in the presence and absence of 300 μM extracellular Ba2+. Current–voltage relations for transfected and control neurons were calculated by recording whole-cell currents under voltage clamp. Voltage steps in 10-mV increments were applied every 1 s. Evoked synaptic currents were recorded using pipettes whose internal solution was supplemented with QX-314 (4 mM), and in the presence of 50–100 μM picrotoxin in the extracellular medium to isolate excitatory synapses. Brief 1-ms pulses were applied to the entire field of neurons using platinum wires separated by about 5 mm. The applied voltage was systematically varied for each recording until the response amplitude became saturated, indicating reliable activation of all axons innervating the recorded neuron. Responses were measured at a holding potential of ~70 mV to record action-potential firing, without disturbing the intracellular environment, we used the cell-attached patch-clamp method. Pipettes similar to those for whole-cell recordings were used, and tight seals were obtained without break-in. Current recordings (under voltage clamp at ~70 mV) allowed unambiguous discrimination of spikes with high signal-to-noise ratios.

**FM4-64 imaging**

To identify functional presynaptic terminals, we labelled recycling synaptic vesicles using 10 μM FM4-64 (Molecular Probes). Neurons were depolarized for 60 s using hyperkalemic solution (in mM): 78.5 NaCl, 60 KCl, 10 HEPES, 10 glucose, 2 CaCl2, 1 MgCl2, 0.05 APS (1--2--5--aminophosphonovl acid), 0.005 CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), and 0.001 TTX. Coverslips were then washed in regular extracellular medium without FM4-64 for 10 min before imaging to reduce the background fluorescence caused by non-internalized dye binding to the cell membrane. This protocol has been shown to provide an estimate of the total recycling pool of vesicles. Cells were then imaged in dye-free buffer containing blockers. Earlier experiments also included a de-staining step to release dye from vesicles, and images after de-staining were subtracted from the initial image. At the initial fluorescence and releasable fluorescence were strongly correlated, in most experiments we skipped the de-staining step. Image stacks (of 2 μm, 7–10 steps) were obtained using a confocal microscope (Olympus Fluoview attached to a BX51WI, × 0.6–0.8NA water-lens). EGFP and FM4-64 signals were acquired simultaneously using 488-nm excitation, and 510–550-nm bandpass and 585 long-pass emission filters, respectively. Transmitted light images were taken separately to identify cell bodies and processes of non-transfected neurons. We chose to measure the density of presynaptic terminals in the proximal regions of the dendrites (about 100 μm from the soma) as these could be identified unequivocally by the EGFP fluorescence. Image analysis was performed in a blind manner with respect to Kir2.1 and mutKir2.1 from the soma as these could be identified unequivocally by the EGFP fluorescence.

**Analysis**

The values for all variables reported were estimated for each cell and averaged across all cells in each group. Errors are reported as standard error of the mean. The Kolmogorov–Smirnoff test was used for all statistical comparisons.

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To test myostatin blockade in vivo, 4-week-old male mdx mice were treated with weekly intraperitoneal injections of blocking antibodies (dose 60 mg kg$^{-1}$; treated mdx group), and vehicle alone (control mdx group) for 3 months. Animals were weighed weekly and growth curves were plotted. Treated mice gained weight faster than controls and weighed significantly more than controls after 3 months of treatment (Fig. 1b), which is consistent with the predicted biological effect of myostatin blockade in vivo.

To estimate energy expenditure in live animals, we performed indirect calorimetry. Treated mice had a greater caloric output than controls (Fig. 1c), which is consistent with an increase in muscle mass and body size due to myostatin blockade. To determine whether the increase was functional, we used a Rota-rod to assess whole body muscle strength. Mdx mice have previously been shown to have an impaired ability to maintain grip and suspend themselves against gravity on the Rota-rod. Treated mice performed better than controls (Fig. 1d), which is consistent with increased functional muscle mass and intact neuromuscular coordination in vivo.

To quantify the increase of muscle mass, animals were killed and extensor digitorum longus (EDL) muscles were dissected and weighed. EDL muscles from treated mice weighed significantly more than those from controls (Fig. 2a). Interestingly, the degree of gain of muscle mass was greater than the degree of increase in body weight (Fig. 2b), suggesting that myostatin blockade had a greater proportional effect on muscle than on other organ systems of mdx mice. Weights of other muscles including gastrocnemius, tibialis anterior and quadriceps were similarly increased (data not shown). We quantified functional improvement by analysing physiological properties of muscle (Table 1). Treated mice had significantly increased force production during twitch and tetanic contraction (Fig. 2c–f). This increase in muscle strength was proportional to the degree of increase in muscle mass and offers physiological evidence for a functional improvement in mdx muscle produced by myostatin blockade in vivo.

To determine whether the increase in muscle mass and strength occurred because of hypertrophy or hyperplasia we performed morphometric examination of the EDL muscle (Table 1). Significant increases were noted in whole-muscle cross-sectional area (CSA) and single-fibre area of EDL from the treated mice, indicating true hypertrophy at the single-myofibre level. Frequency histograms of single-fibre areas revealed an overall shift of distribution towards larger areas (Fig. 2g). No significant difference was noted in the number of muscle fibres, total number of nuclei or the nuclei/fibre ratio in either group, suggesting that the increase in muscle mass and size occurred as a result of hypertrophy and not hyperplasia, as has been noted in dominant-negative myostatin mice. A slight increase was noted in the number of centrally nucleated fibres (CNF) in treated mice, indicating fibres that had undergone either regeneration and/or changes in the state of progenitor cell commitment. Because CNF are considered to be more resistant to necrosis, their greater proportion might be mechanistic in the improvement in muscle function noted in this study. Interestingly, increased regeneration and/or myogenesis in extraocular muscles has recently been suggested as an important mechanism for the

Table 1: Comparison of physiological and morphometric properties of EDL muscle

<table>
<thead>
<tr>
<th></th>
<th>C57BL/10</th>
<th>Control mdx</th>
<th>Treated mdx</th>
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<tbody>
<tr>
<td>CSA (mm$^2$)</td>
<td>1.4 ± 0.6 (6)</td>
<td>1.5 ± 0.5 (12)</td>
<td>2.0 ± 0.4 (12)**</td>
</tr>
<tr>
<td>Absolute force (mN)</td>
<td>356.3 ± 126.8 (10)</td>
<td>370.7 ± 66.5 (12)</td>
<td>491.2 ± 56.5 (12)**</td>
</tr>
<tr>
<td>Specific force (mN mm$^{-2}$)</td>
<td>152.6 ± 58.0 (10)</td>
<td>138.1 ± 30.5 (12)</td>
<td>141.5 ± 22.9 (12)</td>
</tr>
<tr>
<td>ECC force decrease (% of control)</td>
<td>40.0 ± 11.9 (7)</td>
<td>39 ± 4.6 (7)</td>
<td>53.4 ± 3.8 (4.71)**</td>
</tr>
<tr>
<td>CNF (%)</td>
<td>3.5 ± 1.3 (1,554)</td>
<td>4.16 ± 10.3 (4.661)</td>
<td>53.4 ± 3.8 (4.71)**</td>
</tr>
<tr>
<td>Single-fibre area (μm$^2$)</td>
<td>1,050.5 ± 578.6 (4.661)</td>
<td>1,329.1 ± 785.1 (4.71)**</td>
<td></td>
</tr>
<tr>
<td>Number of myofibres</td>
<td>n.d.</td>
<td>786.2 ± 167.0 (6)</td>
<td>777.8 ± 124.1 (6)</td>
</tr>
<tr>
<td>Number of nuclei</td>
<td>n.d.</td>
<td>2,266 ± 339.6 (6)</td>
<td>2,158 ± 1,058.6 (6)</td>
</tr>
</tbody>
</table>

Results are presented as means ± s.d.; numbers in parentheses are n; asterisks, statistical significance (**P < 0.01). CSA, cross-sectional area; ECC, Eccentric contraction; CNF, centrally nucleated fibres; n.d., not determined.
clinical and pathological sparing of this muscle group in DMD\textsuperscript{17,18}. To examine for histological evidence of improvement, we sectioned the diaphragm in addition to EDL, because the diaphragm rather than EDL shows degeneration and fibrosis by 16 weeks\textsuperscript{19}. Histological analysis revealed insufficient foci of degeneration in EDLs of either group to allow comment on whether improvement had occurred (data not shown). However, diaphragm from treated mice (Fig. 3b) showed a decrease in degenerative changes and cellular infiltration compared with controls (Fig. 3a). The inset in Fig. 3b shows diaphragm from normal C57BL/10 mice for comparison. Immunohistochemistry (Fig. 3c) revealed normal concentrations of utrophin enrichment at the synaptic regions of the muscle, suggesting that myostatin blockade ameliorated the dystrophic phenotype via a utrophin-independent mechanism\textsuperscript{20,21}. No differences in utrophin expression were detected by immunoblotting either (data not shown). To ascertain improvement in the pathological status of the whole skeletal musculature, we analysed serum creatine kinase (CK). Elevated CK concentrations are consistently noted with dystrophin deficiency in \textit{mdx} mice and humans owing to sarcolemmal damage\textsuperscript{4,22}. At the start of the trial both groups had marked elevations of serum CK compared with normal C57BL/10 mice. However, after 3 months of myostatin blockade \textit{in vivo} there was a marked decline in serum CK concentrations in treated mice (Fig. 3d), to almost normal. These decreases in muscle degeneration and serum CK offer histological and biochemical evidence for a functional improvement in \textit{mdx} muscle produced by myostatin blockade \textit{in vivo}.

**Figure 2** Increase in muscle mass and strength by myostatin blockade. Comparisons of EDL weight (a), EDL/body weight index (b), twitch force (c, d), tetanic force (e, f) and single-fibre areas (g), between treated \textit{mdx} mice (red) and control \textit{mdx} mice (blue). Treated mice had significantly increased EDL weight (19.72 ± 1.76 versus 14.63 ± 2.42 mg; \(n = 12; P < 0.0001\)), had an increased EDL/body weight index (0.6 ± 0.08 versus 0.5 ± 0.07; \(n = 12; P < 0.014\)), generated greater twitch force (c, representative traces; d; 177.32 ± 28.95 versus 132.38 ± 43.07 mN; \(n = 12; P < 0.03\)) and tetanic force (e, representative traces; f; 491.23 ± 56.54 versus 370.74 ± 66.47 mN; \(n = 12; P < 0.003\)), and had larger single-fibre area distribution (g), than controls.

**Figure 3** Decreased muscle damage resulting from myostatin blockade. a, b, Control \textit{mdx} mice (a) had significantly greater pathological changes (arrowheads) in the diaphragm than treated mice (b), as evidenced by a lack of cellular infiltration and fibrosis. Inset, normal C57BL/10 mice. Haematoxylin–eosin staining; scale bar, 100 μm. c, Treated mice (right panels) show no increase in utrophin compared with controls (left panels). Synapses revealed by bungarotoxin staining. Scale bar, 50 μm. d, Treated mice (red) had significantly decreased CK concentrations compared with controls (blue; 1907.3 ± 907.37 versus 6356.9 ± 3046.57 U l\(^{-1}\); \(n = 6; P < 0.005\)). The decrease approached C57BL/10 concentrations (yellow; 1241.07 ± 539.77 U l\(^{-1}\); \(n = 4\)).
Here we have demonstrated that myostatin blockade in vivo in mdx mice achieved by pharmacological means resulted in a functional improvement of the dystrophic phenotype, by anatomical, physiological and biochemical criteria. The regimen did not completely reverse dystrophic changes; physiological parameters evaluated by ex vivo tests, such as susceptibility to damage by lengthening contractions\(^{22,24}\) were not improved. This might be related to initiating myostatin blockade 1 month after birth rather than at birth, an inadequate dosage, or an inherent limitation of the strategy itself. The nature of the improvement indicates that myostatin blockade might be beneficial for a variety of primary and secondary myopathies such as the muscular dystrophies, ageing and muscle loss due to chronic infections or immobilization. Additionally, it might prove to be a useful adjuvant for the management of systemic metabolic disorders such as obesity and diabetes mellitus\(^27\).

In comparison with conventional transgenic, cell and gene therapy approaches\(^{29,30}\), the current study used a pharmacological approach (administration of a blocking antibody) to improve the dystrophic phenotype functionally. Delivery of blocking antibodies by simple parenteral injection therefore circumvents the generation of specialized viral vectors to deliver gene products. Furthermore, our approach may obviate the problems concerning toxicity or immune response against the vector, which has been of concern in recent therapeutic trials using conventional gene therapy\(^{29,30}\).

## Methods

### Physiological studies on isolated muscle

Physiological properties of muscle were analysed with intact ex vivo muscle from 16-week-old male mdx (C57BL/10ScSn-DMD\(^{−/−}\)) mice, as described previously\(^{24, 26}\). In brief, muscle length was adjusted to achieve maximal twitch response, and this length (\(L_a\)) was measured with vernier calipers. Eccentric contraction (ECC) force decrease was calculated from the difference of isometric force generation during the first and fifth tetani of the standard ECC protocol (supramaximal stimulus of 700 ms, 500 ms isometric phase, measured with vernier calipers. Eccentric contraction (ECC) force decrease was calculated from the difference of isometric force generation during the first and fifth tetani of the standard ECC protocol (supramaximal stimulus of 700 ms, 500 ms isometric phase, 200 ms eccentric phase; total lengthening \(L_a\); lengthening velocity 0.5 \(L_a\) s\(^{−1}\)). At the end of physiological studies, muscles were flash-frozen in isopentane cooled in liquid nitrogen, and stored at \(−80\) °C before being sectioned.

### Antibodies and histology

The mouse anti-myostatin monoclonal blocking antibody (clone JA16; Wyeth Research/ Genetics Institute) was generated against recombinant myostatin and inhibits the binding of myostatin to its receptor ActRIIB with a half-maximal inhibitory concentration of 500 nM (refs 9, 10, 12). Serial frozen sections (7–12 \(\mu\)m) were cut at mid-belly of muscle. Sections were examined after haematoxylin–eosin staining or labelling with anti-laminin antibodies and DNA-binding dye Hoechst 33825 for quantifying nuclear content and other morphological indices. Scion Image 4.02 software (http://www.scioncorp.com) was used for morphometric measurements. All cross-sectional myofibres (9,372) and antibody complexes detected using enhanced chemiluminescence (Pierce).

### SDS–polyacrylamide gel electrophoresis

Laminin antibodies and DNA-binding dye Hoechst 33825 for quantifying nuclear content and other morphological indices. Scion Image 4.02 software (http://www.scioncorp.com) was used for morphometric measurements. All cross-sectional myofibres (9,372) and antibody complexes detected using enhanced chemiluminescence (Pierce).

### Immunoblotting

Cultured C2C12 cells were treated with anti-myostatin antibody (11.5 \(\mu\)g ml\(^{−1}\); rhodamine-conjugated bungarotoxin (4 \(\mu\)g ml\(^{−1}\); Molecular Probes) and Alexa Fluor® 488 secondary antibodies (Molecular Probes) were used for immunochemistry. Differential effects of dystrophin and utrophin gene transfer in immunocompetent muscle dystrophy (mdx) mice. Physiol. Genom. 5, 393–397 (1991).

### Biochemical and functional assessment of muscle

Serum CK was measured with the indirect CK colorimetric assay kit and standards (Sigma). The Rota–rod apparatus was designed and built in the laboratory (details available from the authors on request). The apparatus consisted of a hollow plastic rod 6 mm in diameter attached horizontally to a direct-current motor. Rotation speed was calibrated to 18 r.p.m. by using a potentiometer and stopwatch. Indirect calorimetry was performed with the Oxymax Equallow system (Columbus Instruments). Mice were acclimated to the test chamber on a 12-h light–dark cycle (light on at 0700h) for 2 days, and night-time energy expenditure was measured at 15-min intervals. Settings used per cycle were as follows: airflow, 500 ml min\(^{−1}\); sample flow, 400 ml min\(^{−1}\); set time, 120 s; measuring time, 60 s; temperature, 22 °C. The caloric output (kCal h\(^{−1}\)) was calculated as 3.815 + 1.232 x respiratory exchange ratio (RER). RER is the ratio between carbon dioxide generated and oxygen consumption.

### Statistical analysis

Student’s t-test was used for determining statistical significance throughout the study. Graphical representation of data uses the following convention: mean ± s.d.; treated mdx mice in red; control mdx mice in blue; dashed line represents data from age-matched C57BL/10 normal mice.

Letters to nature

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## Competing interests statement

The authors declare competing financial interests: details accompany the paper on Nature’s website (http://www.nature.com/nature).

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