Denervation in murine fast-twitch muscle: short-term physiological changes and temporal expression profiling

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Denervation in murine fast-twitch muscle: short-term physiological changes and temporal expression profiling. Physiol Genomics 25: 60–74, 2006. First published December 27, 2005; doi:10.1152/physiolgenomics.00051.2005.—Denervation deeply affects muscle structure and function, the alterations being different in slow and fast muscles. Because the effects of denervation on fast muscles are still controversial, and high-throughput studies on gene expression in denervated muscles are lacking, we studied gene expression during atrophy progression following denervation in mouse tibialis anterior (TA). The sciatic nerve was cut close to trochanter in adult CD1 mice. One, three, seven, and fourteen days after denervation, animals were killed and TA muscles were dissected out and utilized for physiological experiments and gene expression studies. Target cDNAs from TA muscles were hybridized on a dedicated cDNA microarray of muscle genes. Seventy-one genes were found differentially expressed. Microarray results were validated, and the expression of relevant genes not probed on our array was monitored by real-time quantitative PCR (RQ-PCR). Nuclear- and mitochondrial-encoded genes implicated in energy metabolism were consistently downregulated. Among genes implicated in muscle contraction (myofibrillar and sarcoplasmic reticulum), genes typical of fast fibers were downregulated, whereas those typical of slow fibers were upregulated. Electrophoresis and Western blot showed less pronounced changes in myofibrillar protein expression, partially confirming changes in gene expression. Isometric tension of skinned fibers was little affected by denervation, whereas calcium sensitivity decreased. Functional studies in mouse extensor digitorum longus muscle showed prolongation in twitch time parameters and shift to the left in force-frequency curves after denervation. We conclude that, if studied at the mRNA level, fast muscles appear not less responsive than slow muscles to the interruption of neural stimulation.

denervation; cDNA microarray; mitochondria; myosin

*MUSCLE ATROPHY IS AN ADAPTIVE RESPONSE TO REDUCED CONTRACTILE

activity, reduced mechanical load, or modified environmental

conditions (hypoxia, uremia, endotoxin action, etc.). The hall-

marks of any type of atrophy are decreased muscle mass and

reduced contractile force. In the last few years, several global

expression studies carried out in various models of muscle

atrophy have revealed the molecular changes that accompany

the muscle alterations in response to food deprivation (33),

aging (71), disuse (4, 62), systemic diseases (40), or micro-

gravity (51). Interestingly, a small subset of genes is implicated in

all atrophy models, whereas most differentially expressed
genes are specific to various forms of atrophy (40).

It is well known that neural stimulation is one of the most

important determinants of gene expression in skeletal muscles

(for a review, see Refs. 19 and 54) and this makes it very

interesting to study how gene expression changes when neural

control is removed. In slow postural muscles such as soleus,

the low-frequency neural discharge is the essential factor in
determining and maintaining the phenotype, whereas in fast

muscles, the role of neural control is more controversial (54).

Actually, fast muscle fibers receive ~100-fold less neural

stimuli than slow muscle fibers do (25).

Denervation causes atrophy and change in fiber phenotype in

fast as well as in slow muscles. The molecular mechanisms

responsible for these changes are only partially understood; it

is, however, clear that atrophy in response to denervation is
different in slow and fast muscles. In denervated rat soleus,

both slow and fast fibers undergo a very rapid atrophy (17),

which is associated with a marked transformation of the muscle
toward a fast phenotype (28, 41). In denervated extensor
digitorun longus (EDL) and tibialis anterior (TA) muscles of

adult rats, atrophy is not less severe, but it affects specifically

fast fibers, whereas slow fibers seem more resistant (9, 17).

In the same muscles, a progressive shift of myosin heavy chain

(MyHC) isoform expression along the transition pathway

2B

2A

3

3

2A

3

1 has been reported (28). In a recent study on rat EDL
denervation (21), we have observed early changes in twitch
time course that were accompanied by changes in sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA), parvalbumin, and calsequestrin expression. Electron microscopy has shown in rat fast muscle a proliferation of the junctional structures (triads and diads) that occurs a few days after denervation (58, 63). Thus, despite the limited amount of nervous stimuli received, fast muscles also respond to denervation, and the excitation-contraction coupling mechanism seems to be more sensitive than the contractile mechanisms to removal of neural control. Mitochondria and energy production systems are also affected by denervation in fast as well as in slow muscles (43, 72, 79). Evidence is, however, still fragmentary, as each study has focused on a single specific functional or structural aspect.

The development of the adaptive response requires several
days or even weeks, and this makes necessary the identification
of the precise time frame in which gene expression is studied.
For instance, it is known that denervation of fast muscles
produces a rapid upregulation of myogenic regulatory factors
(MRFs) (6, 69), whereas the gradual transition in fiber type

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occurs more slowly (74). It is also important to take into account that data on denervation are mostly derived from studies on young adult rat muscles, and results obtained in other species (e.g., rabbit) suggest that the denervation-induced changes may be species, muscle, or developmental stage specific (15).

Many genes differentially expressed in denervated muscle have been identified so far (6, 64). However, only one high-throughput study on gene expression in denervation has been published and is focused on comparing gene expression in denervated neonatal rat muscles (16 days after denervation) with gene expression in space flight atrophy (51). A more complete mapping of the expression changes in an appropriate time window might provide information relevant to understanding the mechanisms leading to atrophy and fiber phenotype change after denervation in muscles.

In this study, we extended our previous work (21) on early changes induced by denervation in fast muscles. By means of real-time quantitative PCR (RQ-PCR) and cDNA microarray expression profiling, we followed changes in the expression of ~2,000 genes in murine TA for 2 wk after surgical denervation. In particular, we focused on genes implicated in contractile and metabolic functions. A few essential genes were further analyzed at the protein level, and the possible functional relevance was also tested with physiological experiments. The results provided the first general timeline of changes in gene expression after denervation in a fast muscle and revealed that removal of neural control determines coordinated and sequential changes in gene expression.

METHODS

Animals. Denervation was performed in adult (3-mo old, n = 32) CD1 mice anesthetized by an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (20 mg/kg). The sciatic nerve was cut unilaterally at the level of trochanter. About 0.5–1 cm of the peripheral nerve stump was removed, and the proximal stump was sutured into a superficial muscle to avoid reinnervation and obtain a permanent denervated muscle. The experiments were performed on young adult rat muscles, and results obtained in other species (e.g., rabbit) suggest that the denervation-induced changes may be species, muscle, or developmental stage specific (15).

Microarray hybridization. Total RNA for each sample (8 μg) was reverse transcribed with SuperScript II (Invitrogen) and oligo-d(T)21 as primer in the presence of either Cy3-dCTP or Cy5-dCTP (Amersham Biosciences, Uppsala, Sweden). Labeled cDNAs were further purified with a GenElute PCR Clean-Up kit (Sigma). Target cDNAs destined for paired analysis (i.e., denervated vs. contralateral) were mixed and precipitated with ethanol and salts. Competitive hybridizations were carried out in a dual slide chamber (HybChamber; GeneMachines, San Carlos, CA). Pellets of purified, labeled cDNAs were dissolved in 40 μl of hybridization buffer (Northern Max, Ambion) and applied on the microarrays, covered with a 22 × 22-mm glass coverslip. Hybridization proceeded overnight at 42°C by submersion of the chamber in a high-precision water bath (W28; Grant, Cambridge, UK). At the end of hybridization, slides were dived for 5 min sequentially in buffer A (0.1 × SSC-0.1% SDS) at 65°C, in buffer B (0.1 × SSC-0.1% SDS) at room temperature, and finally in 0.1 × SSC.

Analysis of expression data. Digital images were generated in a GSI Lumonics LITE dual confocal laser scanner (ScanArray Microarray Analysis software) and processed with QuantArray Analysis software (GSI Lumonics, Ottawa, Canada). Intensity values were processed at the SNOMAD web site (http://pevsnerlab.kennedykrieger.org/sномадinput.html). Normalized values were calculated for each spot and converted to a logarithmic scale. Final values correspond to log2 ratio of the normalized intensities; positive numbers correspond to RNA overexpressed in denervated muscles. The microarray data have been deposited in the GEO database (series accession no. GSE1893). To identify differentially expressed genes, we performed comparison statistical tests implemented in significance analysis of microarray (SAM) (68). SAM analyses were performed on the level of cDNA probes, thus grouping replicate data of the same probe (4 spots on the array). The following criteria were adopted to filter the row data. 1) After visual inspection of MA scatter plots generated by the SNOMAD software, spots with a weak hybridization signal (normalized meanlogint values < -5, which corresponded approximately to absolute intensities <700 in at least 1 channel) were considered not reliable. 2) Empty spots and cDNA probes with a defective signal were not analyzed. The multiclass SAM analysis was performed on a selected set of 2,056 cDNA probes as follows: four
different classes have been set, one for each time point, and genes differentially expressed across all classes (namely, genes differentially expressed in at least 1 class) were selected with a false discovery rate (FDR) <1%. On the other hand, a one-class SAM analysis allowed selection of genes up- and downregulated at each single time point. Results of the two tests were consistent. Probes associated with higher FDRs are shown in Supplemental Materials (see below).

**Real-time quantitative PCR.** Real-time quantitative PCR (RQ-PCR) based on the SYBR Green chemistry (Applied Biosystems, Foster City, CA) was carried out as described in a previous study (12). Total RNA (pooled individuals) from each time point was reverse transcribed with SuperScript II and oligo-dT(21). Diluted cDNA was amplified in 10 μl of PCR reactions in a GeneAmp 9600 thermocycler, coupled with a GeneAmp 5700 Sequence Detection System (Applied Biosystems). Gene-specific primers were selected with Primer 3 software; sequences of distinct exons were chosen to avoid amplifying contaminant genomic DNA. For each time point, we amplified samples and controls (denervated and contralateral TA) from multiple serial dilutions of the cDNA input. Differences in gene expression were evaluated by a relative quantification method, as described by Pfaffl (55). Values were normalized to the expression of the β2-microglobulin internal reference, whose abundance did not change under our experimental conditions. Normalized ratios were converted in logarithmic scale, and standard deviation was calculated according to the mathematical methodologies described by Marino et al. (42).

**Supplemental data.** The cDNA sequences, as well as more information about the Mouse MuscleArrays, are available as Supplemental Materials (available at the Physiological Genomics web site). All information relative to this study is available as Supplemental Materials: 1) RQ-PCR data; 2) a full list of differentially expressed genes detected by cDNA microarray analysis; 3) Supplemental Fig. S8 (nuclear genes with mitochondrial functions); 4) additional tables showing expression data of genes with biological functions relevant to the present study; these genes have been grouped according to Gene Ontology categories, and we noted that several muscle-specific genes have incomplete annotation.

In the Supplemental Tables, genes have been ranked according to their FDR: the lower the FDR associated, the higher the significance of the expression data. We identify five different classes: extremely significant genes (***) with a probability of being a false positive <1%; moderately significant genes (****) with probability <5% or (****) <10%; and low significant genes (**) with a probability <15% or (*) <20%. Only genes of the first class (****) are shown (see Fig. 3 and Table 2).

**Gel electrophoresis and Western blot.** Small muscle fragments from control and denervated TA [3 for each time point, also used for succinate dehydrogenase (SDH) staining and single-fiber dissection] were shattered with a ceramic pestle in liquid nitrogen and dissolved in a concentration of 2 mg/ml in SDS-PAGE solubilization buffer (62.5 mM Tris, pH 6.8, 2.3% SDS, 5% 2-mercaptoethanol, 10% glycerol). MyHC isoform composition was determined by SDS-PAGE on a 4% stacking gel and on an 8% separating gel. The fiber segments were transferred into the subsequent solutions with progressively increasing calcium concentrations. Tension was normalized to tension developed at the highest calcium concentration (pCa = 4.8) and plotted vs. the pCa values. The resulting sigmoid curve was fitted by the Hill equation: relative tension = 100/[1 + 10(pCa - pK) × n]. The parameters pK, corresponding to the pCa value at which relative tension is 50% of the maximum, and n, corresponding to the Hill slope of the curve, were determined with a least-squares fitting procedure. Myosin composition was assessed by single-fiber gel electrophoresis (66).

**Physiological analysis on intact EDL.** Intact EDL muscles were dissected from the denervated and contralateral legs of seven mice for each time point (7 and 14 days after denervation). Isometric properties were tested in vitro at 37°C. Details of the setup and the registration system have been given previously (47). Muscle responses were recorded via an AT-MIO 16 AD card, and data were analyzed by a virtual instrument created with the LabView computer program (National Instruments, Austin, TX). Twitch parameters, tension (P), contraction time (CT), and half-relaxation time (HRT), were measured. Force-frequency curves were determined by stimulating EDL muscles at 30-60-100-120-130 Hz for 500 ms. Force recorded at each stimulation frequency was normalized to the value at 130 Hz. Fatigue was induced with a 6-min-long protocol consisting of short tetani at low frequency (60 Hz for 300 ms) delivered every 3 s. Force recorded during the fatigue protocol was normalized to the initial value.

**Statistical analysis.** Data are expressed as means ± SE unless stated differently. Nonparametric Mann-Whitney test was used for paired comparison between denervated and contralateral legs. Variance analysis (ANOVA) was used for comparison between control and denervated muscles at various times. Pearson correlation coeffi-
RESULTS

Denervation atrophy in mouse TA muscle. Murine TA is almost entirely composed of fast-twitch fibers, predominantly expressing 2B and 2X MyHC isoforms with a minor component of MyHC 2A, as shown by SDS-PAGE analysis (Fig. 1A). Slow fibers, although identified by cryosection immunostaining in the deep part of the TA (data not shown), did not contain a sufficient amount of MyHC to reach the resolution level of SDS-PAGE (~3%, see Ref. 10). After resection of the sciatic nerve, there was a progressive and considerable atrophy of the denervated TA muscles, reaching ~34% of the contralateral muscles after 14 days (Table 1). The cross-sectional area of the single fibers containing MyHC 2B isolated for mechanical studies showed an even more dramatic reduction (see Fig. 4C).

To study changes in gene expression, total RNA was extracted from denervated and contralateral TA at four time points, namely 1, 3, 7, and 14 days after the surgical treatment. The total RNA content of muscles was not reduced by denervation in the time interval examined (Table 1), as reported by others (72). RQ-PCR experiments clearly demonstrated that muscle atrophy was associated with a strong early induction of the best-characterized muscle-specific ubiquitin ligases, atrogin-1/MAFbx and MuRF-1, although their expression declined after 3 days (Fig. 2A).

MyHC isoform composition of denervated TA. RQ-PCR was further used to analyze the expression of the adult sarcomeric MyHC genes. Initially, all mRNAs appeared equally reduced (Fig. 2C). Later on, MyHC 2B and, to a lesser extent, MyHC 2X remained downregulated, whereas MyHC 2A became overexpressed. Also, the slow MyHC 1 isoform appeared slightly induced (Fig. 2C). The relative amount of each MyHC isoform at the protein level was quantified by SDS-PAGE (Fig. 1B). Corresponding to the transitions observed at the mRNA level, MyHC 2A expression was significantly increased, whereas only minor and insignificant decreases of the MyHC 2B isoform were observed during the time interval examined (Fig. 1, A and B).

MRF expression. Because expression of myogenin in adult fast muscles is strongly regulated by neural activity (30, 37), we tested by RQ-PCR the expression of all MRFs. Myogenin was in fact the most strongly induced MRF on denervation; mRNAs for MyoD and MRF4 were also upregulated, although not as strongly as myogenin, while expression of Myf5 did not change (Fig. 2B).

Microarray and differentially expressed genes. To extend our analyses to other genes relevant for muscle functions, we took advantage of the microarray technology. The Mouse MuscleArrays used in this study are muscle-specific microarrays that contain a total of 2,061 cDNA probes, mostly representing mRNAs expressed at high or intermediate levels in skeletal muscle (see METHODS). Due to the composition of the probe set, this microarray provided a reliable description of genes coding for proteins involved in contractile and metabolic functions, whereas less information was available about regulatory genes controlling transcription, cellular growth, proliferation, and signaling cascades (see DISCUSSION). Total RNAs from denervated and contralateral TA muscles of the same animal were directly labeled during reverse transcription, and the cDNA target was subjected to competitive hybridizations.

After processing normalized data with a multiclass comparison statistical test, we found extremely significant expression changes (i.e., with FDR <1%; see METHODS) in 83 cDNAs, which were assigned to 71 unique gene products (Fig. 3). The genes relevant for muscle contractile and metabolic functions are listed in Table 2, whereas a full list of all differentially expressed genes can be found in the Supplemental Materials (see METHODS). To confirm the general validity of the microarray results, the expression of several differentially expressed genes was tested by RQ-PCR, with the finding of a good correlation with microarray data (Table 3).
Muscle fiber type-specific genes. Microarray analysis showed a significant downregulation of the gene (Myh4) coding for MyHC 2B at day 7, in agreement with results of RQ-PCR and found strongly reduced after 7 days (Supplemental Materials). Calmodulin expression was also generally reduced (Supplemental Materials).

To complete the analysis of the changes affecting the contractile mechanism, the expression of the Ca-calmodulin-dependent kinase Mlk, which is responsible for regulatory myosin light chain phosphorylation, was assessed by RQ-PCR and found strongly reduced after 7 days (Supplemental Materials). Calmodulin expression was also generally reduced (Supplemental Materials).

To assess whether the changes in myofibrillar gene expression were accompanied by functional changes, the contractile performance of single skinned fibers was analyzed. Despite the dramatic reduction of cross-sectional area (see Fig. 4C), tension (force/cross-sectional area) developed during maximal activation by fast fibers containing MyHC 2B was not reduced (Fig. 4A). The response to activator calcium was, however, decreased, as indicated by the shift to the right of the pCa-tension curve (Fig. 4B). No changes in troponin I and T expression at the protein level were found (see Fig. 4D, III, for troponin I; data not shown for troponin T). Therefore, the reduced calcium sensitivity might be explained by a lower phosphorylation of myosin light chain in view of the decreased expression of calmodulin and myosin light chain kinase.

Because our previous data (21) were derived from denervated rat muscles, and no information was available on denervated murine fast muscles, it was necessary to examine changes in physiological properties after denervation on fast murine muscles. Murine EDL muscles were chosen as more suitable than TA for in vitro studies (see DISCUSSION) and showed that, after denervation, twitch time parameters were progressively prolonged (see Fig. 5A). This variation was similar to that seen in denervated rat EDL (21). Accordingly, the force-frequency curve was shifted to the left, indicating that fusion occurred at lower frequencies (Fig. 5B). Taken together, these functional changes suggest that the altered expression of genes implicated in calcium kinetics (e.g., SERCA1 and parvalbumin) has immediate effects at the protein level and corresponding effects on function. Interestingly, fatigue curves show changes in the initial days after denervation (Fig. 5C). The transitory increase in tension development during the initial phases of repetitive stimulation disappears, and this can be explained by the reduced expression of calmodulin and myosin light chain kinase genes. Actually, the transient enhancement in tension development at the start of a repetitive stimulation is typical of fast muscles and is attributed to phosphorylation of myosin light chain-2 (22).

The atrophy-related genes, called "atrogenes" by Goldberg and co-workers (40, 59), include only a very few muscle-specific proteins: myosin light chain peptides, parvalbumin, and Zasp/Ldb3, a LIM domain-binding protein (40). Our study revealed that genes coding for other muscle-specific proteins including desmin, lamin A/C, myomesin, titin, and tropomyosin-β might be considered differentially expressed, if higher FDRs were accepted in the statistical test (see Supplemental Materials). Among genes with highly reliable changes in expression (FDR <1%), we found some muscle-specific genes significantly during the first 2 wk after denervation, with the exception of the slow troponin I, which was overexpressed at late stages. Among myosin-binding proteins (MyBPs), MyBP-H was persistently upregulated, whereas MyBP-C isoforms were instead downregulated: the fast isoform progressively, the slow isoform only around day 3. Myomesin 2 was also significantly downregulated.
whose roles in muscle are still controversial, as they share a nuclear and myofibrillar localization. For example, four-and-half LIM domain 1 (Fhl1 or SLIM1), B-crystallin and cysteine and glycine-rich protein 3 (Csrp3 or muscle LIM protein), which are generally more expressed in slow fibers, showed upregulation starting from day 3, whereas myozenin 1 (alias FATZ-1, calsarcin-2), a protein typical of fast fibers, showed downregulation at all time points (see Fig. 3 and Table 2).

**Energy production.** Reduced transcription of genes involved in ATP synthesis or glucose utilization is a common feature of the rapid atrophy induced by fasting or systemic diseases (40). Ten differentially expressed genes involved in energy production are listed in Table 2, and all were downregulated after denervation. Five genes (Atp5b, mt-Co1, mt-Cytb, mt-Nd2, mt-Nd5) encode for proteins of the inner mitochondrial membrane (4 of them are mitochondrial encoded), taking part in electron transport and/or ATP synthesis, and three genes (Mdh2, Cs, Aco2) encode for enzymes participating in the tricarboxylic acid (TCA) cycle. The mRNAs of several glycolytic enzymes were reduced, with the muscle-specific isoforms of phosphofructokinase (Pfk) and enolase (Eno3) reaching significant levels (Supplemental Materials). Adenylate kinase and mitochondrial creatine kinase (Ckm2) were downregulated as well (Table 2). Interestingly, Pfk, enolase, and adenylate kinase (Aki) localize to the M band, where myomesin is also localized, pointing to the M band as a specific target for denervation atrophy. Myoglobin gene expression also showed significant changes, decreasing at 3 days and increasing at 14 days after denervation. A clear decrease was detected also at the protein level (Fig. 4D, II).

**Mitochondrial enzymes.** In mammals, genes on the H-strand of the mitochondrial genome are transcribed as a polycistronic precursor molecule that is later processed to give individual mRNAs for 12 different polypeptides. Because all genes in the mitochondrial genome were represented on the array (in most cases with >1 probe), we checked whether the probes that did not reach statistical significance according to the most severe criteria (FDR < 1%) also exhibited the same expression profile observed for the four genes described above (mt-Co1, mt-Cytb, mt-Nd2, mt-Nd5). The mRNAs of all 12 mitochondrial products shared a common expression profile, being progressively reduced up to 7 days after denervation (Fig. 6). At 14 days, a
Table 2. Differentially expressed genes identified by cDNA microarrays (partial list)

<table>
<thead>
<tr>
<th>Gene Symbol and Name</th>
<th>Gene ID</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Gene Ontology</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy production</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>Mitochondri</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome c oxidase 1, mitochondrial</td>
<td>17708</td>
<td>0.064±0.103</td>
<td>-1.086±0.465</td>
<td>-1.964±0.093</td>
<td>-1.167±0.186</td>
<td>mitochondria, i.m. electron transport</td>
<td></td>
</tr>
<tr>
<td>Cytochrome b, mitochondrial</td>
<td>17711</td>
<td>0.128±0.213</td>
<td>-0.739±0.220</td>
<td>-1.354±0.475</td>
<td>0.188±0.088</td>
<td>electron transport mitochondrial, i.m. electron transport</td>
<td></td>
</tr>
<tr>
<td>NADH dehydrogenase 2, mitochondrial</td>
<td>17717</td>
<td>0.232±0.124</td>
<td>-0.474±0.245</td>
<td>-1.879±0.428</td>
<td>-0.598±0.146</td>
<td>electron transport mitochondrial, i.m. electron transport</td>
<td></td>
</tr>
<tr>
<td>NADH dehydrogenase 5, mitochondrial</td>
<td>17721</td>
<td>0.380±0.225</td>
<td>-0.304±0.325</td>
<td>-1.316±0.440</td>
<td>0.035±0.234</td>
<td>electron transport mitochondrial, i.m. electron transport</td>
<td></td>
</tr>
<tr>
<td>ATP synthase, beta subunit</td>
<td>11947</td>
<td>-0.087±0.253</td>
<td>-1.370±0.224</td>
<td>-1.181±0.168</td>
<td>0.211±0.236</td>
<td>ATP synthase mitochondrial TCA cycle</td>
<td></td>
</tr>
<tr>
<td>Aconitase 2</td>
<td>11429</td>
<td>0.066±0.066</td>
<td>-1.231±0.168</td>
<td>-1.286±0.097</td>
<td>-0.270±0.153</td>
<td>mitochondrial TCA cycle</td>
<td></td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>12974</td>
<td>0.218±0.115</td>
<td>-1.085±0.177</td>
<td>-1.244±0.203</td>
<td>-0.498±0.320</td>
<td>mitochondrial TCA cycle</td>
<td></td>
</tr>
<tr>
<td>Malate dehydrogenase 2, alias Morl</td>
<td>17448</td>
<td>-0.124±0.167</td>
<td>-1.269±0.497</td>
<td>-1.194±0.407</td>
<td>-0.824±0.215</td>
<td>mitochondrial TCA cycle</td>
<td></td>
</tr>
<tr>
<td>Enolase 3, beta muscle</td>
<td>13808</td>
<td>-0.274±0.093</td>
<td>-2.981±0.230</td>
<td>-1.974±0.185</td>
<td>-2.285±0.180</td>
<td>cytosol, M-band glycolysis</td>
<td>(33a)</td>
</tr>
<tr>
<td><strong>Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenylate kinase 1</td>
<td>11636</td>
<td>0.098±0.102</td>
<td>-1.625±0.321</td>
<td>-1.348±0.259</td>
<td>-0.899±0.175</td>
<td>cytosol, M-band purine metabolism</td>
<td>(38a)</td>
</tr>
<tr>
<td>Creatine kinase, mitochondrial 2</td>
<td>76722</td>
<td>-0.237±0.115</td>
<td>-1.850±0.568</td>
<td>-1.590±0.356</td>
<td>-1.471±0.302</td>
<td>mitochondrial amino acid metabolism</td>
<td></td>
</tr>
<tr>
<td><strong>Mitochondrial proteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Myosin-binding protein H</td>
<td>53311</td>
<td>-0.214±0.205</td>
<td>1.357±0.350</td>
<td>2.094±0.340</td>
<td>1.866±0.242</td>
<td>thick-filament muscle contraction</td>
<td></td>
</tr>
<tr>
<td>Myosin-binding protein C, slow type (8030451F13Rik)</td>
<td>109272</td>
<td>-0.018±0.113</td>
<td>-1.553±0.390</td>
<td>-0.214±0.287</td>
<td>0.621±0.436</td>
<td>thick-filament muscle contraction</td>
<td></td>
</tr>
<tr>
<td>Myosin-binding protein C, fast type</td>
<td>233199</td>
<td>-0.029±0.082</td>
<td>-0.422±0.157</td>
<td>-0.975±0.216</td>
<td>-1.602±0.427</td>
<td>thick-filament muscle contraction</td>
<td></td>
</tr>
<tr>
<td>Myosin, heavy polypeptide 4 (MyHC 2B)</td>
<td>17884</td>
<td>0.062±0.145</td>
<td>-0.876±0.378</td>
<td>-2.863±0.426</td>
<td>-0.979±0.646</td>
<td>thick-filament muscle contraction</td>
<td></td>
</tr>
<tr>
<td>ATPase, Ca^++ transporting, fast twitch 1 (SERCA1)</td>
<td>11937</td>
<td>0.183±0.253</td>
<td>-0.757±0.427</td>
<td>-1.777±0.238</td>
<td>-0.523±0.188</td>
<td>sarcoplasmic reticulum regulation of muscle contraction</td>
<td></td>
</tr>
<tr>
<td>Caldesmon 1</td>
<td>109624</td>
<td>0.293±0.152</td>
<td>-0.668±0.273</td>
<td>-1.730±0.433</td>
<td>-0.307±0.209</td>
<td>membrane fraction regulation of muscle contraction</td>
<td></td>
</tr>
<tr>
<td>Tropomyosin 1, skeletal, slow 1</td>
<td>21952</td>
<td>-0.100±0.162</td>
<td>-0.197±0.311</td>
<td>2.691±3.416</td>
<td>2.888±2.778</td>
<td>muscle thin-filament muscle contraction</td>
<td></td>
</tr>
<tr>
<td>Myoglobin</td>
<td>17189</td>
<td>0.010±0.392</td>
<td>-0.928±0.475</td>
<td>0.451±0.326</td>
<td>1.594±0.413</td>
<td>muscle contraction</td>
<td>TCA cycle</td>
</tr>
<tr>
<td>Small muscle protein, X-linked</td>
<td>66106</td>
<td>-0.180±0.110</td>
<td>-0.222±0.214</td>
<td>0.413±0.308</td>
<td>1.573±0.513</td>
<td>M-band, I-band, nucleus muscle contraction</td>
<td>(33b,51a)</td>
</tr>
<tr>
<td>Fhl1: four and a half LIM domains 1</td>
<td>14199</td>
<td>-0.573±0.146</td>
<td>-1.099±0.389</td>
<td>2.051±0.165</td>
<td>2.556±0.274</td>
<td>muscle contraction nucleus, focal adhesions</td>
<td>(57a)</td>
</tr>
<tr>
<td>Myoz1: myozin 1 alias caloscan-2 (FATZ-1)</td>
<td>59011</td>
<td>-0.222±0.140</td>
<td>-1.902±0.192</td>
<td>-2.113±0.105</td>
<td>-1.536±0.239</td>
<td>muscle development</td>
<td>Z-band muscle development</td>
</tr>
<tr>
<td>Cryab: crystallin, alpha B</td>
<td>12955</td>
<td>-0.946±0.225</td>
<td>1.766±0.241</td>
<td>1.307±0.275</td>
<td>1.652±0.140</td>
<td>muscle development</td>
<td>Z-disc muscle development</td>
</tr>
<tr>
<td>Cxcr3: cysteine and glycine-rich protein 3 (MLP)</td>
<td>13009</td>
<td>-2.154±0.121</td>
<td>1.008±0.373</td>
<td>3.430±0.470</td>
<td>2.420±0.323</td>
<td>muscle development</td>
<td>Z-disc, nucleus myogenesis</td>
</tr>
</tbody>
</table>

Gene ID, Entrez Gene database [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene]. Expression, mean log2 ratio ± SD of technical replicates. Values that were significant according to 1-class significance analysis of microarray (SAM) are shown in bold. Gene Ontology, cellular localization (top; i.m., inner membrane) and biological process (bottom). Ref. No., for more information, see REFERENCES.

Partial recovery of mitochondrial gene expression occurred. To explain this, we must take into account that variations of mitochondrial mRNAs might reflect not only changes in gene transcription but also the overall density of mitochondria in the muscle fibers. Of note, SDH staining revealed that denervation induced an altered intracellular distribution of mitochondria. In particular, subsarcolemmal mitochondria were reduced compared with controls, while the SDH activity became higher in the intermyofibrillar region (Fig. 7A). A similar pattern of mitochondria location had been observed also in muscles of spaceflight rats (51). Using a well-characterized antibody that reacts only with the 13-kDa component of the complex III
Table 3. Validation of cDNA microarrays data by RQ-PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene ID</th>
<th>Method</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Probe</th>
<th>(R^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enolase 3, beta muscle</td>
<td>NM_007933</td>
<td>Microarray</td>
<td>-0.274±0.093</td>
<td>-2.981±0.230</td>
<td>-1.974±0.185</td>
<td>-2.285±0.180</td>
<td>MM1-014A09</td>
<td>0.929</td>
</tr>
<tr>
<td>Growth arrest and DNA damage-inducible 45 alpha (Gadd45a)</td>
<td>NM_007836</td>
<td>Microarray</td>
<td>0.419±0.197</td>
<td>3.831±0.206</td>
<td>2.322±0.466</td>
<td>5.272±0.270</td>
<td>MM1-017A08</td>
<td>0.971</td>
</tr>
<tr>
<td>Myosin-binding protein C, fast type</td>
<td>NM_178067</td>
<td>Microarray</td>
<td>-0.209±0.082</td>
<td>-0.422±0.157</td>
<td>-0.975±0.216</td>
<td>-1.602±0.427</td>
<td>MM1-002H05</td>
<td>0.927</td>
</tr>
<tr>
<td>Myosin-binding protein H</td>
<td>NM_016749</td>
<td>Microarray</td>
<td>-0.394±0.000</td>
<td>-1.098±0.004</td>
<td>-3.260±0.009</td>
<td>-3.744±0.131</td>
<td>MM1-012A10</td>
<td>0.759</td>
</tr>
<tr>
<td>Myozenin 1 alias calsarcin-2 (FATZ-1)</td>
<td>NM_021508</td>
<td>Microarray</td>
<td>-0.222±0.140</td>
<td>-1.902±0.192</td>
<td>-2.113±0.105</td>
<td>-1.536±0.239</td>
<td>MM1-002B05</td>
<td>0.911</td>
</tr>
</tbody>
</table>

Comparison of results obtained with microarray and real-time quantitative PCR (RQ-PCR) for 5 representative genes. Pearson correlation coefficient \( (R^2) \) has been calculated by comparison between pairs of cDNA microarray (specified probe) and RQ-PCR gene expression values. Scatter plots of RQ-PCR vs. cDNA microarray data are shown at [http://muscle.cribi.unipd.it/microarrays/atrophy](http://muscle.cribi.unipd.it/microarrays/atrophy). All \( R^2 \) values show a good level of correlation between the 2 methodologies. Gene ID, GenBank database. Microarray, mean \( \log_2 \) ratio ± SD of technical replicates. RQ-PCR, \( \log_2 \) ratio ± intra-assay SD, relative to β2-microglobulin mRNA used as internal reference (see METHODS). Experiments were performed with pooled RNA samples. Bottom: comparison of results of RQ-PCR obtained with RNA samples from single individuals and pooled from several individuals (comparison between pooled samples and average of individual samples). Standard RQ-PCR curves were first generated for the nonregulated reference gene (β2-microglobulin) and the target gene (Eno3) from serial dilutions of PCR templates. The mRNA abundance was estimated by interpolating each quantity from the corresponding standard curve. Values are \( \log_2 \) ratio ± SD of 3 replicate experiments. Values in pooled samples are close to the arithmetic average of values exhibited by single individuals. Similar results were obtained with the other 2 genes (adenylatekinase and aconitase).
proteins (7), the proteins of the mitochondrial fraction were examined by Western blot. A reduction of complex III proteins was apparent during the first week but did not reach statistical significance (Fig. 7B).

DISCUSSION

This study represents the first high-throughput analysis of the expression changes after denervation in fast muscles. Although the effects of denervation on skeletal muscles have been the object of many studies over the last 40 years, only one global expression study on denervation (51) has been published. In that case, only one time point was considered, whereas in the present study, the time course of the expression changes of ~2,000 genes was monitored during 2 wk after denervation. Denervation has distinct and, in some cases, opposite effects on slow and fast muscles. Available evidence (see Refs. 19, 54) suggests that the neural stimulation is essential to induce the slow type of gene expression, whereas it is controversial how much it is relevant to determine gene expression in a fast muscle. The present study was focused on the changes induced by the removal of nerve stimulation in fast muscles, and the TA of the mouse was chosen as a model. Before the current study, the response of murine muscles to denervation was not widely studied. Our analysis of the changes at the protein level based on electrophoresis and Western blot, plus the determination of some functional parameters, accompanied the gene expression profile obtained with cDNA microarrays, and RQ-PCR provided novel information about gene expression in response to denervation. The results show that, after the first 2 wk of TA denervation, a large number of genes changed their expression in a coordinated way, while the changes at the protein level and in the functional properties were limited.

Before a discussion of the expression data, some important features of our approach need to be pointed out. First, total RNA was extracted from whole muscle homogenates, and therefore we could not discriminate among changes occurring in specific cell types. This problem is common in expression studies on muscle tissue (24) and cannot be overcome until single-fiber analyses are feasible. Second, the cDNA microarrays (Mouse MuscleArray) chosen for the expression analysis are derived from muscle cDNA libraries, and therefore the probe set is particularly reliable for assessing changes of transcripts typical of muscle cells. Thus the emerging view of muscle plasticity is centered on contractile and metabolic functions, whereas less information can be extracted about other important classes of proteins, like components of the proteasome subunits, membrane channels implicated in electrical activity, or families of transcription factors (see Supplemental Materials). The peculiar features of the cDNA probes on Mouse MuscleArray (see METHODS) make hybridizations more specific compared with arrays using full-length cDNAs. In fact, we found a good correlation between results of microarrays and RQ-PCR for all the transcripts tested (Table 3). To aid in the comparison of our data with other global gene expression studies, the microarray data have been deposited in the GEO database (series accession no. GSE1893).

Within the time window explored, denervated TA showed clear signs of a transition from fast to slow phenotype involving several myofibrillar proteins. Combined microarray and
RQ-PCR analysis indicated a decrease in MyHC 2B mRNA and an increase in MyHC 2A mRNA, in agreement with previous results of ribonuclease protection assays in TA muscles of rats (28). In substantial agreement with changes in gene expression, a significant increase in MyHC 2A protein expression and little variation of MyHC 2B protein were detected 2 wk after denervation. It is not surprising that changes at the protein level are less pronounced than changes at the mRNA level, particularly in view of the long half-life (14.7 days) of MyHC 2B (65). A generally accepted scheme of reversible fiber type transitions assumes that fast-to-slow transforming muscles undergo a gradual replacement of the fast isoform MyHC 2B by MyHC 2A through the intermediate MyHC 2X (53, 60). The RQ-PCR results indicating overexpression of the slow MyHC isoform should be interpreted with caution, as the expression of MyHC 1 is highly dependent on the neural
discharge [see Spangenberg and Booth (61) for a review]. In the rat, a substantial increase in MyHC 1 expression has been observed only 5 wk after denervation (46). Slow fibers in the deep part of TA are more resistant to atrophy than fast fibers in the superficial part (5, 9, 17). Taking into account that, in the denervated soleus, type 1 fibers undergo a massive atrophy (17), one might speculate that slow fibers in fast muscles such as TA have a different sensitivity to neural stimulation compared with slow fibers in soleus muscle, because they are committed during early myogenesis in a nerve-independent way (50), and for this reason they remain relatively insensitive to neural influences. The greater resistance to atrophy of slow fibers might explain why genes typical of slow fibers appear overexpressed without the need to assume a change in gene expression in fast fibers. Aside from MyHC isoforms, the expression of other genes coding for myofibrillar proteins differentially expressed in fast and slow fibers also suggests a fast-to-slow transition: for example, troponin I slow is overexpressed, whereas MyBP-C fast is downregulated.

The fast muscle phenotype is not only determined by the expression of fast isoforms of myofibrillar proteins but also by specific expression of genes implicated in cytosolic Ca^{2+} regulation. The transition toward a slower muscle phenotype is evident also at this level, as parvalbumin and SERCA1 expression decreases. Among genes coding for proteins involved in excitation-contraction coupling, the gene coding for the γ1-subunit of dihydropyridine receptor (DHPR) showed a significantly increased expression (see Supplemental Materials), whereas mRNA coding for ryanodine receptor (RyR) or other triad components did not vary significantly. The lack of variation of RyR expression is in contrast to the expectations based on electron microscope observations of a rapid doubling of the contact areas between T tubules and terminal cisternae in denervated muscles (58, 63). These ultrastructural findings contrast with the lack of changes in RyR expression (52) in EDL after long-term denervation (25–50 days). A recent paper by Radzyukevich and Heiny (56) shows an increase of mRNA of DHPR in various models of disuse, including denervation of fast muscle. Other studies (57), however, do not report any variations in DHPR expression. A possible explanation of the ultrastructural findings is that triad morphological changes represent a reorganization in response to the early reduction of myofibrillar mass that likely results in only minor transcriptional changes. The changes in gene expression are, on the whole, suggestive of slower kinetics of intracellular calcium, which is consistent with the prolonged time to peak and the leftward shift of the force-frequency curve observed in denervated murine EDL (Fig. 5), a finding that is in agreement with the our previous observations in rat denervated EDL (21). The choice to analyze contraction kinetics in vitro in EDL and not in TA was due to the great thickness and the lack of a proximal tendon of the TA, which make impossible a reliable analysis in vitro. Also, a determination of contractile performance of TA in vivo is made difficult when the nerve supply is interrupted and direct electrical stimulation of the muscle is needed. Because of these difficulties, twitch time parameters, force-frequency curve, and fatigue curve were determined in EDL muscles of the same animals used for RNA preparation. EDL
can be considered a good model of TA because it is very similar to TA in its function (they are both foot dorsal flexor muscles without much of a postural role), innervation (they are both innervated by peroneus nerve), and fiber type composition: MyHC isomer analysis shows that predominant isoforms were 2X and 2B in control TA with 35 and 65%, respectively (see Fig. 1), and in control EDL with 16 and 81%, respectively.

A dramatic reduction in mitochondrial enzyme activities in denervated muscles has been reported in previous studies (26) (72). Our microarray data also show changes consistent with the view that denervation has pronounced effects on mitochondrial function and point to the transcriptional nature of those effects. In the first 3 days after denervation, all genes encoded by the mitochondrial DNA and several nuclear-encoded mitochondrial genes involved in energy production were markedly downregulated. However, during the progression of atrophy, we observed a tendency to rescue mitochondrial gene expression. After performing a cluster analysis of all genes present on the Mouse MuscleArrays with proved mitochondrial localization, we selected 50 genes that exhibited similar expression profiles even if they did not reach statistical significance (Supplemental Fig. S8). Among those genes, soluble enzymes (particularly of the TCA cycle) and membrane complexes involved in energy production through oxidative phosphorylation were particularly represented. Interestingly, some molecular components of the permeability transition pore complex, like voltage-dependent anion channel (VDAC) or adenine nucleotide translocase (ANT), were also identified by these criteria.

Distinct gene expression programs may be activated during the progression of atrophy to achieve a coordinated variation of mitochondrial functions. We noted that nuclear-encoded genes are mostly underexpressed 3 days after denervation, while the mitochondrial-encoded genes reach the lowest expression levels after 1 wk (Table 2). Analysis by computational approaches of the promoter region of the selected genes is underway to identify putative transcription factor-binding sites and modules responsible for a common gene regulation. The pathway based on peroxisome proliferator-activated receptor G co-activator, or PGC-1, appears a likely candidate. Actually, it is known that Ca2+-calmodulin-dependent kinases (CaMK) are implicated in the regulation of mitochondrial gene expression of skeletal muscle through the expression of the master regulator of mitochondrial biogenesis, PGC-1 (76). PGC-1 causes induction of nuclear respiratory factor-1 (NRF-1) and NRF-2 gene expression and co-activates the transcriptional function of NRF-1 on the promoter of the mitochondrial transcription factor TFAM (77). A role for calmodulin and CaMK in activating expression of transcription factor NRF-1 has been demonstrated also in cultured cardiomyocytes (78). Our data show that all calmodulin genes are downregulated after denervation, and cytosolic Ca2+ levels are known to be low in denervated muscles (27).

Although the major focus of the study was on genes related to energy production and contractile responses, the present results provide some information on transcriptional mechanisms responsible for denervation atrophy. In agreement with previous studies (6), we found that denervation of TA induced the rapid overexpression of muscle-specific F-box (atrogin-1/MAFbx) and RING (MuRF-1) proteins that cause accelerated proteolysis through the ubiquitin-proteasome system. Recent data point either to Forkhead box O (FOXO) (59) or to NF-kB (31) as transcriptional regulator for ubiquitin ligases in atrophying muscles. Gene-silencing studies have further shown that distinct components of the NF-kB family are recruited during atrophy of fast or slow fibers (31). Interestingly, the overexpression of atrogin-1/MAFbx and MuRF-1 peaked at day 3 and thereafter declined, suggesting that other signaling pathways and other proteolytic mechanisms might become predominant at later times, in general agreement with previous observations on disuse-induced atrophy (62).

The transcription factors of the MRF family are major regulators of the muscle phenotype, although their role in adult muscle has not yet been investigated in detail. Our RQ-PCR data on denervated TA are in good agreement with mRNA expression levels of myogenin and MyoD mRNA in the mouse TA muscle (32, 69). It is well known that MRF expression differs among various hindlimb muscles, correlating with their respective differences in fiber type composition (69); old animals have fewer satellite cells and spontaneous muscle fiber denervation, and this may in part account for differential expression levels among muscle types (37). The strong increase of myogenin mRNA after denervation is well documented (Ref. 37 and references therein), and immunohistochemical studies showed that myogenin protein is localized in nuclei of both adult fibers and satellite cells (32). The expression of MRF in satellite cells may be related with their activation, which is known to occur during early phases after denervation (see Ref. 8 for a recent review). Apparently, the overexpression of two potent inhibitors of cell proliferation, Gadd45a and Cdkn1a (or p21), also reported by Calozzo et al. (11) in denervated laryngeal muscles, might regulate but not inhibit satellite cell activation.

TA denervation was followed by upregulation of genes like Csrp3 (see Fig. 3) and Ankrd2 (RQ-PCR data not shown), which, in accordance with previous studies (2, 67), are involved in transition toward slow phenotype. These proteins are normally expressed in slow muscles (13, 75) and can mediate protein-protein interactions through LIM or ankyrin domains, respectively (48, 70). Their possible role, however, remains controversial because of their dual localization. While in the nucleus, the CRP3/MLP protein may influence the myogenic activities of the MRFs through a direct physical interaction, as demonstrated for MyoD and myogenin (36). On the other hand, CRP3/MLP can bind several cytoskeletal proteins (14), and these interactions could be important for the rearrangement of the Z-disc during redifferentiation of adult muscle fibers (73). More recently, a multiple role has been suggested for the ANKR2 protein, based on its ability to bind Z-disc proteins (e.g., telethonin) and to act as gene regulator (34). TA denervation also caused upregulation of αβ-crystallin, which is known to be more expressed in slow than in fast muscles (13). This small heat shock protein displays chaperone-like properties and might be involved in the remodeling of myofibrillar structures (18). On the whole, the upregulation in denervated TA of genes expressed at nearly undetectable levels in fast muscles with 35 and 65%, respectively (see Fig. 1), and in control EDL with 16 and 81%, respectively.
affecting mitochondrial and aerobic-oxidative metabolism. Our findings that removal of nerve supply has a great impact on gene expression in fast muscles were unexpected and open important questions concerning the signals and their intracellular mediation. Electrical activity, neurotrophic factors, and load might be the signals relevant to explain transcriptional changes.

1) The membrane electrical activity is an important regulator of the activity of Ca\(^{2+}\)-dependent transcription factors via calcium/calmodulin-regulated enzymes such as calcineurin (phosphatase 2B), CaMK, and myosin light chain kinases. In particular, activated calcineurin promotes nuclear translocation of nuclear factor of activated T cells (NFAT), which in turn controls the expression of slow fiber type genes (44). Calcineurin activity is not only influenced by intracellular calcium but also by a family of calcineurin-interacting proteins called filamin-actinin-telothonin-binding protein of the Z disc (FATZ)/calsarcins (14). Gene silencing of the slow isoform of FATZ (calsarcin-1) resulted in constitutively enhanced calcineurin signaling and an excess of type I fibers in skeletal muscles (20). Similarly, the marked downregulation of the Myoz1 gene (see Fig. 3 and Table 2), encoding the fast isoform of FATZ (calsarcin-2), might eventually lead to increased calcineurin activity in denervated TA muscles. Marked slow-to-fast transformation occurs after denervation in the soleus muscle (28), whereas only partial fast-to-slow transitions occur in the denervated TA. The huge difference in the amount of neural stimulation delivered to a slow compared with a fast muscle (~100-fold, see Ref. 25) might account for this discrepancy. Interestingly, there are also some common effects, for example the upregulation of MyHC 2A in both denervated slow muscles (28) and fast muscles (this study). Common to fast and slow muscles is the appearance of a new type of electrical activity: fibrillation appears in denervated rat TA after 54–55 h (45) and might cause an increase in electrical activity above that experienced by fast muscle fibers in physiological conditions. Among fast MyHC isoforms, the MyHC 2A promoter was shown to be by far the most responsive to intracellular calcium (1).

2) Neurotrophic factors might contribute to the control of transcription. The neuromuscular junction is a potential source for such signals. Recent studies have shown clear differences between the lack of nerve-muscle contact and the lack of the mere nerve electrical activity, suggesting a role for the ciliary neurotrophic factor to blunt muscle atrophy (32).

3) The load experienced by the leg muscles is dramatically reduced after sciatic nerve interruption. Both flexors and extensors of the foot were paralyzed, and the load applied by tonic and phasic activity of ankle extensors on TA disappeared. In this respect, it is paradoxical to note that several genes typical of slow muscles found upregulated in the present study (e.g., Ankr2d, Cryab, Csrp3) were induced in the TA muscle even after a single bout of eccentric contraction (3). It is tempting to speculate that those genes are implicated in a load-related intracellular signaling that would cause remodeling of myofibrillar structures, although the molecular mechanisms by which muscle cells are sensing such mechanical stimuli remain elusive at present. It must be underlined, however, that reduction of mechanical load is known to shift gene expression toward the fast and not the slow phenotype (29).

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