video camera (Infinity Camera, Lumenera Corp., Canada) using image processing software. Sarcomere length was measured by counting 10 consecutive sarcomeres on a calibrated scale on the acquired images. Stimuli and fiber length changes were controlled by custom-written software (LabView, NI, USA) which was also used to record force and length at sampling speed up to a maximum of 200 kHz.

Before the start of each experiment, fibers were maintained at optimal sarcomere length for 15 min in control solution without electrical stimulation. Twitch and tetanic tension (control tetanic stimulation: 70 Hz frequency, 300 ms duration, and 90 Hz, 250 ms for FDB and EDL, respectively) levels were measured. Tetani were given at an interval of 2 min for a period of equilibration of
about 8-12 min in which \( P_0 \) remained stable. If \( P_0 \) decreased by >15%, the fibers were discarded. In

the experiments with S1P supplementation, viability test was made before and after 30 min of

incubation with 1 \( \mu \)M S1P. Untreated or treated fibers where subjected to a fatigue protocol

consisting of 105 tetani (70 Hz, 300 ms every 1.5 s, for FDB; 90 Hz, 350 ms every 3 s, for EDL).

Recovery from fatigue was followed for 30 min in EDL or 60 min in FDB by applying tetani with

the same characteristics every 2 min.

Stiffness

To estimate the number of attached crossbridges we measured fiber stiffness (43, 45) by applying

small (about 0.2% \( l_0 \)) sinusoidal length changes (dl) to one end of the activated fibers and measured

the resulting force oscillations (dp) at the other end. The oscillation frequency was 2.5 and 6.5 kHz

for EDL and FDB, respectively. The measurements and calculations were made as previously

reported in detail (43, 45). Briefly, fiber stiffness was calculated as the ratio dp/dl at any tension

over dp/dl measured at \( P_0 \). Stiffness data presented here refer to the relative changes of this ratio

during tetanus rise and during fatigue respect to the values measured at plateau before the fatigue.

Surgical procedures

All surgical procedures were performed in a single session under general anesthesia, carried out by

the intraperitoneal injection of tiletamine and zolazepam (7 mg/kg, Virbac, Carros) and xylazine (14

mg/kg, Bayer). The sciatic nerve was cut monolaterally at the level of trochanter. About 0.5-1 cm of

the peripheral nerve stump was removed to avoid re-innervation and obtain a permanent

denervation of the lower hind limb. Mice were euthanized 7 or 14 days after denervation by neck

dislocation. Denervated and contralateral EDL muscles were used for functional, biochemical and

molecular studies while denervated and contralateral tibialis anterior (TA) muscles were used for

mitochondrial analyses.
**Histological and immunofluorescence analysis**

Muscles were isolated and quickly frozen in liquid nitrogen in a slightly stretched position. Serial cross sections (8-μm thick) were cut in a cryostat microtome (Slee, London, UK) set at -24 ± 1°C. Hematoxylin-eosin (HE) and succinate dehydrogenase (SDH) staining were performed on transverse muscle sections to examine the general morphology and overall mitochondrial content (54). To better evaluate the mitochondrial content, SDH staining images were processed with Adobe Photoshop setting the SDH intensity of individual fibers on an arbitrary 0-255 grey scale. Then fibers with similar SDH intensity were assembled in ten discrete groups. Laminin staining was carried out to determine the cross-sectional area (CSA) of individual fibers (69). Muscle sections were incubated for 1 hour at 37°C with the polyclonal antibody specific for laminin (L9393, Sigma, St. Louis, MO) diluted 1:150 in 5% fetal bovine serum. Laminin was revealed with an anti-rabbit FITC-labelled secondary antibody (F0382 Sigma) diluted 1:200 in PBS incubated for 1 hour at 37°C. Muscle sections were examined in a Leica RD100 fluorescence microscopy equipped with a digital camera. Muscle fiber CSA was measured on digital photographs by the ImageJ NIH software. More than 600 fibers per muscle were evaluated.

**Western blotting and SDS-PAGE analysis**

Western immunoblotting was performed on muscle fragments dissolved in SDS-PAGE buffer supplemented with Complete protease inhibitors cocktail (Roche, Basel, Switzerland). Muscle lysates (25-30 µg each) were electrophoresed on 10% SDS-PAGE gels. Electroblotting was performed as previously described (69). Nitrocellulose filters were probed with the selected primary antibody incubated overnight at 4°C at the conditions above described.

The following rabbit polyclonal antibodies were used: Phospho-Akt (Cell Signaling technology; 1:2000 in 5% BSA and 0.1% Tween-20 in TBS), Akt (Cell Signaling technology; 1:500 in 5% BSA and 0.1% Tween-20 in TBS), Atrogin-1 (ECM Bioscences; 1:1000 in 5% low-fat milk, 0.04% Tween-20 in TBS), MuRF1 (ECM Bioscences; 1:500 in 5% low-fat milk, 0.04%
Tween-20 in TBS), FoxO3 (Cell Signaling; 1:250 in 5% low-fat milk, 0.1% Tween-20 in TBS), P-
FoxO3, (Cell Signaling; diluted 1:500 in 5% low-fat milk, 0.1% Tween-20 in TBS), cytochrome c
(Cell Signaling; 1:1000 in 5% low-fat milk, 0.1% Tween-20 in TBS), GAPDH (GenTex; 1:5000 in
2% BSA, 0.2% Tween-20 in TBS), sarcomeric actin (Sigma; 1:5000 in 5% low-fat milk, 0.2%
Tween-20 in TBS), LC-3 and P62 (Sigma; 1:1000 in 5% low-fat milk, 0.1% Tween-20 in TBS),
PGC-1α (Abcam; 1:1000 in 5% low-fat milk, 0.1% Tween-20 in TBS). The secondary antibody
utilized was an anti-rabbit peroxidase-conjugated antibody (Chemicon) diluted 1:5,000 in the same
buffer as the cognate primary antibody and incubated for 1 hour.

Moreover, the following mouse monoclonal antibodies were used: myogenin (F5D,
Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) 1:100 in 10% low-fat
milk, 0.1% Tween-20 in TBS, α-actinin (EA-53, Sigma, St. Louis, MO) 1:1000 in 5% low-fat milk,
0.2% Tween-20 in TBS, incubated for 1 hour. The secondary antibody was an anti-mouse
peroxidase-conjugated (Dako, Glostrup, Denmark) at 1:10000 dilution (in the same buffer as the
cognate primary antibody), incubated for 1 hour. Visualization of reaction bands was performed
either by tetramethylbenzidine staining or by ECL chemiluminescence (Amersham Pharmacia
Biotech, Buckinghamshire, UK). Signal intensities were evaluated by densitometry.

Analysis of whole muscle MyHC isoforms was performed by the SDS-PAGE method of
Talmadge and Roy, as previously described (12). Small muscle fragments were solubilized in SDS-
PAGE sample buffer (62.5 mM Tris, pH 6.8, 2.3% SDS, 5% 2-mercaptoethanol, 10% glycerol).
Muscle protein samples (10 µg each) were electrophoresed on 8% SDS-PAGE slab gels. MyHC
protein bands were revealed by Coomassie brilliant blue staining. MyHC isoform percentage
composition was determined by densitometry of gels by using a Bio-Rad Imaging Densitometer
(GS-670).

Cell fractionation

Mouse muscles were homogenized on ice in 15 volumes of 10 mM Tris-HCl, 0.25 M sucrose, 0.2
mM EDTA, pH 7.8, containing protease inhibitor cocktail. The homogenates were centrifuged at 2,000 x g for 8 min at 4°C. Pellets contained myofibrils, while the supernatant contained mitochondrial, cytosolic and membrane proteins. The supernatant was then centrifuged at 12,000 x g for 15 min at 4°C. The pellet contained mitochondria while the supernatant contained cytosolic and membrane proteins. The mitochondrial fraction was resuspended in 10 mM Tris-HCl, 0.25 M sucrose, 0.2 mM EDTA, pH 7.8 (with protease inhibitor cocktail) and subjected repeatedly (3-4 times) to centrifugation at 12,000 x g for 15 min at 4°C. After the last centrifugation, the pellet was snap frozen in liquid nitrogen and kept at -80°C for future analysis (18).

**Real-Time PCR**

Total RNA (1 μg), extracted with TRI Reagent from EDL, was reverse transcribed using the High Capacity cDNA Reverse Transcription kit, according to the manufacturer’s instructions (Life Technologies, Carlsbad, CA, USA). The quantification of S1P receptors (S1P1, S1P2, and S1P3) mRNA level was performed by Real-Time PCR employing TaqMan Gene Expression Assays (S1P1 Mm00514644_m1; S1P2 Mm01177794_m1; S1P3 Mm00515669_m1). Each measurement was carried out in triplicate, using the automated ABI Prism 7700 Sequence Detector System (Life Technologies, Carlsbad, CA, USA), as described previously (15) by simultaneous amplification of the target sequence together with the housekeeping gene 18S rRNA. Results were analyzed by ABI Prism Sequence Detection Systems software, version 1.7 (Life Technologies, Carlsbad, CA, USA). The $2^{−ΔΔCT}$ method was applied as a comparative method of quantification (35) and data were normalized to ribosomal 18S RNA expression.

**Statistical analysis**

All values are means ± SEM. Comparisons of mean values were performed after analysis of variance (ANOVA) and the Newmann-Keuls post-hoc test. In the Real-Time PCR experiments results are expressed as means ± SEM of fold changes according to the $2^{−ΔΔCt}$ method, utilizing
18S rRNA as housekeeping and each S1P receptor in wild type innervated EDL as calibrator.

Statistical significance of Real-Time PCR data was assessed with two-way ANOVA followed by Bonferroni’s post-hoc test. In all the statistical analyses differences were considered significant at the $P < 0.05$ level.
RESULTS

Characterization of S1P3-null EDL muscle

To examine the role of S1P3 and its downstream signaling in modulating muscle properties, we utilized transgenic mice lacking S1P3 (27, 28). The absence of S1P3 apparently did not affect the normal growth of mice, as the body weight of three-month old male S1P3-null mice was similar to that of age-matched wild type controls (Fig. 1A). Moreover, the muscle mass and the ratio between muscle mass and body weight of fast-twitch EDL isolated from S1P3-null mice were similar to that of wild type muscle (Fig. 1A). Coherently, the mean CSA of S1P3-null muscle fibers was similar to that of wild type (Fig. 1A). Morphology of EDL muscle did not show evident differences between wild type and S1P3-null mice (Fig. 1B). However, SDH staining, an estimate of mitochondria content, showed a higher intensity in the S1P3-null EDL compared to wild type (Figs. 1C and 1D). Coherently, the amount of cytochrome c in the total muscle homogenate from S1P3-null EDL was larger (13.2 ± 1.6 a.u., n = 11) than that in wild type (8.1 ± 1.2, a.u., n = 7, P < 0.05).

To evaluate whether the absence of S1P3 may affect the overall muscle phenotype, the expression level of MyHC isoforms was analyzed by SDS-PAGE. The densitometric analysis of MyHC composition showed no major differences between wild type and S1P3-null EDL except for a slightly higher content in the slow type 1 MyHC isoform in the transgenic muscle.

We next examined whether the absence of S1P3 affects the expression level of the S1P receptors physiologically expressed in skeletal muscles (13, 26). We quantitated, by Real-Time PCR, the transcript level of S1P1 and S1P2 receptors in EDL muscle. The data show that the expression of S1P1 and S1P2 was enhanced in the S1P3-null EDL (Fig. 1F).

Then, we analyzed the contractile properties of EDL muscle deprived of S1P3 receptor. Twitch tension of S1P3-null EDL was significantly smaller (P < 0.05) compared to wild type (Fig. 2A and 2C), while time-to-peak tension (15.7 ± 0.9 vs. 15.0 ± 0.7 ms) and half-relaxation time (15.3 ± 1.7 vs. 11.7 ± 1.2 ms) were similar (Fig. 2A). The tetanic tension of S1P3-null EDL was not significantly
different than that of wild type (Fig. 2B and 2C). The force/frequency curve of S1P3-null EDL shifted to the right, with significantly reduced tension at low stimulation frequencies (Fig. 2D).

Moreover, we measured the resistance to and recovery from fatigue in muscle fibers isolated from wild type and the S1P3-null EDL (Fig. 3A and 3B). At the end of the fatigue protocol, in wild type fibers force decreased to 0.42 ± 0.09 \( P_0 \) while S1P3-null fibers were largely \( (P < 0.05) \) more fatigable than wild type fibers starting from the 42nd tetanus. All S1P3-null fibers endured the full fatiguing stimulation protocol but the mean force in the last tetanus was 0.22 ± 0.03 \( P_0 \). After 2 min of resting, tetanic force reached 0.56 ± 0.10 and 0.30 ± 0.03 of the pre-fatigue value, respectively in wild type and in S1P3-null fibers (Fig. 3A and 3B). Both wild type and S1P3-null fibers reached the maximum force recovery value in few minutes after the end of fatigue and then remaining almost constant. However, at the end of the recovery period the tetanus force was significantly higher \( (P < 0.05) \) in wild type fibers \( (0.61 ± 0.07 \ P_0) \) compared to that of S1P3-null fibers \( (0.35 ± 0.05 \ P_0) \) (compare Fig. 3A with 3B). As shown in Fig. 3C, the average time course of stiffness drop during fatigue was similar to tension decrease in both wild type and S1P3-null fibers reaching at the end of the fatigue protocol 0.60 ± 0.07 and 0.39 ± 0.03 \( P_0 \) of the pre-fatigue value, respectively.

Then, we examined the effects of S1P supplementation on fatigue responses of wild type and S1P3-null EDL fibers. In the resting state, twitch properties and tetanic tension of wild type and S1P3-null EDL were not affected by the treatment. S1P supplementation did not influence fatigue development and recovery of wild type EDL (Fig. 3A). Vice versa, in the presence of S1P, fatigue development of S1P3-null EDL fibers was significantly reduced, making the fatigue time course similar to that of untreated wild type (compare Fig. 3A with Fig. 3B). In fact, the tetanic force of S1P3-null EDL fibers at the last tetanus was 0.22 ± 0.03 \( P_0 \) without S1P and 0.44 ± 0.07 \( P_0 \) in the presence of the lipid, a value very close to that of untreated wild type \( (0.42 ± 0.09 \ P_0) \). Moreover, after 2 minutes of resting, in the presence of S1P the tetanic force of S1P3-null EDL moved from 0.44 ± 0.07 \( P_0 \) (last fatiguing tetanus) to about 0.67 ± 0.10 \( P_0 \), with the recovery force curve almost identical to that of the wild type (compare Fig. 3A with Fig. 3B).
To better evaluate the tension decrease during fatigue, we measured the fiber stiffness-tension relation during fatigue and tension rise in FDB fibers that are a more reliable, reproducible and less variable mechanical model than EDL muscle fibers, in particular for the stiffness measurements (45). In FDB fibers the fatigue time-course showed, more clearly than in EDL fibers, a first rapid decay and a second more slowly decrease of tension (43). As in the S1P3-null EDL, in the absence of S1P3 the FDB fibers showed a higher fatigability than wild type (Fig. 4A). At the end of the fatigue protocol, the force of FDB fibers decreased to 0.66 ± 0.03 P0 in wild type and to 0.49 ± 0.02 P0 in S1P3-null fibers. Comparing the EDL and FDB fatigue profiles (Figs. 3 and 4), it is clear that FDB, having a more oxidative phenotype, is basically more resistant to fatigue than EDL. As in the EDL, S1P supplementation did not influence force decrease in fatigued wild type fibers (Fig. 4A). On the contrary, the higher fatigability of S1P3-null FDB fibers was significantly reduced by S1P supplementation (Fig. 4A), with the final tension value (0.63 ± 0.03 P0) similar to that of untreated wild type fibers (0.66 ± 0.03 P0). As in EDL, S1P treatment did not make the null fibers more resistant to the early fatigue but it decreased the loss of tension during the later phase (Figs. 3B and 4A).

In FDB, the recovery of wild type and S1P3-null fibers, without or with S1P supplementation, was similar, reaching a high value of force in few minutes to then slowly enduring. At the end of the recovery protocol, tetanic force reached 0.97 ± 0.02 of the pre-fatigue value in wild type fibers, 0.98 ± 0.01 in S1P-treated wild type fibers, 0.92 ± 0.03 in S1P3-null fibers and 0.94 ± 0.02 in S1P-treated S1P3-null fibers. It is worth noting that in the presence of S1P the recovery seems improved: in S1P3-null fibers became as in the untreated wild type and in the wild type fibers reached the pre-fatigue tension value already in the first minutes (Fig. 4A).

As in EDL (Fig. 3C), the stiffness of S1P3-null FDB fibers decreased in proportion to tension and S1P supplementation decreased stiffness in the later phase of fatigue (Fig. 4B). The data of Fig. 4B were also plotted as a stiffness-tension relation and compared to those obtained on the same fibers during tetanus rise, a condition in which the force development is considered proportional to
crossbridge number (Fig. 4C). The stiffness-tension relation during the rise was not linear and
deviated from the direct proportionality (showed by the dashed line) due to the myofilament and
tendon stiffness presence (3, 9, 43, 45). In S1P₃-null fibers, treated or not with S1P, the stiffness-
tension relationship during fatigue had a more pronounced downward curvature and deviated even
more from linearity so that, especially at high tension, fiber stiffness was greater than during the
tetanus rise.

**Denervation atrophy**

We previously reported that denervation of rat soleus muscle modifies the expression of S1P receptors, causing a decrease of both S1P₁ and S1P₃ (69). The analysis of S1P receptors (S1P₁₋₃) transcripts in 14-day denervated EDL muscle of wild type mice confirms an overall decrease of all S1P receptors after denervation. In S1P₃-null mice, denervation caused a significant decrease of both S1P₁ and S1P₂ receptors (Fig. 5).

To examine the possible role of S1P₃ in the regulation of muscle mass, we analyzed the effects of muscle denervation in S1P₃-null transgenic mice. We measured the extent of muscle atrophy as the percentage change of the mean CSA of fibers from 7- and 14-day denervated EDL with respect to fibers from the contralateral innervated muscle. Denervation caused a progressive atrophy in wild type EDL, whose mean fiber CSA was reduced by 15.3 % after 7 days and 41.2 % after 14 days. In S1P₃-null mice, denervation produced a smaller atrophy in EDL fibers, whose mean CSA was in fact reduced by 9.7 % at 7-day and 26.8% ($P < 0.05$) at 14-day (Figs. 6A, 6B and 6C).

Denervation is known to cause a fast-to-slow transformation in wild type EDL, with the progressive increase of both type 1 and type 2A MyHC isoforms and the decrease of type 2B MyHC (22, 40). Similarly, denervation induced a comparable transformation to S1P₃-null EDL, with the progressive increase of type 1 MyHC isoform and the decrease of type 2B MyHC, whereas the proportion of type 2A MyHC was not different compared to the innervated muscle (Fig. 6D).
Atrophy-related genes

Muscle atrophy is the consequence of the unbalance between protein synthesis and degradation (35). To evaluate whether the smaller atrophy induced by denervation to the S1P3-null EDL was associated to a different activation of atrophy-related genes, we analyzed the expression level of atrophy-associated E3 ligases MuRF1 and atrogin-1. As expected (4), expression of MuRF1 progressively increased during denervation of wild type and S1P3-null EDL (Fig. 7A). Eventually, 14 days after denervation the expression level of MuRF1 was similarly increased in wild type and S1P3-null EDL (2.5 and 2.4 times, respectively). The analysis of the other E3 ligase atrogin-1 shows that denervation did not increase the expression of atrogin-1 both in wild type and S1P3-null EDL (Fig. 7B).

In the early phases of denervation, the expression of E3 ubiquitin ligases is modulated by transcription factor myogenin (63). In innervated EDL, the basal protein expression level of myogenin was significantly higher in the S1P3-null than in wild-type EDL (Fig. 7C). As expected, denervation up-regulates the expression of myogenin. Eventually, 14 days after denervation the expression level of myogenin was increased 2.7 and 2.3 times in wild type and S1P3-null EDL, respectively.

The Forkhead Box O (FoxO) family of transcription factors also plays a critical role in the atrophy program. Since FoxO is downstream of the insulin-Akt-mTOR pathway (55), we have evaluated expression levels of these proteins during denervation. The extent of activated Akt (P-Akt) progressively increased in both denervated wild type and transgenic EDL, apparently more slowly in the S1P3-null EDL. However, the ratio between P-Akt and total Akt did not change appreciably during denervation of both wild type and S1P3-null EDL (Fig. 7E). The analysis of P-FoxO3 vs. FoxO3 protein ratio shows no major changes in wild type and S1P3-null EDL muscle during denervation (Fig. 7D).

Denervation is not only associated to the progressive reduction of muscle mass but also to the variation of mitochondrial content, either as an adaptation to the reduced fiber volume and energy...
request (67) and/or as an event concurring to the atrophy program (5, 6). The higher SDH staining and level of total cytochrome c indicates that S1P3-null EDL muscle contains more mitochondria than wild type. We thus investigated the expression of PGC-1α, a key regulator of mitochondria biogenesis (34). Basal PGC-1α expression level was higher in innervated S1P3-null EDL compared to wild type (Fig. 8A). Denervation of wild type and S1P3-null EDL caused no major changes of PGC-1α protein level. Nevertheless, PGC-1α protein level was significantly higher in 7- and 14-day denervated S1P3-null EDL compared to the respective values of wild type EDL (Fig. 8A).

Recently, it has been proposed that in the denervated muscle, cytoplasmic cytochrome c is released from fragmented mitochondria to start apoptosis (48, 60). A first step is activation of caspase 3 that, among other targets, seems to cleave myofibrillar actin to generate a 14-kDa fragment, which is then ubiquitinated and degraded (16). To investigate this occurrence, we measured the cytoplasmic cytochrome c level in TA muscle instead of EDL because of its larger mass. Our results did not show a clear increase of cytosolic cytochrome c level after denervation in wild type and S1P3-null TA muscle. However, it is worth reporting that cytosolic cytochrome c slightly increased in the denervated wild type TA (from 3.31 ± 0.71 to 4.37 ± 1.13 a.u., +32%, n = 6) and slightly decreased in the S1P3-null muscle (from 3.72 ± 0.6 to 2.93 ± 0.93 a.u., -21%, n = 7). We then measured the amount of the 14-kDa actin fragment possibly generated by caspase 3 activity. Denervation induced an increase of 14-kDa actin fragment in wild type but had no effects on S1P3-null EDL (Fig. 8B).

Denervation stimulates autophagic signaling pathways in muscle (46). To evaluate in the denervated muscle the possible role of S1P signaling in the activation of autophagy gene expression and flux, we measured the amount of p62 protein, a protein involved in the recognition of autophagy substrates, and the LC3-II/LC3-I ratio, which is important in autophagosome formation (55). Denervation induced a marked increase of p62 protein both in wild type and S1P3-null EDL, with an apparent progressive increase in wild type and a late strong increase in the transgenic muscle (Fig. 8C). Similarly, denervation caused a progressive and marked increase of the LC3-
II/LC3-I ratio in wild-type EDL muscle, whereas the increase was evident only after 7 days in the S1P3-null muscle (Fig. 8D).
DISCUSSION

This work investigated the role of S1P3 in skeletal muscle. We utilized the S1P3-null mouse model to determine the effects of receptor ablation on morphological and physiological characteristics of EDL muscle. The analysis of EDL muscle showed no overt muscle mass and morphological differences between wild type and S1P3-null mice. Minor differences were related to the lower twitch tension and a larger proportion type 1 MyHC and mitochondria in S1P3-null EDL. However, two main differences emerged: 1) the S1P3-null EDL is more fatigable than wild type; 2) during denervation the S1P3-null EDL is less prone to atrophy than wild type.

Typically, force decline during development of fatigue shows a first phase in which force rapidly declines followed by a second slower phase (2, 45). Our previous data obtained in FDB fibers (43, 45) showed that the initial force decline occurred without great reduction of the number of attached crossbridges (or fiber stiffness) but it was attributed to a reduction of the average force per attached crossbridge. In the second phase, the force decrease was accompanied by a proportional stiffness decline and therefore attributable to the reduction of attached crossbridge number. As during the initial force decline, tetanic [Ca^{2+}]_{i} is not reduced but rather increased (66), the first loss of force has been attributed to a fast direct inhibitory effect of the increase of the [P_{i}], that pushes the crossbridge into a low-force generating state (7, 10, 49). On the contrary, the later force decline is attributed to a reduction of Ca^{2+}-release from the sarcoplasmic reticulum (20) and/or a reduced myofilament Ca^{2+}-sensitivity due to high [P_{i}], and/or higher production of ROS/RNS (30).

Present results show that in the absence of S1P3, EDL fibers fatigue largely more than wild type. Tension and stiffness data show that as in FDB (43, 45) also in EDL fibers it is possible to distinguish two different phases during fatigue. In the initial phase, force of wild type and S1P3-null fibers declined more than stiffness, implying on both cases a similar reduced force per attached crossbridge. On the contrary, the average time course of stiffness during late fatigue decreased
similarly to tension both in wild type and S1P3-null fibers (Figs. 3C and 4B), indicating a proportional reduction of crossbridge number. To further inspect the two different mechanisms responsible for the fatigue, we analyzed the stiffness-tension relation in S1P3-null FDB fibers treated or not with S1P. If tension loss during fatigue is due exclusively to the reduction of attached crossbridge number, the stiffness-tension relation during fatigue should be exactly the same as during the tetanus rise (3, 9, 43, 45). On the contrary, our data show that during fatigue the relation has more pronounced downward curvature and deviates even more from linearity so that fiber stiffness is greater than during the tetanus rise. Since filament and tendon stiffness are the same in both conditions this means that at any given force, to justify the greater fiber stiffness, there must be more attached crossbridges during fatigue than during the tetanus rise, which means that each fatigued crossbridge generates less average force. Both in S1P3-null treated or untreated fibers this effect is probably due to the high [Pi]. The tension stiffness relation in FDB fibers indicates a reduction in crossbridge number in the second phase, supporting the EDL results.

This evidence suggests that the higher drop of tension of S1P3-null fibers compared to wild type could be related to a larger reduction of calcium availability. Though we did not performed direct measurements of intracellular calcium, this possibility is consistent with the demonstrated involvement of S1P3 in the S1P-induced increase of intracellular calcium in skeletal (50) and smooth muscle cells (21), suggesting that S1P3 provides the extra calcium needed to attenuate fatigue in wild type EDL or FDB fibers. In wild type EDL, two main S1P receptors, S1P1 and S1P3, are expressed at the surface and in the T-tubules of muscle fibers (13). The localization in the T-tubules, i.e. the site of excitation-contraction coupling, validates a potential role in contractility of the two S1P receptors. However, we cannot exclude the contribution of possible changes of calcium sensitivity of myofilaments and/or altered fiber metabolites.

Similarly to fatigue, the initial fast recovery is likely due to a return to normal levels of [Pi] (43, 68), whereas the late force recovery depends on the slower return to normal condition of calcium release and/or myofilament calcium sensitivity (43). The smaller recovery of the EDL
S1P3-null with respect to wild type fibers is probably related to the dramatically higher drop of force at the end of the fatigue protocol.

We also examined the effects of supplemented S1P on fatigue responses of wild type and S1P3-null EDL fiber bundles. In contrast with previous data (11), S1P supplementation did not ameliorate fatigue development and recovery of wild type EDL. The divergence could be due to different experimental protocols (whole muscle vs. fiber bundles, frequency stimulation and temperature, 30 vs. 24 °C) and/or the different mice strain utilized in the experiments (CD1 vs. C57BL/6J). Notably, S1P supplementation strongly reduced the loss of force in the second phase of fatigue of S1P3-null EDL, apparently indicating that exogenous S1P stimulates an increased amount of calcium release from the sarcoplasmic reticulum, possibly operated by S1P1. However, stimulation of S1P1 is known to inhibit the release of calcium (29). On the other hand, over-stimulation of S1P receptors, and in particular of S1P1, is known to cause internalization of the receptor followed by either ubiquitin-directed degradation of the protein, resulting in the termination of receptor signaling, or alternatively trafficking of the receptor and activation of intracellular signals (65). Thus, internalization of S1P1 could abrogate its putative negative action on fatigue making the muscle less fatigable. Future work is necessary to discriminate between the possible functions of S1P1 receptor in muscle fatigue.

Finally, our results also show that twitch tension amplitude is reduced in the S1P3-null EDL, without changes in the time-to peak and half-relaxation times. The amplitude of twitch tension is essentially associated with the rate and amount of calcium released and re-accumulated from and into the sarcoplasmic reticulum. Also this result demonstrates that in the absence of S1P3 the excitation-contraction coupling process is perturbed. It is worth noting that S1P3-null EDL has a slightly higher content of type 1 MyHC (up to 6%), which could contribute to reduce the amplitude of twitch tension. However, we exclude this possibility because contraction and half-relaxation times as well as the maximal rate of rise of tetanic tension were unmodified (data not shown).
Moreover, the higher content of type 1 MyHC and of mitochondria in S1P3-null EDL did not compensate the effects on fatigue caused by the absence of the receptor.

We have recently demonstrated that S1P1 and S1P3 receptors are localized both in the T-tubules and in the sarcolemma (13). Here, we propose that the different localization of these receptors could serve two different functional roles. As indicated above, S1P receptors in the T-tubules are probably involved in the modulation of contractility. We hypothesize that sarcolemmal S1P receptors could modulate distinct signaling pathways, such as that regulating muscle mass.

Past studies demonstrated that S1P signaling exerts a trophic role in rat soleus denervation atrophy. In fact, boosting or decreasing circulating S1P ameliorated or worsened, respectively, denervation atrophy (69). Moreover, denervation down-regulated the expression of S1P1 and S1P3 receptors, that could contribute to muscle atrophy by reducing the trophic influence of S1P. Present results show that in the absence of S1P3, in denervated EDL, atrophy is substantially less than in wild type muscle. In wild type EDL, S1P1 and S1P3 may contribute to regulate muscle mass, either in the same direction and/or by balancing opposite actions. However, the data indicate that the absence of S1P3 is beneficial for denervated EDL and attenuates the atrophy program, suggesting that signaling pathways downstream S1P3, implicated in the onset and development of atrophy, are less active in the S1P3-null muscle than in wild type.

The absence of S1P3 has distinct effects on the expression of key proteins involved in the muscle atrophy program as a consequence of denervation. First, unexpectedly, despite the reduced atrophy, the protein level of the E3 ligase MuRF1 similarly increased in denervated wild type and S1P3-null EDL and increased more in the 14-day denervated S1P3-null EDL than in the wild type. Despite the known increase of atrogin-1 mRNA in the early phases of muscle atrophy (31), our data show that at 7 and 14 days after denervation atrogin-1 protein level is similar to that of innervated muscles both in wild type and S1P3-null EDL. Thus, apparently, the smaller atrophy of denervated S1P3-null EDL does not seem to involve these basic atrophy players.

Compared to the denervated wild type, no difference was observed in the S1P3-null EDL in
With regard to Akt, myogenin and FoxO3 protein levels, known key players in denervation atrophy. Of note, though myogenin expression, as expected (63), increased upon denervation similarly in wild type and S1P3-null EDL, the basal level of myogenin was higher in S1P3-null EDL compared to wild type.

Consistent with the higher content of mitochondria, the basal expression of PGC-1α protein, a key regulator of mitochondria biogenesis (34), is higher in innervated S1P3-null EDL compared to wild type. Recently, it has been demonstrated that EDL atrophy is largely reduced in transgenic mice over-expressing PGC-1α (6, 51, 56, 64). Therefore, the higher basal level of PGC-1α in the S1P3-null EDL could provide an explanation for why the muscle is, at least partly, protected from denervation atrophy. However, further research is required to understand the actual role of PGC-1α in muscle atrophy.

Apoptotic and autophagic signaling pathways are activated in muscle denervation atrophy to adequate myonuclear and mitochondrial content to the reduced fiber size (47). Moreover, mitochondria also undergo a partial fragmentation that liberates cytochrome c into the cytoplasm (1). The release of cytochrome c into the cytoplasm is known to activate caspase 9 and 3 (48, 60), which seems involved in the initial fragmentation of myofibrillar actin, a necessary step for the subsequent actin degradation by E3 ligases (16). Our data confirm the increase of cytoplasmic cytochrome c in the denervated wild type TA but show a small decrease in the S1P3-null TA, suggestive of a different trend between the two muscles. Moreover, we confirm the increase of 14-kDa actin fragment during denervation of wild type EDL. Conversely, the level of the 14-kDa actin fragment does not change during denervation of S1P3-null EDL. This finding suggests that in S1P3-null EDL, MuRF1, though elevated, is not able to tackle the dismantling of myofibrils as in wild type, and could provide an explanation for the smaller atrophy of the transgenic muscle.

During denervation, autophagy and autophagy-related genes (i.e., LC3-II, p62, Beclin1) are up-regulated, with the extra LC3-II being preferentially associated to mitochondria (47). We here confirm that LC3-II levels progressively increase in denervated wild type EDL, while in S1P3-null
EDL autophagy seems to progress less precisely, suggesting that the absence of S1P3 perturbs autophagy.

In conclusion, we propose that S1P3 plays a role in attenuating the development of EDL muscle fatigue as well as in controlling the mass of the denervated fast-twitch muscle. We propose that the first action is due to the T-tubule-associated receptor while the second is to be ascribed to the sarcolemmal one. The protective role against fatigue is most likely associated to a modulatory role of S1P3 in EC-coupling and eventually in intracellular calcium mobilization. The role of S1P3 in controlling muscle mass and the mechanisms involved appear to be more complex. The finding that S1P3 ablation has a protective role against denervation-induced muscle atrophy, strongly suggests that this receptor is implicated in autophagy.
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Legends to Figures

Figure 1. Characterization of S1P3-null EDL muscle. A. Body mass (n = 24), muscle mass (n = 11), muscle-to-body mass ratio (n = 11), and muscle fiber CSA (μm², n = 11) of 3-month old male mice from wild type (WT) and S1P3-null mice. B. Hematoxylin-eosin of EDL muscle from WT and S1P3-null mice. Bar = 20 µm. C. SDH staining of EDL whole muscle from WT and S1P3-null mice. Bar = 100 µm. D. SDH staining intensity of EDL muscle fibers from WT and S1P3-null mice. See Methods for details. E. Myosin heavy chains (MyHC) isoform composition in EDL muscle from WT and S1P3-null mice. Data are reported as mean ± SEM. Six-to-ten experiments were carried out in each group. F. Expression of S1P1 and S1P2 receptors transcripts in EDL muscle isolated from WT and S1P3-null mice. S1P receptors in each S1P3-null muscle were measured by Real Time PCR and compared to that in wild type muscles. Results are expressed as fold changes according to the 2^(-∆∆Ct) method, utilizing S1P1 or S1P2 genes in wild type muscle as calibrator. Data are reported as mean ± SEM of three independent experiments performed in triplicate. *, P < 0.05 compared to WT.

Figure 2. Contractile properties of S1P3-null EDL. A, Typical twitch tension curves of wild type (WT) and S1P3-null EDL. B, Typical tetanic tension curves of wild type and S1P3-null EDL. C, Mean twitch (Pt) and tetanic (P0) tensions of wild type and S1P3-null EDL. D, Force/frequency curves of wild type (open circles) and S1P3-null (grey circles) EDL. Force is expressed relative to the maximum tetanic force. Data are reported as mean ± SEM. Four experiments were carried out in each group. *, P < 0.05 compared to wild type.

Figure 3. Fatigue characteristics of EDL fibers. A, Tension development during fatigue and recovery of EDL fibers from wild type mice (WT, n = 8) without and with S1P supplementation (1 μM, black circles). B, Tension development during fatigue and recovery of EDL fibers from S1P3-
null mice (n = 5) without and with 1 µM S1P supplementation (+ S1P, black circles). *, P < 0.05 compared to the corresponding wild type values of the untreated muscles shown in A; a, P < 0.05 compared to untreated S1P3-null fibers. C, Tension and stiffness drop during fatigue in WT (n = 5) and S1P3-null (n = 4) EDL fibers. Stiffness and tension are expressed relatively to their plateau value before fatigue. For fatigue and recovery protocols see Methods. Data are reported as mean ± SEM.

Figure 4. Fatigue characteristics of FDB fibers. A, Tension development during fatigue and recovery of FDB muscle fibers from wild type (WT, white circles, n = 8) and S1P3-null mice (grey circles, n = 4). The effects of 1 µM S1P supplementation (+ S1P) was measured in WT (black circles, n = 8) and S1P3-null fibers (black squares, n = 4). *, P < 0.05 compared to the corresponding wild type values of the untreated fibers. B, Effects of S1P on tension (circles) and stiffness (square) drop during fatigue in S1P3-null (n = 4) FDB fibers. Tension data are the same as in A. C, Comparison of the stiffness-tension relations during fatigue and tetanic tension rise (TTR). Stiffness and tension are expressed relatively to their plateau value before fatigue. The dashed straight line indicates the direct proportionality between tension and stiffness. Measurements on the TTR (downward triangles) and fatigue (upward triangles) were made on the same fibers, therefore the absolute fiber stiffness at plateau was the same in both cases. For fatigue and recovery protocols see Methods. Data are reported as mean ± SEM.

Figure 5. Expression of S1P receptors transcripts in wild type (WT) and S1P3-null EDL muscle after 14 days of denervation. Expression of S1P receptors in each muscle was measured by Real Time PCR and compared to that in wild type innervated muscles. ND, not detected. Results are expressed as fold changes according to the 2^(-ΔΔCt) method, utilizing murine S1P1, S1P2 or S1P3 genes in WT innervated EDL muscle as calibrator. Data are reported as mean ± SEM of three
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Figure 6. Effects of denervation on wild type (WT) and S1P3-null EDL muscle. A, Laminin staining of innervated and 14-denervated EDL muscle from WT and S1P3-null mouse. Bar = 50 µm. B, Changes of muscle fiber cross-sectional area (CSA) in EDL muscle. The comparison was done between each denervated muscle and its contralateral innervated muscle. Four-to-six experiments were carried out in each group. Data are reported as mean ± SEM. *, $P < 0.05$ compared to WT. C, Mean CSA distribution of 14-day denervated EDL from WT and S1P3-null mice. D, Changes of Myosin heavy chains (MyHC) isoform composition in denervated EDL from wild type (WT) and S1P3-null mice. Data are reported as mean ± SEM. Four-to-six experiments were carried out in each group. *, $P < 0.05$, compared to the innervated muscle.

Figure 7. Protein expression level of atrophy-related genes in innervated and in 7 and 14 day-denervated wild type (WT) and S1P3-null EDL (a.u., arbitrary units). Intensity of protein signal in each sample was normalized to that of GAPDH or α-actinin. Examples of Western blots are given above each plot. A, MuRF1 protein level; *, $P < 0.05$, compared to innervated EDL; a, $P < 0.05$, compared to 14-day denervated WT EDL. B, Atrogin 1 protein level. C, Myogenin protein level; *, $P < 0.05$, compared to the innervated muscle; a, $P < 0.05$, compared to innervated WT EDL. D, P-FoxO3/FoxO3 ratio. E, Protein changes of total Akt, activated P-Akt and of the P-Akt/Akt. *, $P < 0.05$, compared to the innervated muscle. Data are reported as mean ± SEM. Four-to-ten experiments were carried out in each group.

Figure 8. Protein expression level of atrophy-related genes in innervated and in 7- and 14-day-denervated wild type (WT) and S1P3-null EDL (a.u., arbitrary units). Intensity of protein signal in each sample was normalized to that of GAPDH. Examples of Western blots are given above each
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