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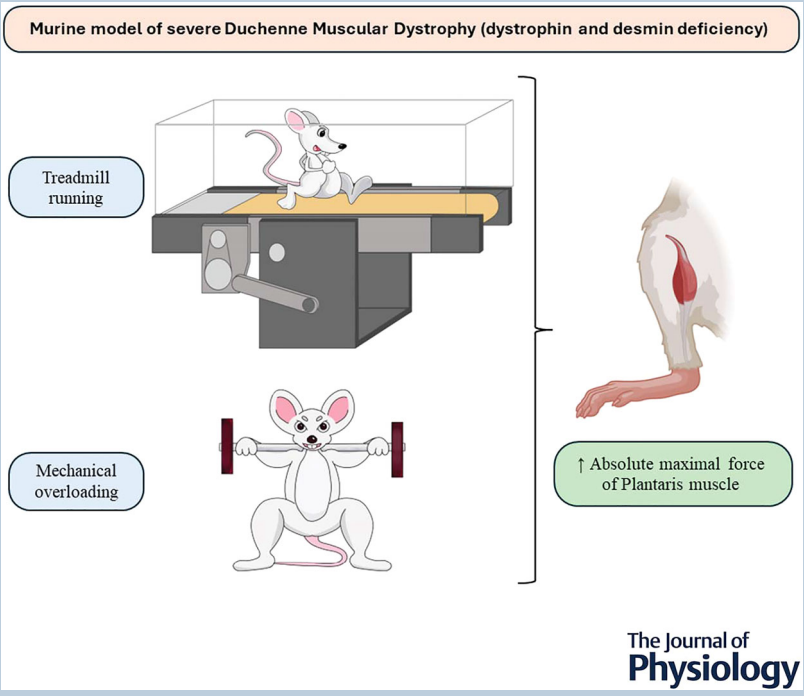

Abstract Limited knowledge exists regarding the chronic effect of muscular exercise on muscle function in a murine model of severe Duchenne muscular dystrophy (DMD). Here we determined the effects of 1 month of voluntary wheel running (WR), 1 month of enforced treadmill running (TR) and 1 month of mechanical overloading resulting from the removal of the synergic muscles (OVL) in mice lacking both dystrophin and desmin (DKO). Additionally, we examined the effect of activin receptor administration (AR). DKO mice, displaying severe muscle weakness, atrophy and greater susceptibility to contraction-induced functional loss, were exercised or treated with AR at 1 month of age and *in situ* force production of lower leg muscle was measured at the age of 2 months. We found that TR and OVL increased absolute maximal force and the rate of force development of

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the plantaris muscle in DKO mice. In contrast, those of the tibialis anterior (TA) muscle remained unaffected by TR and WR. Furthermore, the effects of TR and OVL on plantaris muscle function in DKO mice closely resembled those in *mdx* mice, a less severe murine DMD model. AR also improved absolute maximal force and the rate of force development of the TA muscle in DKO mice. In conclusion, exercise training improved plantaris muscle weakness in severely affected dystrophic mice. Consequently, these preclinical results may contribute to fostering further investigations aimed at assessing the potential benefits of exercise for DMD patients, particularly resistance training involving a low number of intense muscle contractions.

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Abstract figure legend Beneficial effect of exercise in a mouse model of severe Duchenne muscular dystrop.

Key points

- Very little is known about the effects of exercise training in a murine model of severe Duchenne muscular dystrophy (DMD). One reason is that it is feared that chronic muscular exercise, particularly that involving intense muscle contractions, could exacerbate the disease.
- In DKO mice lacking both dystrophin and desmin, characterized by severe lower leg muscle weakness, atrophy and fragility in comparison to the less severe DMD *mdx* model, we found that enforced treadmill running improved absolute maximal force of the plantaris muscle, while that of tibialis anterior muscle remained unaffected by both enforced treadmill and voluntary wheel running.
- Furthermore, mechanical overloading, a non-physiological model of chronic resistance exercise, reversed plantaris muscle weakness.
- Consequently, our findings may have the potential to alleviate concerns and pave the way for exploring the prescription of endurance and resistance training as a viable therapeutic approach for the treatment of dystrophic patients. Additionally, such interventions may serve in mitigating the pathophysiological mechanisms induced by physical inactivity.

Introduction

Skeletal muscle contraction constitutes a highly intricate phenomenon, involving a myriad of structures and processes that operate in a coordinated manner to generate external force. Maximal force and rapid force development capacity are paramount for various activities of daily living, particularly among individuals with compromised functional capacity, such as patients with Duchenne muscular dystrophy (DMD). Decreased leg muscle function in ambulatory DMD patients would cause, for example, a reduction in walking speed and increase the risk of falling following a slip. DMD is a severe neuromuscular disease caused by mutations in the gene encoding dystrophin, a costameric protein playing a role in lateral force transmission and muscle integrity (Gumerson & Michele, 2011; Hughes et al., 2015; Lynch, 2004; Ramaswamy et al., 2011). In murine models of DMD, dystrophic skeletal muscle exhibits weakness, that is reduced maximal force capacity (Deconinck et al., 1998;

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Hakim et al., 2017; Head et al., 1994; Moens et al., 1993), and absolute lower maximal rate of force development (Nelson et al., 2018). Additionally, murine dystrophic muscle displays a greater fragility with respect to maximal contractions, in particular eccentric contractions (braking contractions), resulting in an increased immediate loss of muscle function and eventually muscle histological damage (Brussee et al., 1997; Deconinck et al., 1998; Hakim et al., 2017; Head et al., 1994; Moens et al., 1993). Owing to this greater fragility, physicians hesitate to prescribe exercise to DMD patients due to concerns that the repetitive cycles of muscle degeneration/regeneration induced by chronic exercise might deplete satellite cells, impeding muscle regeneration and potentially accelerating disease progression (degeneration) (Sacco et al., 2010).

Not surprisingly, dystrophin deficiency leads to a decline in voluntary locomotion in the 'classic' *mdx* mouse, a murine model with mild DMD (C57BL/10ScSn-*Dmd mdx*/J), characterized by hypertrophy (increased muscle weight) rather than muscle atrophy (Dupont-Versteegden et al., 1994; Hara et al., 2002). Given the established role of physical activity in shaping skeletal muscle physiological characteristics, it is plausible that the observed reduced maximal force capacity in *mdx* mice is, in part, attributable to their relative inactivity. Supporting this notion, studies have demonstrated that experimentally induced inactivity exacerbates lower leg muscle weakness in *mdx* mice (Hourde et al., 2013). Conversely, increased physical activity is anticipated to alleviate this dystrophic trait. Indeed, chronic voluntary wheel running (WR) and chronic enforced treadmill running (TR) of low to moderate intensity have been reported to enhance lower leg muscle function in *mdx* mice, albeit with inconsistencies in the specific muscles affected (Frinchi et al., 2021; Hyzewicz et al., 2015; Markert et al., 2011; Spaulding & Selsby, 2018). Nevertheless, conflicting evidence exists, as some studies indicate a reduction in lower leg muscle function with TR in *mdx* mice (Frinchi et al., 2021; Hyzewicz et al., 2015; Markert et al., 2011; Spaulding & Selsby, 2018). These diverse outcomes may be contingent upon the muscle under investigation, the running protocol employed and the age of the mice. To illustrate this variability, we previously reported an increase in the absolute maximal force of the tibialis anterior (TA) muscle following 4 months of WR initiated in 1-month-old *mdx* mice (Hourde et al., 2013). However, this effect was not observed with a 1 or 3 month WR regimen in older *mdx* mice (Ferry, Benchaouir, et al., 2015; Monceau, Delacroix, et al., 2022). Regardless, it can be criticized that these studies did not use a murine model of severe DMD, with a functional deficit more closely resembling that of DMD patients. A new murine DMD model exhibiting more pronounced weakness, the D2-*mdx* mouse, has recently been developed. Unlike *mdx* mice, D2-*mdx* mice demonstrate reductions in both absolute and specific (absolute maximal force relative to muscle size) maximal isometric forces (Hakim et al., 2017; Hammers et al., 2020; Moutachi et al., 2023). It was recently found that 6 months of TR increased both absolute and specific maximal forces of the TA muscle in D2-*mdx* mice (Zelikovich et al., 2019) whereas 1 month of WR did not produce similar effects (Monceau, Moutachi, et al., 2022). However, D2-*mdx* mice do not fully replicate a key dystrophic feature, severe muscle wasting, as some lower leg muscles in these mice are not significantly atrophied (Hakim et al., 2017; Moutachi et al., 2023). To our knowledge, the effect of chronic running on muscle function in a murine model of severe DMD exhibiting very pronounced weakness and notable atrophy (McGreevy et al., 2015), such as mice lacking both dystrophin and utrophin (*mdx*-utr), or both dystrophin and MyoD (*mdx*:myoD), or both dystrophin and desmin, remains undetermined. It was previously reported that *mdx*-utr and *mdx*:myoD mice were not able to perform voluntary exercise (Landisch et al., 2008; Mangner et al., 2012).

Compared to endurance-like training, a more limited number of studies have investigated the effects of resistance-like training, characterized by high-intensity muscle contractions, on locomotor muscle function in murine DMD models (Call et al., 2011; Ferry, Parlakian, et al., 2015; Joanne et al., 2012; Lindsay et al., 2019; Terada et al., 2012). Theoretically, chronic resistance exercise holds the potential for marked beneficial effects on muscle function, provided such exercise regimens do not exacerbate the dystrophic processes in skeletal muscle. Reports indicate that the function of lower leg muscles is enhanced by chronic resistance-like training in *mdx* mice (Call et al., 2011; Ferry, Parlakian, et al., 2015; Joanne et al., 2012; Lindsay et al., 2019). For instance, studies have demonstrated a substantial increase in the absolute maximal force of plantaris muscle in *mdx* mice in response to mechanical overloading (OVL), a non-physiological model simulating chronic resistance training (Roy & Edgerton, 1995), and the gain of performance is sustained even after 6 months of OVL (Ferry, Parlakian, et al., 2015; Joanne et al., 2012). To the best of our knowledge, the impact of chronic resistance exercise on muscle function in a murine model of severe DMD, characterized by pronounced weakness and atrophy, remains unexplored.

The general purpose of the present study was to determine whether TR, WR and OVL could improve lower leg muscle function (i.e. absolute maximal force and the rate of force development) in a recently generated murine model of severe DMD lacking both dystrophin and desmin (DKO mice) (Ferry et al., 2020). Previous

findings from our group indicated a 67% reduction in absolute maximal force of TA muscle and a 59% decrease in muscle weight in DKO mice compared to *mdx* mice (Ferry et al., 2020). In addition, DKO mice exhibited greater susceptibility to eccentric contraction-induced functional loss than *mdx* mice (Ferry et al., 2020). Given the markedly reduced lifespan of dystrophin–desmin double knockout mice (Banks et al., 2014), the different forms of chronic exercise (TR, WR and OVL) were conducted in the present study between 1 and 2 months of age. This time frame corresponds to a peak of fibre degeneration/regeneration in *mdx* mice and D2-*mdx* mice (Duddy et al., 2015; Hammers et al., 2020; Vallese et al., 2013), although confirmation of this pattern in DKO mice remains pending. Furthermore, we compared the effects of TR and OVL between DKO and *mdx* mice to determine whether they could be affected by disease severity. Additionally, we analysed the effect of activin receptor administration (AR), known to increase muscle growth in murine dystrophic mice (Hoogaars et al., 2012), to evaluate its potential for improving absolute maximal force in DKO mice. Our results indicated that both TR and OVL increased absolute maximal force of the plantaris muscle in DKO mice, while that of the TA muscle remained unchanged by TR and WR. The effects of TR and OVL in DKO mice closely resembled those observed in *mdx* mice. Furthermore, AR also increased absolute maximal force of TA muscle in DKO mice, but to a lesser extent than OVL in the case of the plantaris muscle.

Methods

Ethical approval

The authors understand the ethical principles under which *The Journal of Physiology* operates, and the work presented in this study complies with the animal ethics checklist as outlined in Instructions to Authors. All experiments conform to the principles and regulations as described by Grundy (2015). They were carried out according to the guidelines laid down by the institution's animal welfare committee, in compliance with National and European legislation, under the licence APAFIS#21554-2019071912051421 v13 (French Ministry of National Education, Higher Education and Research).

Animals and experimental design

Breeding and housing of the animals were conducted in the departmental animal facility, with free access to water and rodent laboratory chow. The animal facility was specific pathogen-free, maintaining a 12 h light/12 h dark cycle. We previously generated desmin-deficient *mdx* mice (Ferry et al., 2020) by crossing previously generated desmin knock-out (DesKO) (Li et al., 1996, 1997) (C57Bl/6) with *mdx* mice (C57BL/10ScSn-*mdx*/J). Female double knock-out (DKO) mice, lacking both dystrophin and desmin, were obtained by inbreeding *mdx*:Des$^{+/-}$ mice. Genotyping was determined using standard PCR as previously described (Li et al., 1996; Shin et al., 2011). Due to the reduced lifespan of DKO mice (Banks et al., 2014; Ferry et al., 2020), female DKO mice with a hybrid background (C57Bl/6 × C57Bl/10) were utilized at 1 month of age and studied at the age of 2 months. Female DKO mice were chosen empirically due to their observed survival beyond 2 months, unlike their male counterparts. The study of Banks et al. (2014) reported a median survival of 76 days for male *mdx4cv* mice lacking desmin. No notable mortality or morbidity was observed during the duration of the present experiment in any of the mouse groups. Similar to males, dystrophin-deficient female mice exhibited dystrophic features compared to wild-type mice (lower specific maximal force, greater susceptibility to contraction-induced functional loss and increased fibrosis) (Hakim & Duan, 2012; Hourdé et al., 2013). Four separate experiments were conducted to assess the effects of TR (Fig. 1*A*), WR (Fig. 1*B*), OVL (Fig. 1*C* and *D*) and AR (Fig. 1*E*), each lasting 1 month, on the function of the lower leg muscle (tibialis anterior or TA muscle, and plantaris muscle). In each experiment, 1-month-old mice were randomly assigned to different control and experimental groups (exercise or AR treatment) as shown in Fig. 1. In certain experiments, female *mdx* mice of the same age and background (Mdx) were included to compare the effects of chronic exercise (TR and OVL) between DKO and Mdx mice (Fig. 1*A* and *D*).

Enforced treadmill running (TR)

Throughout the 1 month training period, the duration of the enforced treadmill running session and running speed were progressively increased from 10– to 60 min and from 5– to 20 cm/s, respectively, with the aim to avoid the exhaustion of the DKO mice (DKO+TR mice, $n = 5$). Both female DKO+TR and Mdx+TR ($n = 6$) mice completed five sessions per week (Fig. 1*A*). During the acclimatization period to treadmill running, the duration was 10 min at a speed of 5 cm/s. Training duration was 3 weeks. By the end of the 2nd, 3rd and 4th weeks, DKO+TR mice successfully ran for 45 min at speeds of 10, 15 and 20 cm/s respectively. Mdx+TR mice followed an identical exercise regimen to DKO mice, as they ran together on the same motorized treadmill. The assessment of lower leg muscle function (TA and plantaris muscles) in DKO+TR, Mdx+TR, DKO ($n = 6$) and Mdx ($n = 4$) mice was conducted 1 month after the initiation of enforced running.

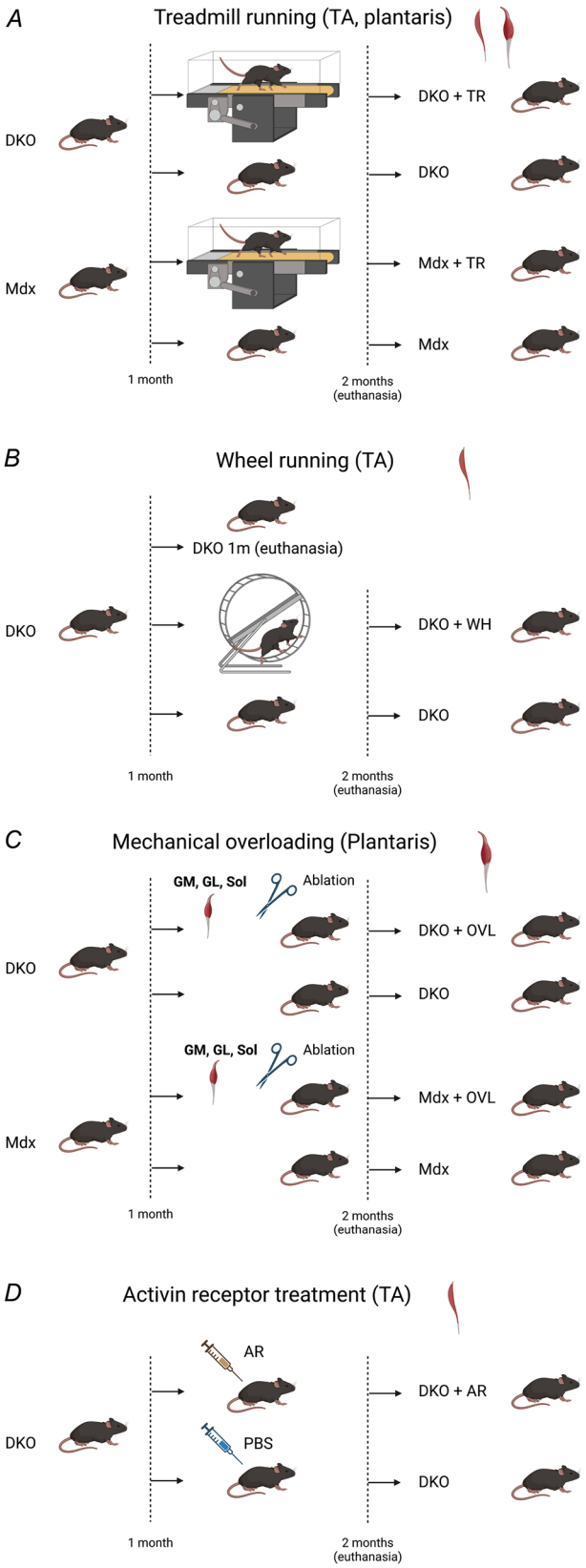

Figure 1. Experimental design of the study
The four experiments of the study were performed to determine the effects of treadmill running (*A*), wheel running (*B*), mechanical overloading (*C*) or activin receptor administration (*D*) on muscle function in DKO mice. AR: activin receptor administration; GL: lateral gastrocnemius muscle; GM: medial gastrocnemius muscle; OVL: muscle overloading; PBS: phosphate-buffered saline; Sol: soleus muscle; TA: tibialis anterior muscle; TR: treadmill running; WR: wheel running.

Voluntary wheel running (WR)

Female DKO mice were individually housed in cages equipped with a running wheel and they were either allowed (DKO+WR) to run freely for 1 month or kept sedentary. TA muscles from DKO+WR ($n = 6$) and DKO ($n = 4$) mice were measured 1 month after the initiation of voluntary running. Additionally, we measured the TA muscle function of 1-month-old DKO mice ($n = 4$) (Fig. 1*B*).

Mechanical overloading (OVL)

For the mechanical overloading (OVL) procedure, female mice were anaesthetized with isoflurane. Plantaris muscles of both legs in DKO+OVL ($n = 7$) and Mdx+OVL ($n = 7$) mice were subjected to mechanical overloading for 1 month through surgical removal of soleus muscles, along with a significant portion of the medial and lateral gastrocnemius muscles, as described (Joanne et al., 2012, 2021) (Fig. 1*C*). Plantaris muscle function was measured 1 month after the surgery in female DKO+OVL, Mdx+OVL, DKO ($n = 4$) and Mdx ($n = 5$) mice.

Activin receptor administration (AR)

Soluble ligand-binding domain of type IIb activin receptors fused to the Fc domain of IgG (AR) was synthesized to inhibit myostatin/activins and stimulate muscle growth, following previously established protocols (Hoogaars et al., 2012; Hulmi et al., 2013). In female DKO mice, AR or phosphate-buffered saline (PBS) was intraperitoneally injected once a week with a 5 mg/kg dose of AR (DKO+AR). TA muscle function was assessed 1 month after the initiation of the first injection in female DKO+AR ($n = 6$) and DKO ($n = 5$) mice (Fig. 1*D*).

Lower leg muscle function

Lower leg muscle function was assessed by measuring the *in situ* TA or plantaris muscle contraction in response to nerve stimulation, as described previously (Ferry et al., 2014; Roy et al., 2016). The plantaris muscle serves as an ankle plantar flexor, exhibiting a distinct function compared to the plantar extensor TA muscle. In one experiment (TR), both TA and plantaris muscles

were measured, in the same animal (Fig. 1*A*). Force measurement for the plantaris muscle was conducted a few minutes after that of the TA muscle. Mice were anaesthetized using pentobarbital (60 mg/kg, I.P.). Body temperature was maintained at 37°C through radiant heat. Knee and foot were secured with pins and clamps and the distal tendon of the muscle was connected to a lever arm of a servomotor system (305B, Dual-Mode Lever, Aurora Scientific, Bristol, UK) using a silk ligature. Sciatic nerve was proximally crushed and distal stimulation was applied by a bipolar silver electrode utilizing supramaximal square wave pulses of 0.1 ms duration. We measured absolute maximal force that was generated during isometric contractions in response to electrical stimulation (frequency of 75–150 Hz, train of stimulation of 500 ms). Absolute maximal isometric force (P0) was determined at L0 (length at which maximal tension was achieved during the tetanus). Furthermore, absolute maximal force was normalized to muscle mass providing an estimate of specific maximal force (sP0). The ability to rapidly generate muscle force was also assessed, using two parameters. It is an important functional parameter, which allows a subject, for example, to avoid an obstacle or a fall. First, the rate of force development (RFD), i.e. the slope (dF/dt, mN/s) was measured when the force increased from 0% to 25% of P0. Second, we also determined the time (ms) to achieve 25% of P0, in order to measure this ability independently of the force produced. The slope and the time gives an indication of how fast absolute force can be delivered and the contractile properties of the muscle, respectively.

In certain experiments, susceptibility to contraction-induced functional loss was additionally assessed based on the force drop resulting from eccentric (lengthening) contractions. Sciatic nerve was stimulated for 700 ms (150 Hz). A maximal isometric contraction of the muscle (TA and plantaris muscles) was initiated during the first 500 ms (at L0). Subsequently, muscle lengthening (10% L0) at a velocity of 5.5 mm/s was imposed during the last 200 ms. Muscle length was measured using a caliper. All isometric contractions were performed at the initial L0. Nine eccentric contractions of the muscle were executed, each separated by a 45 s rest period. Maximal isometric force was measured 45 s after each eccentric contraction and expressed as a percentage of the initial maximal force. Following contractile measurements, the animals were killed by cervical dislocation and muscles were weighed.

Histological markers of muscle damage

In select experiments (TR, OVL and AR), a histological evaluation was incorporated into the study. No histology measurements were performed in the WR experiment because of freezer failure. Muscles were dissected and frozen in isopentane cooled in liquid nitrogen. Transverse cryosections (7 or 10 μm thickness) were prepared from the frozen muscles. Some sections were utilized for quantification of the extracellular matrix (ECM) according to a standard protocol for Sirius Red staining, serving as a marker for ECM. For the detection of centronucleated fibres, other sections were fixed using cooled acetone. Muscle fibre contours were visualized through fluorescence excitation at 488 nm with the anti-laminin antibody L0663 (Sigma, St. Louis, MO, USA) under a Leica microscope. Nuclei were revealed by fluorescence excitation at 405 nm with DAPI (Sigma). ECM quantification was carried out using the ImageJ software (W. Rasband, U. S. National Institutes of Health, Bethesda, MD, USA, https://imagej.nih.gov/ij/, 1997–2018). The percentage of centronucleated fibres was determined through manual counting from laminin positive-fibres, using ImageJ software.

Statistical analysis

Statistical analyses were conducted using Prism v8.4.0 software (GraphPad, La Jolla, CA, USA). When two groups of data for a single variable (AR experiment) were compared, either a *t* test, or *t* test with Welch's correction (when variances between groups were different) was utilized. When three groups of data for a single variable (WR experiment) were compared, one-way ANOVA possibly followed by a *post hoc* test (Tukey test) or Brown–Forsythe ANOVA with Dunnet's T3 test (when variances between groups were different) were employed. Differences in variance between groups were assessed using the Brown–Forsythe test. When comparing groups for more than one variable (TR experiment and OVL experiment), two-way ANOVA (exercise × genotype) and Sidak test (when there was an interaction between factors) were applied. No outliers were removed during the analyses. To compare the effects of TR, WR, OVL and AR, the percentages of variation were compared, that is the values of the experimental muscles relative to those of the control muscles. Individual values were graphically presented in graphs, with mean and standard deviation (SD).

Results

Chronic enforced treadmill running had no effect on absolute maximal force of the TA muscle function

The gene encoding desmin (Des) was constitutively inactivated in *mdx* mice, resulting in the generation of double knockout mice (DKO mice) as previously described (Ferry et al., 2020). We studied 2-month-old DKO mice following 1 month of treadmill running

(DKO+TR mice) (Fig. 1*A*). Body weights remained unaffected by TR ($P > 0.05$) (Fig. 2*A*). Additionally, the effect of 1 month treadmill running in Mdx mice (Mdx+TR mice) was examined to determine whether the effect of TR differed between severe and mild murine DMD models. DKO+TR mice and Mdx+TR mice covered a distance of 7.1 km over the 1 month period.

In situ force production of the TA muscle in response to nerve stimulation was measured, to assess muscle weakness, an important functional dystrophic feature. Absolute maximal force (P0) was reduced in DKO mice compared to Mdx mice ($P \leq 0.0001$) (Fig. 2*B*), indicating a marked weakness. We also observed that absolute maximal force was similar in DKO+TR mice and Mdx+TR mice compared with DKO and Mdx mice, respectively (Fig. 2*B*). Moreover, specific maximal force (sP0) was increased by TR in DKO+TR mice ($P \leq 0.05$), but was unchanged in Mdx+TR mice (Fig. 2*C*). The ability to rapidly generate force was another crucial aspect of muscle function. Rate of force development (slope, dF/dt) was lower in DKO mice compared to Mdx mice ($P \leq 0.0001$) and remained unaltered by TR in both DKO+TR mice and Mdx+TR mice (Fig. 2*D*). Time to achieve 25% of P0 was also unchanged by TR (Fig. 2*E*), indicating that the contractile properties did not differ.

An immediate marked force drop was observed following eccentric contractions in 2-month-old DKO mice ($P \leq 0.0001$) (Fig. 2*F*) that was not reported in wild-type mice (Ferry et al., 2020). The force drop following the third eccentric contraction was even greater in DKO mice compared to Mdx mice ($P \leq 0.01$) (Fig. 2*F*), indicating a greater fragility in DKO mice. We also found that the force drop following eccentric contractions in DKO+TR and Mdx+TR mice was not different ($P > 0.05$) compared with DKO and Mdx mice, respectively (Fig. 2*F*). Additionally, muscle weights from DKO+TR mice and Mdx+TR mice did not show significant differences from DKO and Mdx mice, respectively (Fig. 2*G*).

We also assessed potential histological damage in the TA muscle induced by TR. To estimate the number of regenerated fibres, we performed labelling of central myonuclei and laminin to quantify the number of fibres with centrally located nuclei in DKO mice and Mdx mice (Fig. 2*H*). The percentage of fibres with centronuclei in DKO mice was lower compared to Mdx mice ($P \leq 0.0001$) and remained unchanged in DKO+TR mice compared to DKO mice (Fig. 2*I*). Muscle damage can result in an increase in the ECM (i.e. fibrosis). Therefore, we conducted Sirius Red staining to quantify the percentage of muscle surface area occupied by the ECM in cross-sections (Fig. 2*H*). ECM was increased in DKO mice compared to Mdx mice ($P \leq 0.0001$) but was unchanged in DKO+TR mice compared to DKO mice (Fig. 2*H* and *J*). TR had a similar effect on these histological markers in Mdx+TR mice (Fig. 2H and *J*).

Collectively, these results indicated that 1 month of TR did not alter absolute maximal force of the TA muscle in DKO mice, similar to the observed effect in Mdx mice. Moreover, it increased specific maximal force in DKO mice.

Chronic enforced treadmill running improved plantaris muscle function

As the impact of muscular exercise may vary across different muscles (Delacroix et al., 2018; Hayes & Williams, 1996; Vilquin et al., 1998), we extended our investigation to assess the effect of TR (1 month, TR) (Fig. 1*A*) on plantaris muscle function.

Interestingly we observed that both absolute ($P \leq 0.01$) (Fig. 3*A*) and specific ($P \leq 0.01$) (Fig. 3*B*) maximal forces were higher in plantaris muscle of DKO+TR mice and Mdx+TR mice compared to DKO mice and Mdx mice, respectively. When comparing the effect of TR between DKO+TR and Mdx+TR mice, the percentage of increase in absolute maximal force ($+90.4 \pm 73.3\%$ *vs.* $+48.9 \pm 49.9\%$, respectively) and specific maximal force ($+111.0 \pm 80.0\%$ *vs.* $+38.5 \pm 57.4\%$, respectively) did not exhibit differences ($P = 0.214$ and $P = 0.063$, respectively). Moreover, 1 month of TR increased the rate of force development (slope) in both DKO+TR and Mdx+TR mice ($P \leq 0.05$) (Fig. 3*C*). Time to achieve 25% of P0 was unchanged by TR (Fig. 3*D*). Muscle weight (Fig. 3*E*) was also not different in DKO+TR and Mdx+TR mice compared with DKO and Mdx mice, respectively. Additionally, the muscle histological images suggested no dramatic exacerbation of damage markers in DKO+TR mice (Fig. 3*F* and *G*), with $11.4 \pm 2.3\%$ ($n = 5$) of centronucleated fibres. ECM in DKO+TR mice was greater than in Mdx+TR mice ($P \leq 0.0001$) (Fig. 3*F*).

In summary, these results indicated that 1 month of TR increased absolute maximal force, specific maximal force and rate of force development (slope) of the plantaris muscle in DKO mice, akin to the observed effect in Mdx mice.

Chronic voluntary wheel running had no effect on TA muscle function

We also determined the effect of voluntary wheel running as the effects of exercise can vary with the type of running (Delacroix et al., 2018). Wheel running was initiated at the age of 1 month. DKO mice that engaged in wheel running (DKO+WR mice) covered a distance of 12.5 ± 0.8 km over the 1 month period (Fig. 1*B*). We observed that body weight ($P \leq 0.001$) (Fig. 4*A*) (as well as muscle weight) was increased in DKO mice and DKO+WR mice as compared to DKO 1 month (DKO 1m) mice, indicating that DKO mice were still growing between 1 and 2 months of age.

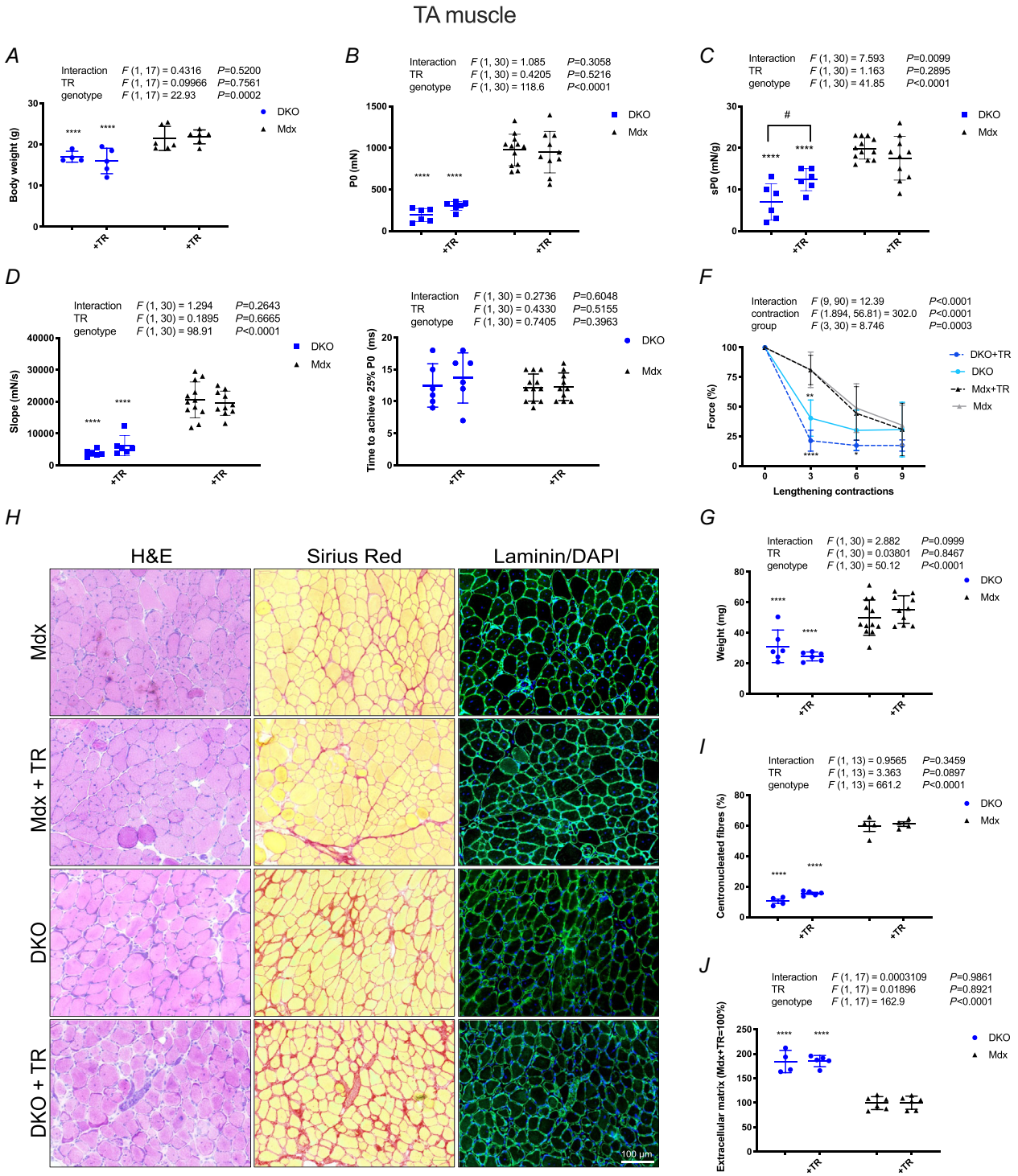

Figure 2. Chronic enforced treadmill running (TR) did not change absolute maximal force of the tibialis anterior (TA) muscle in DKO mice

A, body weight. *B*, absolute maximal force (P0). *C*, specific maximal force (sP0). *D*, rate of force development: slope d*F*/d*t*. *E*, time to achieve 25% of P0. *F*, force drop following eccentric contractions. *G*, muscle weight. *H*, representative images of muscle of DKO mice. *I*, percentage of centronucleated fibres. *J*, amount of extracellular matrix (ECM). n = 6 per group of DKO mice and n = 10–12 per group of Mdx mice for electrophysiological data; n = 4–6 per group for histological data. DKO+TR: DKO mice that ran on a treadmill; DKO: DKO mice that did not run; Mdx+TR: Mdx mice that ran on a treadmill Mdx: Mdx mice that did not run. *, **, ****: significantly different from Mdx mice (main effect of genotype, except in *F*), $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.0001$, respectively; #: significantly different from DKO mice (interaction between the two factors), $P \leq 0.05$.

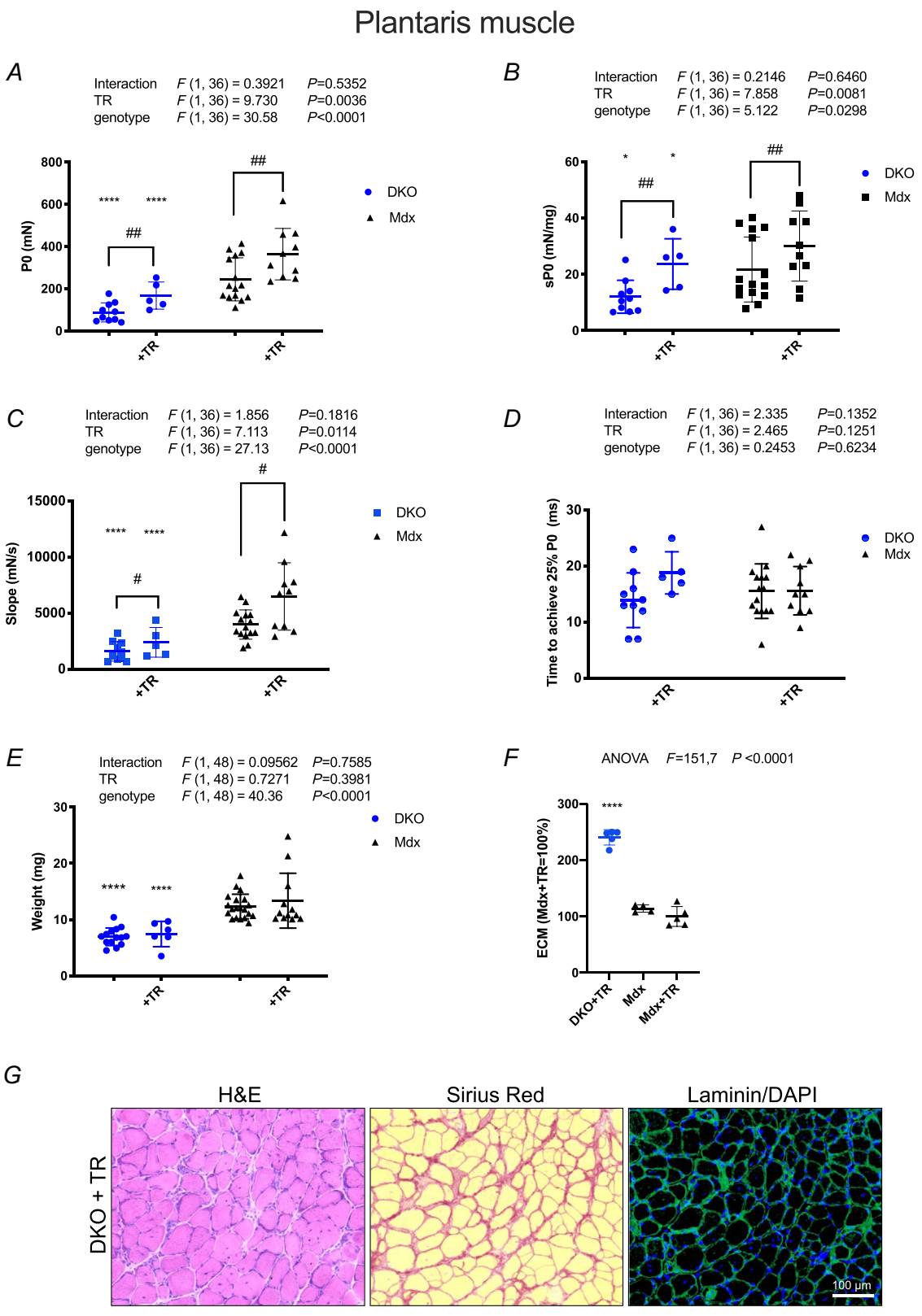

Figure 3. Chronic enforced treadmill running (TR) improved absolute maximal force of the plantaris muscle in DKO mice

A, absolute maximal force (P0). *B*, specific maximal force (sP0). *C*, rate of force development: slope d*F*/d*t*. *D*, time to achieve 25% of P0. *E*, muscle weight. *n* = 5–6 per group of DKO+TR mice, *n* = 10–14 per group of DKO mice,

n = 10–12 per group of Mdx mice and *n* = 15–20 per group of Mdx. *F*, amount of extracellular matrix (ECM). *G*, representative images of muscle of DKO+TR mice. *n* = 5 for histological data. DKO+TR: DKO mice that ran on a treadmill; DKO: DKO mice that did not run; Mdx+TR: Mdx mice that ran on a treadmill; Mdx: Mdx mice that did not run. #, ##: significantly different from mice that did not run (main effect of treadmill running), $P \leq 0.05$, $P \leq 0.01$, respectively. *, ****: significantly different from Mdx mice (main effect of genotype, except in *F*), $P \leq 0.05$, $P \leq 0.0001$, respectively.

Our findings revealed that absolute maximal force (Fig. 4*B*), specific maximal force (Fig. 4*C*), rate of force development (slope) (Fig. 4*D*), time to achieve 25% of P0 (Fig. 4*E*), force drop following eccentric contraction (Fig. 4*F*) and weight (Fig. 4*G*) of the TA muscle showed no differences between DKO+WR mice and DKO mice. In contrast to DKO mice, absolute maximal force (Fig. 4*B*) ($P \leq 0.001$) and the rate of force development ($P \leq 0.01$) (Fig. 4*D*) were increased in DKO+WR mice compared to 1-month-old DKO mice.

These results indicated that TA muscle function of DKO mice was at worst unaffected by WR.

Mechanical overloading markedly improved absolute maximal force in plantaris muscle

At the age of 1 month, ablation of synergic muscles of plantaris muscle (lateral and medial gastrocnemius and soleus muscles) was carried out, and DKO mice (DKO+OVL mice) were examined when 2 months old (Fig. 1*D*). Body weight in DKO+OVL mice was greater than in DKO mice ($P \leq 0.01$) (Fig. 5*A*). We also investigated the effect of OVL in Mdx mice (Mdx+OVL) to draw comparisons with the outcomes observed in DKO mice.

The results indicated that in the plantaris muscle absolute maximal force ($P \leq 0.0001$) (Fig. 5B) and specific maximal force ($P \leq 0.001$) (Fig. 5*C*) were notably increased in DKO+OVL mice compared with DKO mice. Furthermore, force drop following the third eccentric contraction ($P \leq 0.05$) (Fig. 5*D*) and muscle weight (+77%) ($P \leq 0.0001$) (Fig. 5*E*) were increased in DKO+OVL mice compared with DKO mice. Time to achieve 25% of P0 was reduced by TR in DKO+OVL ($P \leq 0.0001$) (Fig. 5*F*). Similar effects of OVL were observed in Mdx+OVL mice regarding absolute ($P \leq 0.0001$) (Fig. 5*B*) and specific maximal forces ($P \leq 0.001$) (Fig. 5*C*), force drop following eccentric contraction (Fig. 5*D*) ($P \leq 0.05$ to $P \leq 0.001$), weight ($P \leq 0.0001$) (Fig. 5*E*) and time to achieve 25% of P0 ($P \leq 0.0001$) (Fig. 5*F*). When comparing the gains induced by OVL between DKO+OVL mice and Mdx+OVL mice, the percentages of increase in absolute maximal force (+202.6 ± 141.6% *vs.* +273.2 ± 108.0%, respectively) and specific maximal force (+85.3 ± 104.4% *vs.* +146.9 ± 79.2%, respectively) were not different ($P = 0.166$ and $P = 0.128$, respectively). However, the rate of force development (slope) was not increased in DKO+OVL mice ($P = 0.0581$), unlike Mdx+OVL mice ($P \leq 0.0001$) (Fig. 5*G*).

Additionally, the percentage of fibres with centronuclei in DKO+OVL mice was lower compared with Mdx+OVL mice ($P \leq 0.0001$) (Fig. 5*H* and *I*). The amount of the ECM was not greater in DKO+OVL mice compared with Mdx+OVL mice (Fig. 5*H* and *J*). These results suggest no dramatic worsening of histological markers of damage in DKO+OVL mice compared to Mdx+OVL mice.

The collective results demonstrated that OVL markedly increased plantaris muscle function in DKO mice. Moreover, the effect of OVL was almost similar in DKO mice and Mdx mice.

Activin receptor administration augmented TA muscle function

In the following investigation, we examined the effect of administering a soluble activin receptor IIB (AR). Previous studies have demonstrated that AR increases muscle weight in *mdx* mice (Hoogaars et al., 2012). To explore whether a possible gain in muscle weight in DKO mice leads to an increase in absolute maximal force, we treated 1-month-old DKO mice with AR (DKO+AR) and assessed them at the age of 2 months (Fig. 1*E*). DKO+AR mice exhibited a higher body weight ($P \leq 0.05$) (Fig. 6*A*).

Our findings revealed that AR increased TA muscle function. Specifically, absolute maximal force ($P \leq 0.01$) (Fig. 6*B*) (+48%) was greater in DKO+AR mice compared to DKO mice, while specific maximal force remained unchanged (Fig. 6*C*). The rate of force development (slope) ($P \leq 0.01$) (Fig. 6*D*), the force drop following the third eccentric contraction ($P \leq 0.001$) (Fig. 6*E*) and muscle weight ($P \leq 0.01$) (Fig. 6*F*) (+38%) were greater in DKO+AR mice compared with DKO mice. Time to achieve 25% of P0 was not changed by AR (Fig. 6*G*). In addition, the percentage of fibres with centronuclei increased in DKO+AR mice compared to DKO mice ($P \leq 0.01$) (Fig. 6*H*), whereas the amount of ECM decreased ($P \leq 0.05$) (Fig. 6*I*).

Together, these results indicated that AR improved TA muscle function in DKO mice.

Discussion

The findings from this study consistently demonstrate that DKO mice represent a more severe murine model of DMD

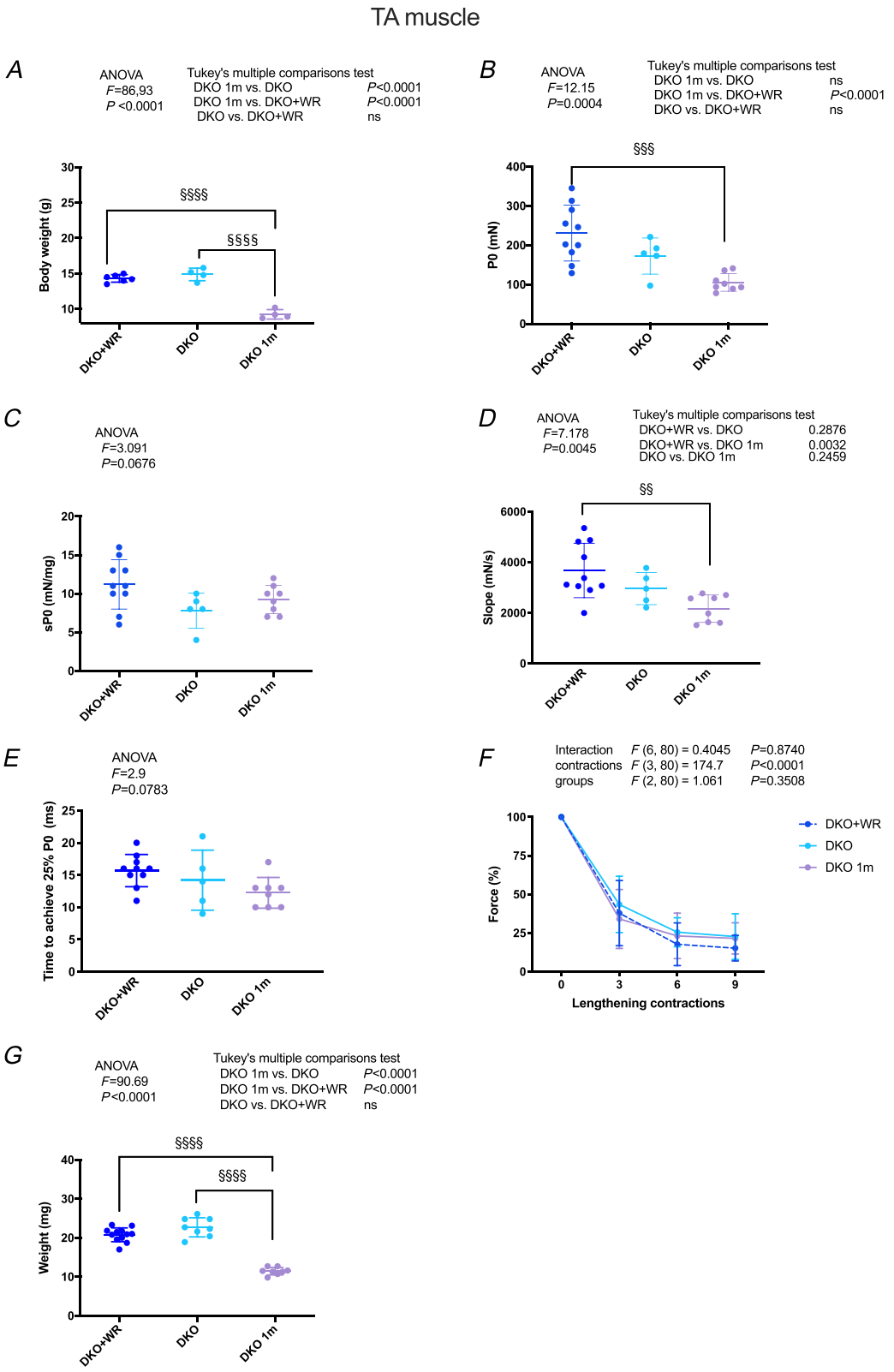

Figure 4. Chronic voluntary wheel running (WR) did not change tibialis anterior (TA) muscle function in DKO mice
A, body weight. *B*, absolute maximal force (P0). *C*, specific maximal force (sP0). *D*, rate of force development: slope d*F*/d*t*. *E*, time to achieve 25% of P0. *F*, force drop following eccentric contractions. *G*, muscle weight. *n* = 10–12

per group of DKO+WR mice, n = 5–8 per group of DKO mice and n = 8 per group of DKO 1m mice. DKO+WR: DKO mice that ran on a wheel; DKO: DKO mice that did not run; DKO 1m: 1-month-old DKO mice. §§, §§§, §§§§: significantly different from DKO 1m (effect of growing), $P \leq 0.01$, $P \leq 0.001$, $P \leq 0.0001$, respectively.

compared to *mdx* mice (Figs 2, 3 and 5). Lower absolute maximal force, reduced muscle weight (atrophy) and greater susceptibility to eccentric contraction-induced functional loss observed in young DKO mice at the initiation of chronic exercise (1 month of age) (Ferry et al., 2020) highlight the heightened severity of the dystrophic features in this model, emphasizing its relevance for studying more severe forms of DMD. Despite this severe phenotype, we found that chronic exercise did have marked beneficial effects in young DKO mice. Indeed, absolute maximal force of plantaris muscle was markedly increased by chronic enforced running and mechanical overload. These results are unique because, to our knowledge, the effect of chronic exercise has not yet been studied in such a severe murine model of DMD. Another interesting finding was that activin receptor administration reduced the deficit in absolute maximal force of the TA muscle in DKO mice.

Chronic running increased absolute maximal force of the plantaris muscle but not TA muscle

The significant increase in absolute maximal force of the plantaris muscle in DKO+TR mice was attributed to a higher specific maximal force (sP0). This observation suggests that the functional quality of the plantaris muscle was improved by TR. The exact cause of the reduced specific maximal force in dystrophin-deficient mice remains unclear (Hernandez-Ochoa et al., 2015). Various factors have been proposed, including a debated decrease in myofibrillar function (Friedrich et al., 2010; Lynch et al., 2000; Schneidereit et al., 2018), reduced action potential-induced calcium release from the sarcoplasmic reticulum (Hernandez-Ochoa et al., 2015), decreased lateral transmission of force which is carried out at the level of the costameres and the dystrophin–glycoprotein complex (Hughes et al., 2015; Ramaswamy et al., 2011) and an increase in the amount of ECM at the expense of muscle fibres. Future investigations are needed to determine whether improvements in one or more of these altered parameters, or potentially other factors, contribute to the observed enhancement in specific maximal force in DKO+TR mice. In particular, it would be interesting to determine whether at least part of the increase in specific maximal force is linked to a possible change in fibre type induced by TR. Furthermore, our findings indicate that the improvement in plantaris muscle function was comparable between DKO+TR mice and Mdx+TR mice, suggesting that the severity of the dystrophic disease did not influence the observed effects of TR. It is noteworthy that the absolute maximal force of the plantaris muscle in DKO+TR mice (mean 167 mN) was comparable to what we previously observed in adult healthy mice (means: 195, 231 and 241 mN) (Joanne et al., 2012, 2021; Stantzou et al., 2021). This suggests that TR could be highly effective in mitigating plantaris muscle weakness in a murine model of severe DMD (Fig. 7*A*), unlike chronic electrostimulation with 10 or 100 Hz at least in dystrophin-deficient and *mdx*-utr mice (Hardee et al., 2021; Yamauchi et al., 2023). Moreover, the ability to rapidly generate muscle force (slope) was also increased in DKO+TR plantaris muscle.

In contrast, the absolute maximal force of the TA muscle was not increased in DKO+TR mice, although specific maximal force was. Unlike the plantaris muscle, the TA muscle of DKO+TR mice therefore had a maximal force much lower than that of healthy mice (Fig. 7*B*). The same was also observed in the TA muscle of DKO+WR mice (Fig. 7*C*). It is conceivable that the TA muscle was less intensively 'trained' during running than the plantaris muscle. Supporting this hypothesis, there is evidence of a differential effect of running between ankle plantar flexor and ankle plantar extensor muscles in adult *mdx* mice (Brussee et al., 1997; Delacroix et al., 2018; Hayes & Williams, 1996). Additionally, it remains to be determined whether a longer duration of the TR protocol could have a beneficial effect, as a study reported that 6 months of TR increased the absolute maximal force of the TA muscle in adult D2-*mdx* mice (Zelikovich et al., 2019). Nevertheless, it is likely that simply increasing the function of the plantaris muscle (and other plantar flexor muscles) would result in improved locomotion, given the crucial role played by plantar flexor muscles in this context.

Given the increased or at worst unchanged lower leg muscle function, along with no notable changes in histological markers of muscle damage, our results suggest that both chronic enforced and voluntary running had no negative impact on the dystrophic process, even in a murine model of severe DMD. Besides the specific positive effects on dystrophic muscle function, it is plausible that chronic running may have other numerous beneficial effects on health, similar to those observed in healthy subjects (Booth et al., 2017).

Weakness of the plantaris muscle was probably fully prevented by mechanical overloading

In this study, we observed a significant increase in the absolute maximal force of the plantaris muscle in DKO+OVL mice. This positive outcome was attributed

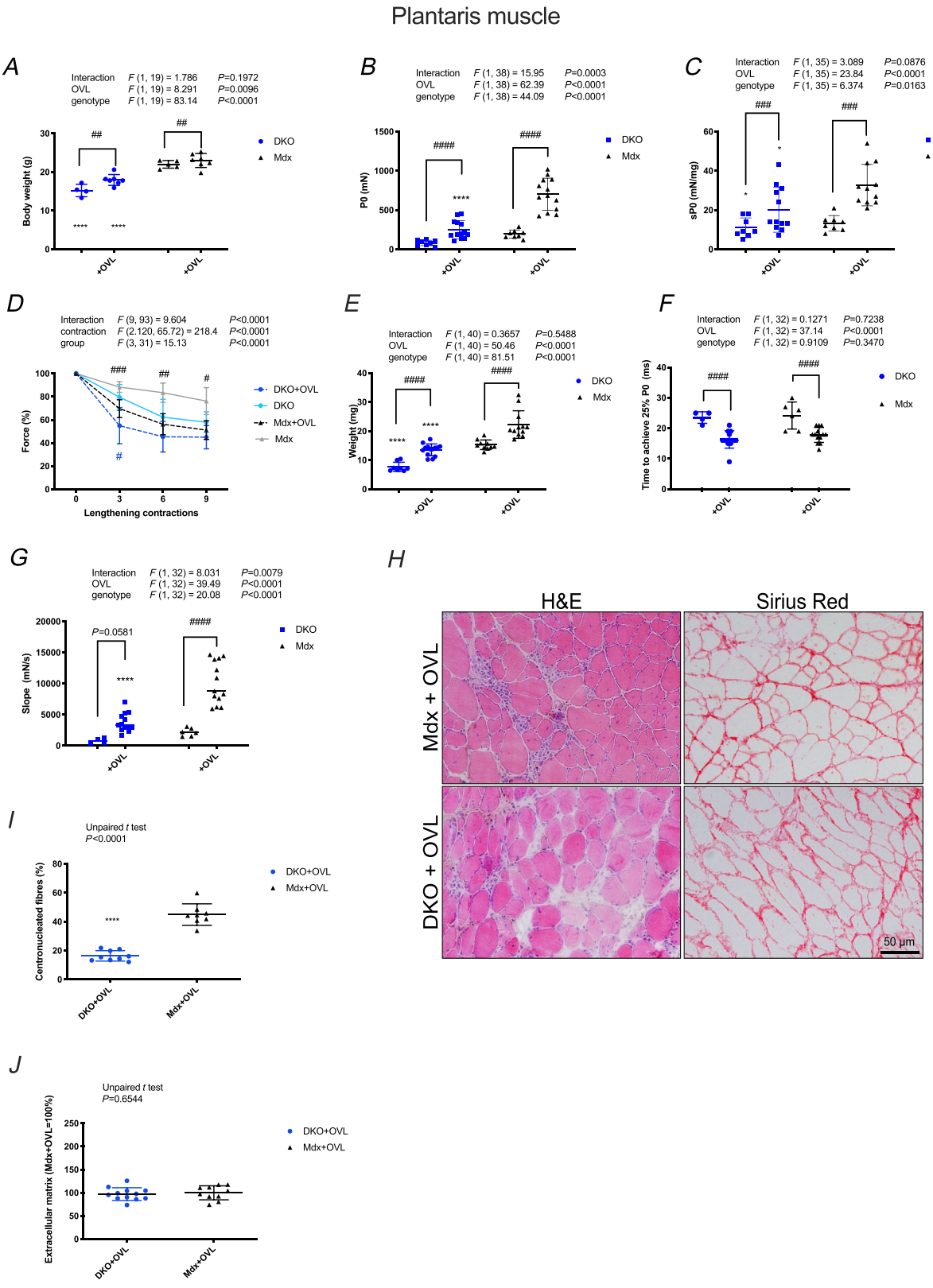

Figure 5. Mechanical overloading (OVL) markedly improved plantaris muscle function in DKO mice
A, body weight. *B*, absolute maximal force (P0). *C*, specific maximal force (sP0). *D*, force drop following eccentric contractions. *E*, muscle weight. *F*, time to achieve 25% of P0. *G*, rate of force development: slope d*F*/d*t*. *H*, representative images of muscle of DKO+TR mice. *I*, percentage of centronucleated fibres. *J*, amount of extracellular matrix (ECM). *n* = 12–14 per group of DKO+OVL mice, *n* = 4–8 per group of DKO mice, *n* = 10–13

per group of Mdx+OVL and n = 5–10 per group of Mdx mice. DKO+OVL: DKO mice with overloaded muscles; DKO: DKO mice with no overloaded muscles; Mdx+OVL: Mdx mice with overloaded muscles; Mdx: Mdx mice with no overloaded muscles. #, ##, ###, ####: significantly different from DKO mice or Mdx mice (main effect of mechanical overload, except in *D* and *G*), $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, $P \leq 0.0001$, respectively. *, ****: significantly different from Mdx mice (effect of genotype), $P \leq 0.05$, $P \leq 0.0001$, respectively.

to both higher specific maximal force and an increase in muscle weight, serving as an indicator of hypertrophy. Our previous findings indicated that the increased muscle weight observed in response to OVL was related to a greater number of fibres in the muscle mid-belly in adult *mdx* mice (Ferry, Parlakian, et al., 2015; Joanne et al., 2012). It was suggested that increased fibre length (Jorgenson & Hornberger, 2019) and hyperplasia

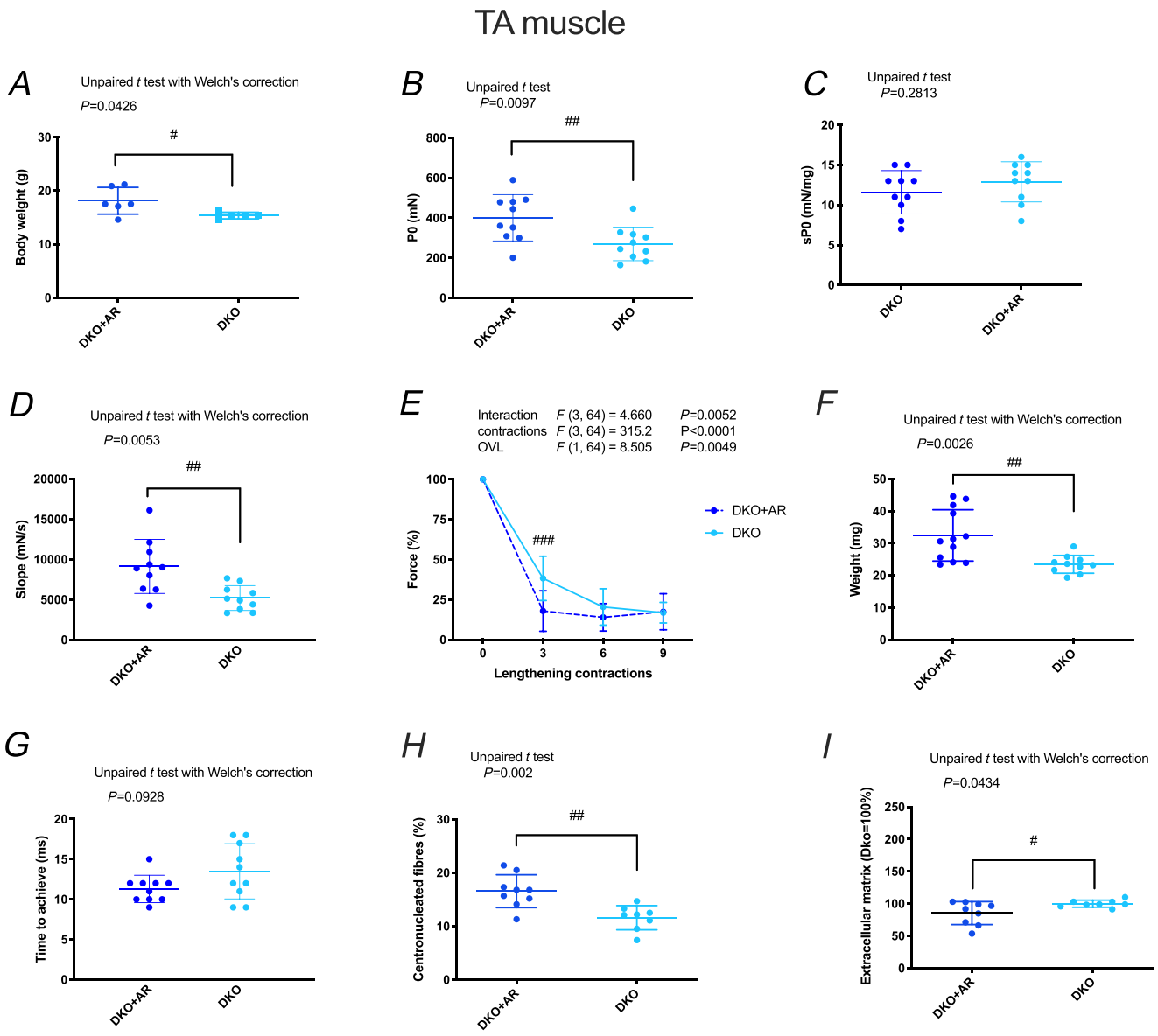

Figure 6. Activin receptor administration (AR) increased tibialis anterior (TA) muscle function in DKO mice
A, body weight. *B*, absolute maximal force (P0). *C*, specific maximal force (sP0). *D*, rate of force development: slope d*F*/d*t*. *E*, force drop following eccentric contractions. *F*, muscle weight. *G*, time to achieve 25% of P0. *H*, percentage of centronucleated fibres. *I*, amount of extracellular matrix (ECM). n = 9–12 per group of DKO+AR mice and n = 8–10 per group of DKO mice. DKO+AR: DKO mice with activin receptor administration; DKO: DKO mice that were not administrated with activin receptor. #, ##: significantly different from DKO mice, $P \leq 0.05$, $P \leq 0.001$, respectively.

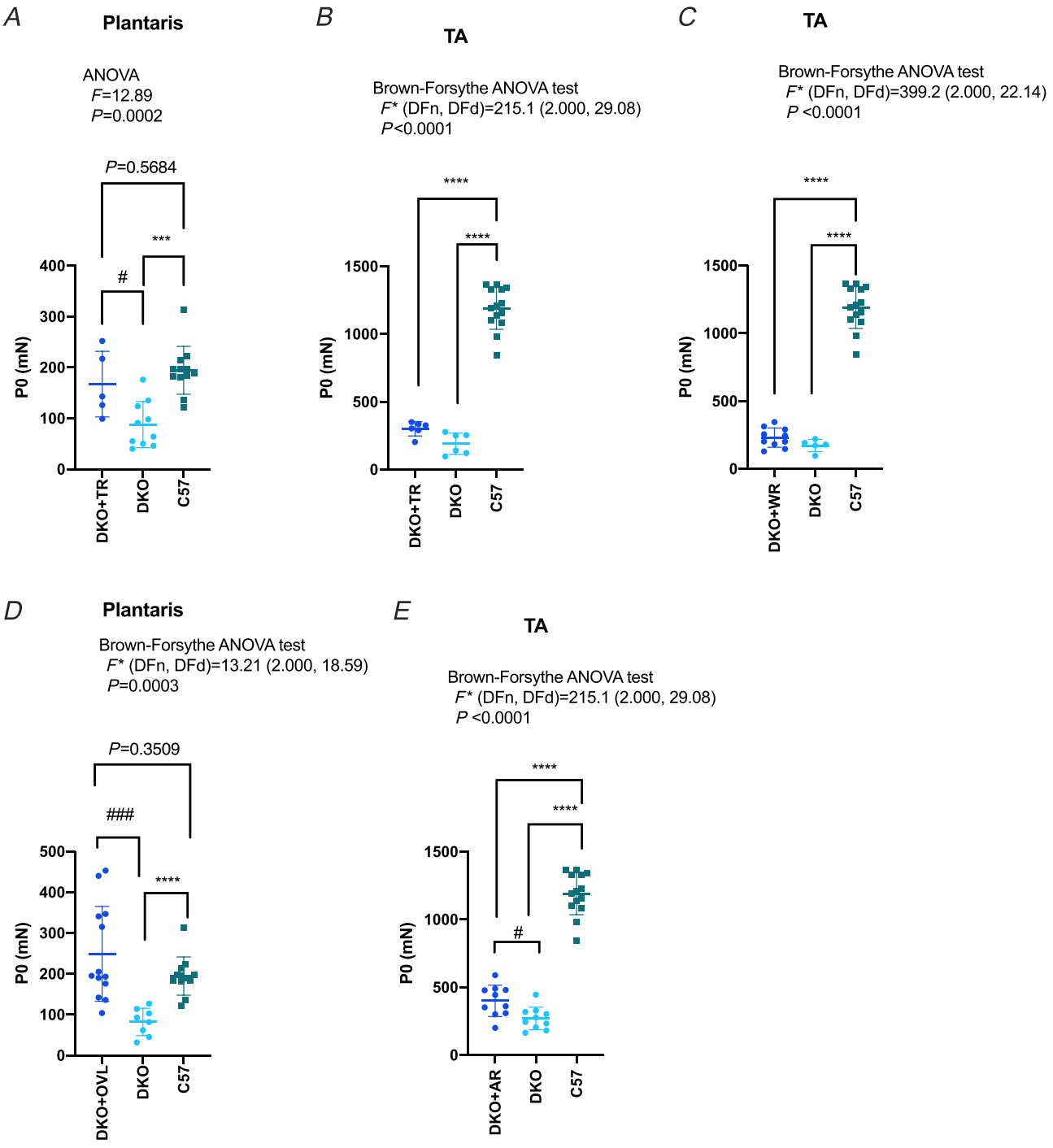

Figure 7. Absolute maximal force of DKO mice compared to healthy mice (C57)
A, plantaris muscle from DKO+TR mice. *B*, TA muscle from DKO+TR mice. *C*, TA muscle from DKO+WR mice. *D*, plantaris muscle from DKO+OVL mice. *E*, TA muscle from DKO+AR mice. Force values of C57 mice were measured in our previous studies (Ferry et al., 2020; Stantzou et al., 2021). DKO+TR, DKO+WR, DKO+OVL and DKO+AR: DKO mice that ran on a treadmill, on a wheel, with overloaded muscles and with activin receptor administration, respectively. ***, ****: significantly different from C57, $P \leq 0.001$ and $P \leq 0.0001$, respectively. #, ###: significantly different from DKO, $P \leq 0.05$, $P \leq 0.001$, respectively.

(Goodman et al., 2011) might contribute to the greater number of fibres in the muscle mid-bell in healthy OVL muscle. Other potential mechanisms include fibre splitting during hypertrophy and incomplete lateral fusion of myotubes during regeneration (Faber et al., 2014; Murach et al., 2019), as supported by the notable increase in branched fibres in at least the soleus muscle of *mdx* mice subjected to OVL (Terada et al., 2012). It remains to be investigated whether these mechanisms were at play in DKO+OVL mice. Moreover, our results suggested that the severity of the dystrophic disease did not influence the response to OVL, except for the ability to rapidly generate muscle force (slope), which did not show a significantly increase in DKO+OVL mice. Moreover, our results suggested that a non-physiological model of chronic resistance training could fully reverse plantaris muscle weakness in a murine model of severe DMD. This suggestion was based on the fact that the absolute maximal force of the plantaris muscle from DKO+OVL mice was comparable to that observed in adult healthy mice in previous studies (Joanne et al., 2012, 2021; Stantzou et al., 2021) (Fig. 7*D*). These preclinical findings suggested the potential benefits of resistance training for dystrophic muscles and would warrant further investigation in patients with DMD. Recently, the effects of mild to moderate muscle contractions have been reported in ambulatory DMD boys (Lott et al., 2021).

It was noteworthy that increased susceptibility to eccentric contraction-induced immediate functional loss resulting from OVL did not result in a dramatic change in histological markers of muscle damage in DKO+OVL mice. Given that the force drop following lengthening contractions is associated with a loss of muscle excitability in *mdx* mice (Call et al., 2013; Roy et al., 2016), it would be interesting to determine whether OVL worsens this parameter in DKO mice. Additionally, OVL did not prevent the gain in absolute maximal force, indicating that the chronic effect of exercise was not predictable based on its acute effect. This aligns with previous reports suggesting that chronic eccentric exercise increased maximal force production in adult *mdx* mice (Call et al., 2011; Pedrazzani et al., 2021). In contrast, OVL and isometric resistance exercise reduced susceptibility to eccentric contraction-induced functional loss in older adult *mdx* mice (Ferry, Parlakian, et al., 2015; Lindsay et al., 2019). The observed differential effect of OVL cannot be attributed to the absence of desmin, known to protect against excessive fragility in the absence of dystrophin (Ferry et al., 2020), as we observed a similar aggravation of force drop in Mdx+OVL mice. Instead, it may be related to the fact that OVL was performed during an active phase of the dystrophic process concomitantly with muscle growth in DKO and Mdx mice, in contrast to older adult *mdx* mice. Moreover, the reduced time to achieve 25% of P0 in DKO+OVL mice suggests an increased percentage of fast fibres which are known to be more susceptible to contraction-induced functional loss (Head et al., 1994; Moens et al., 1993). In contrast, OVL promotes a slower fibre phenotype, at least in older mdx mice (Ferry, Parlakian, et al., 2015; Joanne et al., 2012).

Activin receptor administration increased absolute maximal force of the TA muscle

AR increased absolute maximal force of TA muscle in DKO+AR mice, but it remained lower (mean: 400 mN) than observed in adult healthy mice (means: 1120, 1190 and 1270 mN) (Ferry et al., 2020; Hovhannisyan et al., 2019; Roy et al., 2016) (Fig. 7*E*). The gain

Brown-Forsythe ANOVA test
*F** (DFn, DFd)=9.786 (4.000, 21.76)
P=0.0001

Dunnett's T3 multiple comparisons test

DKO+TR (TA) vs. DKO+TR (Plantaris)	*P*=0.967
DKO+TR (TA) vs. DKO+WR (TA)	*P*=0.8044
DKO+TR (TA) vs. DKO+OVL (Plantaris)	*P*=0.03
DKO+TR (TA) vs. DKO+AR (TA)	*P*=0.9992
DKO+TR (Plantaris) vs. DKO+WR (TA)	*P*=0.6917
DKO+TR (Plantaris) vs. DKO+OVL (Plantaris)	*P*=0.3259
DKO+TR (Plantaris) vs. DKO+AR (TA)	*P*=0.8837
DKO+WR (TA) vs. DKO+OVL (Plantaris)	*P*=0.0093
DKO+WR (TA) vs. DKO+AR (TA)	*P*=0.9964
DKO+OVL (Plantaris) vs. DKO+AR (TA)	*P*=0.0186

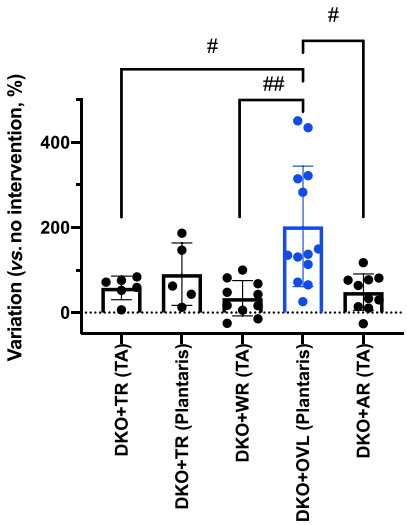

Figure 8. Gains (percentage variation) in absolute maximal force in plantaris muscle from DKO+TR mice, plantaris muscle from DKO+OVL mice and TA muscle from DKO+AR mice
DKO+TR, DKO+WR, DKO+OVL and DKO+AR: DKO mice that ran on a treadmill, on a wheel, with overloaded muscles and with activin receptor administration, respectively. #, ###: DKO+OVL (plantaris) were significantly different from DKO+TR (TA), DKO+WR (TA) and DKO+AR (TA), $P \leq 0.05$ and $P \leq 0.001$, respectively.

(percentage increase) in absolute maximal force induced by AR in DKO+AR mice (TA muscle) was lower than that resulting from OVL in DKO+OVL mice (plantaris muscle) ($P \leq 0.05$) (Fig. 8). AR only increased TA muscle weight, while OVL increased both specific maximal force and weight of plantaris muscle. Previous reports indicated that AR did not increase absolute maximal force of lower leg muscle in adult *mdx* mice (Béchir et al., 2016; Hoogaars et al., 2012). The differential effect of AR between DKO mice and older adult *mdx* mice can be due to an age difference (young *vs*. adult mice), the severity of the disease (severe *vs*. milder), combined deficiency of dystrophin and desmin, or the presence of muscle atrophy (reduced muscle weight *vs*. increased muscle weight). Additionally, the ability to rapidly produce force (slope) was increased in DKO+AR mice but not the time to achieve 25% of P0. These results suggested that the increase in the rate of force development was linked to increased absolute maximal force and not the improvement of one factor determining the slope of the force (activation of myofibrils and series compliance of activated sarcomeres) (Edman & Josephson, 2007; Maffiuletti et al., 2016). The fact that AR reduced the muscle weakness in young DKO+AR mice, despite an increased susceptibility to eccentric contraction-induced functional loss, also supported the idea that an increased functional loss following eccentric contractions did not prevent a gain in muscle function.

Conclusion

In DKO mice characterized by severe lower leg muscle weakness, atrophy and fragility compared with *mdx* mice, we observed that treadmill running and mechanical overloading increased the absolute maximal force of the plantaris muscle while that of the TA muscle remained unchanged with both treadmill and wheel running. Furthermore, the effects of treadmill running, wheel running and mechanical overloading on plantaris and TA muscle function were not influenced by the severity of the disease as they were almost similar between DKO and *mdx* mice. Activin receptor also improved TA muscle function in DKO mice. Since enforced treadmill running, voluntary wheel running and mechanical overloading were not at worst harmful and at best beneficial to lower leg muscle function in DKO mice, it is reasonable to assume that they did not exacerbate the dystrophic process, even in this murine model of severe DMD. Thus, our results could contribute to alleviating concerns about testing the prescription of endurance and resistance training as a potential therapy for dystrophic patients and offering a way to counteract the pathophysiological mechanisms induced by physical inactivity.

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Additional information

Data availability statement

All data supporting the results of the present study are included within the published paper.

Competing interests

All authors declare no conflict of interest.

Author contributions

D.F., Z.L., O.A. and A.F. conceived, coordinated and designed the study. D.M., P.R., M.L., Z.L. and A.F. performed animal experiments. D.M., J.H., P.R., M.L. and A.F. performed and analysed measurements. D.M., J.H., D.B., H.A., O.R., D.F., Z.L., O.A. and A.F. wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Keywords

chronic muscular exercise, Duchenne muscular dystrophy, endurance training, maximal force, mice, resistance training

Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

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