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**Valproic acid reduces muscle susceptibility to contraction-induced functional loss but increases weakness in two murine models of Duchenne Muscular Dystrophy**

Running head: VPA improves fragility of dystrophic muscle

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## **Abstract**

Skeletal muscles in animal models of Duchenne muscular dystrophy (DMD) are more susceptible to contraction-induced functional loss, which is not related to fatigue. Valproic acid (VPA) reportedly improves serological and histological markers of damage in dystrophin-deficient murine muscle. Here, we tested whether VPA would reduce the susceptibility to contraction-induced functional loss in two murine DMD models. Adult

female *mdx* (mild) and D2-*mdx* (severe) DMD murine models were administered VPA (240 mg/kg) or saline for 7 days. Some VPA-treated *mdx* mice also performed voluntary running in a wheel, which is known to reduce the susceptibility to contraction-induced functional loss, that is, isometric force drop following eccentric contractions. *In situ* muscle function was assessed before, during and after eccentric contractions. Muscle utrophin and desmin expression was also evaluated using immunoblotting. Interestingly, VPA reduced the isometric force drop following eccentric contractions in both murine models, without change in the relative eccentric maximal force and in the expression of utrophin and desmin. VPA for 7 days combined to voluntary running had no additive effect compared to VPA alone. Furthermore, VPA reduced the absolute isometric maximal force before eccentric contractions in both murine models. The results of our study indicated that VPA in both murine DMD models reduced the susceptibility to contraction-induced functional loss, but increased muscle weakness.

**Key Words:** Duchenne muscular dystrophy, valproic acid, murine DMD models, muscle function, susceptibility to contraction-induced function loss.

## Introduction

Dystrophin deficiency results in Duchenne muscular dystrophy (DMD), a skeletal muscle disease commonly inherited in boys. DMD is characterized by histological tissue damage (necrosis, fibrosis), weakness, greater susceptibility to contraction-induced functional loss, and ultimately, leads to death. Muscle weakness is defined as a reduction in the absolute and/or specific (absolute maximal force relative to the muscle's cross-sectional area or weight) maximal forces. Although the causes for this weakness remain unknown, muscle wasting, lack of lateral force transmission across the costameres, and greater susceptibility to contraction-induced functional loss have been suggested (1, 2). The greater susceptibility to contraction-induced functional loss is commonly assessed by the immediate isometric maximal force drop following eccentric (lengthening) contractions in murine DMD models, which is a type of muscle contraction that produces very high forces (3, 4). No histological or structural muscle injury immediately following *in vivo* eccentric contractions or reduction in the maximal force of permeabilized muscle fibers have been noted in mild murine DMD models (*mdx* mice) (5). This suggests that the primary cause of the immediate force drop is not impaired myofilament functioning. Another study also reported that there was no force drop following *in vitro* eccentric contractions in permeabilized muscle fibers in *mdx* mice (6). The greater force drop is associated with loss of excitability (5, 7) and the presence of branched fibers (8). Murine DMD model studies revealed that muscle weakness and greater susceptibility to eccentric contraction-induced functional loss are affected by the expression levels of other muscle proteins, such as utrophin and desmin (9–11). Utrophin and desmin levels are higher in *mdx* mice, possibly compensating for the absence of dystrophin; invalidation of *Utr* and *Des* in *mdx* mice results in extreme force drop following eccentric contractions and weakness (9, 10).

A promising therapeutic approach for DMD is the use of gene therapies aimed at restoring dystrophin expression (12). This approach requires supplementation with therapies aimed at reducing the symptoms, such as contraction-induced function loss and weakness. Chronic muscular exercise, particularly voluntary exercise, has been beneficial in murine DMD models (13). Pharmacological agents, particularly glucocorticoids, also reportedly improve some DMD symptoms (12); however, they can produce several side effects (14).

Recently, valproic acid (VPA) was found to be beneficial in murine DMD models (15). In a murine model of severe DMD, lacking both dystrophin and utrophin, VPA administration for 5 weeks (240 mg/kg) reduced the expression of histological markers of muscle inflammation, fibrosis, and degeneration (15). However, the effects of VPA on the susceptibility to contraction-induced functional loss and weakness in murine DMD models remain unknown. VPA, which is used for the treatment of seizures and certain psychiatric disorders, is also an inhibitor of histone deacetylase (HDAC) and can modulate the expression of several genes (16, 17). Other pharmacological agents with HDAC inhibition activity have produced beneficial effects in murine DMD models, reducing histological signs of muscle damage and increasing muscle regeneration (18–25). However, these agents have not shown any effects on the susceptibility to contraction-induced functional loss.

Herein, we aimed to determine the effect of VPA administration on the susceptibility to contraction-induced functional loss in two murine DMD models (*mdx* and D2-*mdx* mice). The histopathological muscle changes (e.g., increased fibrosis, lower regeneration) are reportedly more severe in D2-*mdx* mice than in *mdx* mice, causing marked weakness (26). Specifically, we aimed to determine whether VPA would reduce the immediate maximal force drop following eccentric contractions in the fast-type muscles. Moreover, some mice

were made to perform chronic voluntary exercises to determine whether the potential effects of VPA and voluntary exercise are additive since we previously determined that voluntary running in a wheel reduced the susceptibility to contraction-induced functional loss (27). Finally, as utrophin and desmin are known to markedly modulate dystrophic functional features and VPA is capable of modulating the expression of several genes, we also tested the hypothesis that the VPA effects are linked to the variation in the expression of utrophin and desmin.

## Methods and Materials

### Ethics statement and experimental design

All procedures were performed in accordance with the National and European legislations and approved by the French Ministry of National Education, Higher Education and Research (No: APAFIS 21554-2019071912051421v13). The mice (obtained from Jackson Laboratory) were bred and housed in the departmental animal facility with free access to water and laboratory rodent chow. The animal facility was pathogen free with a 12h-light/12h-dark cycle, and 3–5 mice were housed per cage. We performed three experiments to determine the effect of VPA in moderate (Mdx mice) and severe (D2-mdx) DMD murine models (Supplementary Figure 1). We used adult female mice lacking dystrophin (Mdx) (hybrid background, C57Bl/6 × C57Bl/10) and female D2-mdx mice (D2.B10-Dmd). In some experiments, we also used female age-matched wild-type C57 (C57, n = 6) (Supplementary Figure 1A) and D2 (D2, n = 4) (Supplementary Figure 1B) mice. The mice were randomly divided into control and experimental groups. A VPA dose of 240 mg/kg/day was administered two times per day, as in previous studies (15, 28). In the first experiment (Supplementary Figure 1A), 7-month-old Mdx mice were treated with VPA for 7 days (Mdx+VPA7, n = 7), VPA for 1 day (Mdx+VPA1, n = 5), or saline for 7 days (Mdx and C57, n = 6 each). In the second experiment (Supplementary Figure 1B), 6-month-old D2-mdx mice were treated with VPA (D2-mdx+VPA7, n=3) or saline (D2-mdx and D2, n=3 each) for 7 days. In the third experiment (Supplementary Figure 1C), 4-month-old Mdx mice were treated with VPA (Mdx+VPA7, n = 7) or saline (Mdx, n = 8) for 7 days. In addition, 6 Mdx mice treated with VPA for 7 days were placed in separate cages containing a wheel and



allowed to run for 7 days ad libitum (Mdx+VPA7+wheel). In the three experiments, muscles were measured and collected the day after the last VPA and saline dose.

#### Susceptibility to contraction-induced loss of function

Maximal forces before and after eccentric contractions were evaluated by measuring the *in situ* tibialis anterior (TA) muscle contraction in response to nerve stimulation, as described previously (29, 5). Mice were anesthetized using pentobarbital (60 mg/kg, ip). Body temperature was maintained at 37°C using radiant heat. The knee and foot were fixed with pins and clamps and the distal tendon of the fast-type muscle was attached to a lever arm of a servomotor system (305B, Dual-Mode Lever, Aurora Scientific) using a silk ligature. The sciatic nerve was proximally crushed and distally stimulated by a bipolar silver electrode using supramaximal square wave pulses of 0.1 ms duration. We measured the absolute maximal isometric 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 was determined at L0 (length at which maximal tension was obtained during the tetanus). Absolute isometric maximal force was normalized to the muscle weight as an estimate of specific isometric maximal force, another index of muscle weakness.

Susceptibility to contraction-induced loss of function in Mdx mice was then estimated from the immediate isometric force drop resulting from eccentric contractions. The sciatic nerve was stimulated for 700 ms (frequency of 150 Hz). A maximal isometric contraction of the TA muscle was initiated during the first 500 ms. Then, muscle lengthening (10% L0) at a velocity of 5.5 mm/s (0.85 fiber length/s) was imposed during the last 200 ms. All isometric

contractions were made at an initial length L<sub>0</sub>. Nine eccentric contractions of the TA muscles were performed, each separated by a 45-s rest period. Maximal isometric force was measured after each eccentric contraction and expressed as a percentage of the initial maximal force. In addition, we measured the absolute maximal eccentric force during the first eccentric contraction, and index of the muscle strain. After contractile measurements, the mice were killed with cervical dislocation.

#### Immunoblotting (utrophin, desmin)

TA muscles were snap-frozen in liquid nitrogen immediately after dissection. Frozen muscles were crushed and placed into an ice-cold homogenization buffer containing: 7 M Urea, 2 M thiourea, 2% CHAPS, 50 mM dithiothreitol, 10 mM sodium orthovanadate, 10 mM NaF, 10 mM glycerophosphate and 1% of protease inhibitor cocktail (Thermo Scientific, Courtaboeuf, France) for utrophin, sarcomeric alpha-actinin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and desmin immunoblotting. Samples were homogenized on a wheel for 2h, sonicated for 20 sec, incubated 30 min in ice and then centrifuged at 12,000 rpm for 30 min at 4°C. Protein concentration was measured using the Bradford method with bovine serum albumin as a standard. Equal amounts of protein extracts (6 µg) were distributed and separated in a 3-8% tris-acetate polyacrylamide gel (Thermo Scientific, Courtaboeuf, France), before electrophoretic transfer onto a PVDF membrane (Thermo Scientific, Courtaboeuf, France). Western-blot analysis was conducted using anti-utrophin (1:50, mouse monoclonal, DSHB, Iowa City, USA), anti-sarcomeric alpha-actinin (1:2000, mouse monoclonal, Abcam, Amsterdam, Netherlands), anti-desmin (1:2000, mouse monoclonal, Sigma-Aldrich, Saint-Quentin, France). Proteins bound to primary antibodies were visualised with peroxidase-conjugated secondary antibodies (Tebu-bio, Le Perray-en-Yvelines, France) and a chemiluminescent detection system (Substrat

HRP Immobilon Western, Millipore, Molsheim, France). Bands were quantified by Image Studio Lite software. The ratios of utrophin or desmin to alpha-actinin were calculated. Unlike GAPDH, the level of alpha-actinin does not vary between groups (GAPDH was about 30% increased by VPA). Utrophin and desmin were then normalized to Mdx values.

### Statistical analysis

Groups were statistically compared using the Prism software v8 (GraphPad, La Jolla, CA, USA). Data were tested for normal distribution (Anderson-Darling test, d'Agostino and Pearson Test, Shapiro-Wilk test, and Kolmogorov-Smirnov test) and homogeneity of variance (Brown-Forsythe test and Welch's test). For experiment 1, one-way ANOVA and Tukeys test were used to analyze the following variables: absolute and muscle weight. Kruskal Wallis test and Dunn's test were used to analyze specific maximal force. The force drop following eccentric contractions was analyzed by two-way ANOVA, groups (Mdx+VPA7, Mdx+VPA7+wheel, Mdx+VPA1 Mdx, C57) by eccentric contractions (0, 3, 6, 9), with the repeated measures on eccentric contractions, and Tukeys test. For experiment 2, one-way ANOVA and Tukeys test were used for the following variable: absolute and muscle weight. Specific maximal force was analyzed using Welch's ANOVA test and Dunnett's T3. The force drop following eccentric contractions was analyzed by two-way ANOVA, groups (D2-mdx+VPA7, D2-mdx, D2) by eccentric contractions (0, 3, 6, 9), with the repeated measures on eccentric contractions, and Tukeys test. For experiment 3, one-way ANOVA and Tukeys test were used to analyze the following variables: absolute maximal force, specific maximal force, absolute maximal eccentric force, and relative maximal eccentric force. Kruskal-Wallis tests were performed to analyze utrophin and desmin levels. The force drop following eccentric contractions was analyzed by two-way ANOVA, groups (Mdx, Mdx+VPA7, Mdx+VPA7+Wheel) by eccentric contractions (0, 3,

6, 9), with the repeated measures on eccentric contractions, and Tukeys test. Values were means  $\pm$  SEM (except for utrophin and desmin, with median and interquartile range).

## Results

### 1- VPA reduced the force drop following eccentric contractions and maximal force before eccentric contractions

Mdx mice were treated with VPA for 7 days (Mdx+VPA7), 1 day (Mdx+VPA1), or saline (Mdx) (Supplementary Figure 1A). *In situ* maximal TA muscle force production in response to nerve stimulation revealed an immediate isometric force drop following eccentric contractions in the Mdx mice (Figure 1A). There was no such isometric force drop following 9 eccentric contractions in the C57 mice, indicating a higher susceptibility to contraction-induced functional loss in the Mdx mice. Interestingly, VPA7 reduced the isometric force drop following eccentric contractions (Figure 1A). The drops following the 6<sup>th</sup> and 9<sup>th</sup> eccentric contractions were lower in the Mdx+VPA7 mice than those in the Mdx mice ( $p < 0.05$ ) (Figure 1A). In contrast, isometric force drop following eccentric contractions was not reduced in the Mdx+VPA1 mice ( $p > 0.05$ ) (Figure 1A).

In addition, the absolute maximal isometric force before eccentric contractions was decreased in the Mdx+VPA7 mice as compared to the Mdx mice ( $p < 0.0001$ ) (Figure 1B). Concerning the specific isometric maximal force, it was decreased in the Mdx mice as compared to the C57 mice ( $p < 0.0001$ ) (Figure 1C), indicating weakness in the mouse Mdx DMD model. We found that VPA7 and VPA1 treatments did not affect the specific isometric maximal force (Figure 1C) ( $p > 0.05$ ). These results indicated that the reduced absolute maximal isometric force in the Mdx+VPA7 mice was related to the reduced muscle weight in the Mdx+VPA7 mice as compared to the Mdx mice (Figure 1D) ( $p < 0.01$ ).

Together, we found that although VPA7 reduced the isometric force drop following eccentric contractions in the Mdx mice, VPA1 did not. These results indicated that the effect of VPA was not acute and is only detectable after multiple injections. Moreover, VPA reduced the absolute isometric maximal force, that is, increased weakness.

## **2- VPA induced the same effects regardless of the severity of DMD**

Next, we aimed to determine the effects of VPA7 in a more severe murine DMD model (D2-Mdx mice) (Supplementary Figure 1B). The force drop following eccentric contractions was greater in the D2-mdx mice than in the wild-type D2 mice (Figure 2A) ( $p < 0.0001$ ). VPA7 reduced the isometric force drop following eccentric contractions (Figure 2A) ( $p < 0.0001$ ) in the D2-mdx mice (D2-mdx+VPA7 mice). In addition, the absolute maximal force before eccentric contractions was lower in the D2-mdx mice than in the D2 mice ( $p < 0.0001$ ), which was further reduced by VPA7 (Figure 2B) ( $p < 0.001$ ). VPA also decreased the specific isometric maximal force (Figure 2C) ( $p < 0.05$ ) without affecting the muscle weight (Figure 2D) ( $p > 0.05$ ). Thus, the overall VPA7 effects in the D2-mdx were the same as that in the Mdx mice.

## **3-The beneficial effect of VPA was not increased by voluntary exercise and not related to utrophin and desmin**

In this experiment (Supplementary Figure 1C), we confirmed the beneficial effects of VPA7 on the isometric force drop following eccentric contractions that were observed previously (Figure 3A) ( $p < 0.05$  to  $p < 0.0001$ ). Because the isometric force drop is related to the maximal eccentric force and work (30, 31), we measured the maximal eccentric force before

a reduction in function. We found that the absolute maximal eccentric force in the Mdx+VPA7 mice was lower than that of the Mdx mice (Figure 3B) ( $p < 0.05$ ); the maximal eccentric force relative to the maximal isometric force was not (Figure 3C). In addition, we confirmed the effects of VPA7 on the absolute maximal isometric force before eccentric contractions, although not statistically significant ( $p = 0.056$ ) (Figure 3D), specific maximal isometric force (Figure 3E) ( $p > 0.05$ ), and muscle weight (Figure 3F) ( $p < 0.001$ ). Susceptibility to contraction-induced functional loss is associated with the expression of utrophin (10, 11) and desmin (9). However, immunoblotting analysis did not reveal any effect of VPA7 on the expression of utrophin (Figures 3G, 3H) and desmin (Figures 3I, 3J) ( $p > 0.05$ ).

We also determined whether voluntary exercise further increased the beneficial effect of VPA7 on the susceptibility to contraction-induced functional loss. One week of voluntary exercise reportedly reduces the force drop following eccentric contractions in the *mdx* mice (27, 32). Some of the Mdx+VPA7 mice performed voluntary exercise in a wheel (Mdx+VPA7+wheel), running  $1.7 \pm 1.0$  km/day. We found no difference in the isometric force drop following eccentric contractions (Figure 3A) between the Mdx+VPA7+wheel and the Mdx+VPA7 mice. In addition, there were no differences in the other parameters between the two mice groups (Figures 3B, 3J).

The third experiment confirmed the beneficial effect of VPA7 on the susceptibility to contraction-induced functional loss. Furthermore, the VPA7-induced reduction in force drop following eccentric contractions was not related to a reduction in the relative eccentric maximal force or to an increased in the expression of utrophin or desmin. The results also

showed that VPA7+wheel and VPA7 produced similar effects, indicating that the effects of VPA and voluntary exercise were not additive in the Mdx mice.



## Discussion

To the best of our knowledge, this is the first study to demonstrate that VPA7 reduced the susceptibility to contraction-induced functional loss in dystrophin-lacking fast-type muscles of mice, an effect similar to that observed with *Dmd* gene-based therapy (5, 33) and chronic muscular exercise (27, 32). This beneficial effect of VPA was observed in all three experiments, in both Mdx and D2-mdx mice. The VPA7-induced reduced force drop following eccentric contractions cannot be explained by the decrease in the absolute maximal eccentric force (presumably lower strain) because the relative maximal eccentric force remained unchanged. Moreover, the force drop was not related to increases in the utrophin and desmin levels; these proteins reportedly protect murine dystrophic muscles against a catastrophic loss of maximal force following eccentric contractions (9, 10). Our study results are in concordance with those of a previous study, in that VPA did not affect the expression of alpha 7 integrin, another important protein (34), in a severe murine DMD model (15). In addition to its action as an HDAC inhibitor capable of modulating protein levels (16, 17), VPA also alters the functions of ion channels in healthy individuals (16, 35–37). Thus, VPA could improve susceptibility to contraction-induced functional loss by preserving muscle excitability via ion channels; the force drop following eccentric contractions being also related to defective membrane excitability (5, 7). This hypothesis is supported by the finding that the HDAC inhibitor arginine butyrate restores neuromuscular excitability in *mdx* mice (38).

This study also showed that the beneficial effect of VPA7 on the force drop following eccentric contractions did not vary with the severity of DMD. Thus, VPA could be used in patients with DMD as murine models are less affected than patients. The reduction in the

force drop could contribute to the improvement of the quality of life of patients with DMD; they would suffer lesser functional loss following braking contractions. To avoid the hepatotoxic and teratogenic effects of VPA, its derivatives are currently being tested (17).

The VPA potential is challenged by an increase in muscle weakness. VPA7 reduced the absolute isometric maximal force before eccentric contractions in both murine DMD models. Therefore, VPA was detrimental to muscle function at a dose of 240 mg/kg. A possible explanation for this is that VPA inhibits neuromuscular transmission (35), reducing muscle activity which decreases the maximal force in *mdx* mice (32). VPA use has been considered in other neuromuscular diseases such as amyotrophic lateral sclerosis (39), myotubular myopathy (31), and oculopharyngeal muscular dystrophy (40). Our results suggest that VPA should be cautiously used in patients with neuromuscular diseases, which are already weak.

## Conclusion

VPA application after an active dystrophic process (41), reduced the susceptibility to contraction-induced functional loss in both mild and severe murine DMD models. However, this beneficial effect was not related to an increase in utrophin and desmin expression in fast-type muscles. VPA also reduced the maximal force production in both murine DMD models. Thus, the effects of VPA on functional dystrophic features were more complex than expected, and the potential preclinical benefit of VPA requires further evaluation. For example, it remains to be determined whether the use of low-dose VPA or its derivatives, to reduce the detrimental effect on weakness, would reduce the susceptibility to contraction-induced functional loss. Regardless of the existence of a clinical potential for VPA or its

derivatives, this study is a stepping stone to better combat post-contraction functional loss in DMD, independent of gene therapies aimed at restoring dystrophin.

#### Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

#### Ethics statement

All procedures were performed in accordance with National and European legislations, under the licence APAFIS 21554-2019071912051421v13 (French Ministry of National Education, Higher Education and Research).

#### Author contributions

DF and AF conceived, coordinated and designed the study. DM, ML, CD, and AF performed animal experiments. DM performed immunoblotting, with the contribution of OA. DM, OA, DF, and AF wrote and reviewed the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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## Figure legends

Supplementary Figure 1. Experimental design of the 3 experiments. Mice were treated with valproic acid (VPA) or saline. A group of VPA-treated mice voluntarily ran in a wheel (Experiment 3; Figure 1C). Electrophysiology was used to measure the susceptibility to contraction-induced function loss (Fragility) and maximal isometric force (weakness). Utrophin and desmin protein levels were analyzed by western blotting. VPA: valproic acid. Mdx and D2-mdx: mild and severe murine DMD models. C57 and D2: wild-type mice. TA muscle: tibialis anterior muscle.

Supplementary Figure 2. Body weights were not changed with valproic acid (VPA). Mdx+VPA7: Mdx mice treated with VPA for 7 days. Mdx+VPA1: Mdx mice treated with VPA for 1 day. Mdx: Mdx mice treated with saline for 7 days. D2-Mdx+VPA7: D2-Mdx mice treated with VPA for 7 days. D2-Mdx: D2-Mdx mice treated with saline for 7 days. Mdx+VPA7+wheel: Mdx mice treated with VPA for 7 days, that voluntary exercised. Statistical analyses found no differences between VPA treated mice and saline treated mice.

Figure 1. VPA (valproic acid) for 7 days reduced the isometric force drop following eccentric contractions and absolute maximal force before eccentric contractions in Mdx mice (Supplementary Figure 1A). Electrophysiology was used to measure absolute maximal isometric force before and after eccentric contractions in mild murine DMD model (Figures A-C). (A) Maximal isometric force drop following eccentric contractions. (B) Absolute isometric maximal force. (C) Specific isometric maximal force. (D) Muscle weight. The number of values per group was 10-14 in Figures A-D. Mdx: Mdx mice treated with saline

for 7 days. Mdx+VPA7: Mdx mice treated with VPA for 7 days. Mdx+VPA1: Mdx mice treated with VPA for 1 day. C57: Healthy mice, with dystrophin. a1, a2, a4 (effect of VPA7): significantly different from Mdx mice,  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.0001$ , respectively. b1, b2, b3, b4: significant different from C57 mice (healthy),  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$ ,  $p < 0.0001$ , respectively. c1, c2: significant different from VPA7 mice,  $p < 0.05$ ,  $p < 0.01$ , respectively.

Figure 2. VPA (valproic acid) for 7 days also reduced the isometric force drop following eccentric contractions and absolute maximal force before eccentric contractions in D2-mdx mice (Supplementary Figure 1B). Electrophysiology was used to measure absolute isometric maximal force before and after eccentric contractions in a severe murine DMD model (Figures A-C). (A) Maximal isometric force drop following eccentric contractions. (B) Absolute isometric maximal force. (C) Specific isometric maximal force. (D) Muscle weight. The number of values per group was 6-8 in Figures AD. D2-Mdx: D2-Mdx mice treated with saline for 7 days. D2-Mdx+VPA7: D2-Mdx mice treated with VPA for 7 days. D2: Healthy mice, with dystrophin. a1, a3, a4 (effect of VPA7): significant different from D2-Mdx mice,  $p < 0.05$ ,  $p < 0.001$ ,  $p < 0.0001$ , respectively. b2, b3, b4: significant different from D2 mice (healthy),  $p < 0.01$ ,  $p < 0.001$ ,  $p < 0.0001$ , respectively.

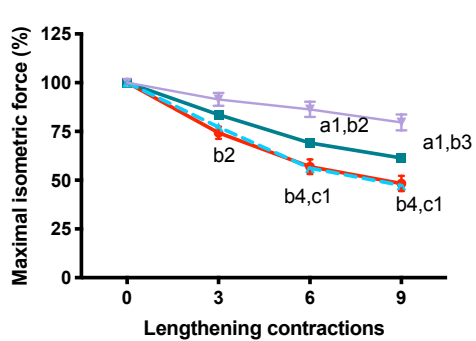
Figure 3. The beneficial effect of VPA (valproic acid) was not increased by voluntary exercise and related to increased utrophin and desmin levels in Mdx mice (Supplementary Figure 1C). Electrophysiology was used to measure absolute isometric maximal force before and after eccentric contractions and absolute maximal eccentric force in a mild murine DMD model (Figures A-E). (A) Maximal isometric force drop following eccentric contractions. (B) Absolute eccentric maximal force during the first eccentric contraction. (C) Relative



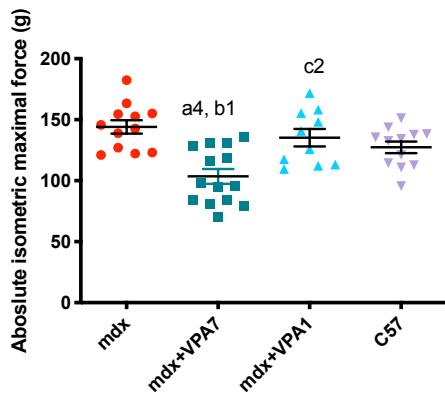
eccentric maximal force (expressed in percentage of isometric maximal force) during the first eccentric contraction. (D) Absolute isometric maximal force. (E) Specific isometric maximal force. (F) Muscle weight. The number of values per group was 12-14 in Figures A-F. Western blotting was used to measure protein levels (Figures G-J). (G) Photograph of a representative immunoblot showing utrophin bands. (H) Utrophin expression. (I) Photograph of a representative immunoblot showing desmin bands. (J) Desmin expression. The number of values per group was 7-11 in Figure G-J. Mdx: Mdx mice treated with saline for 7 days. Mdx+VPA7: Mdx mice treated with VPA for 7 days. Mdx+VPA7+wheel: Mdx mice treated with valproic acid (VPA) for 7 days, that ran for 7 days. kDa: relative molecular weight in kiloDalton. a0, a1, a2, a4 (effect of VPA7): significantly different from Mdx,  $p=0.056$ ,  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.0001$ , respectively.

Figure 1

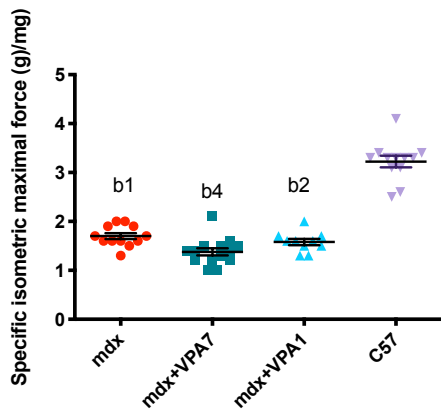
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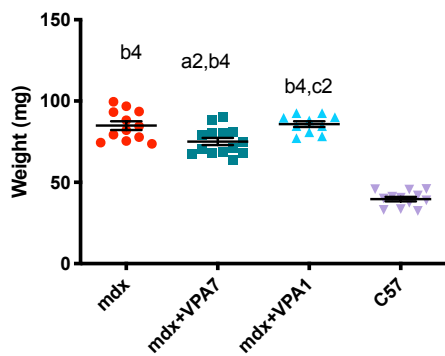
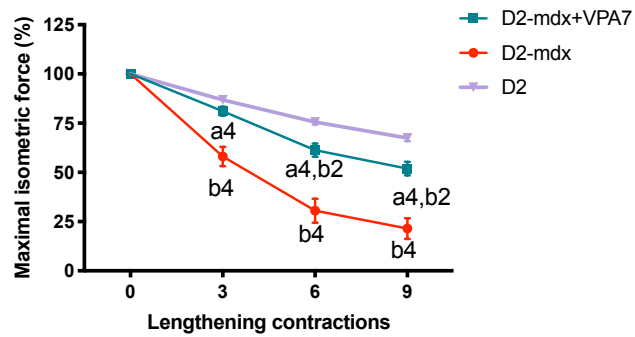
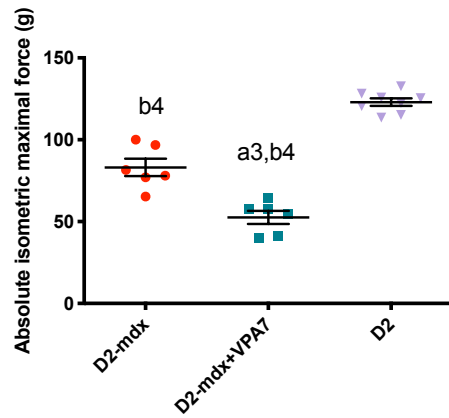


Figure 2

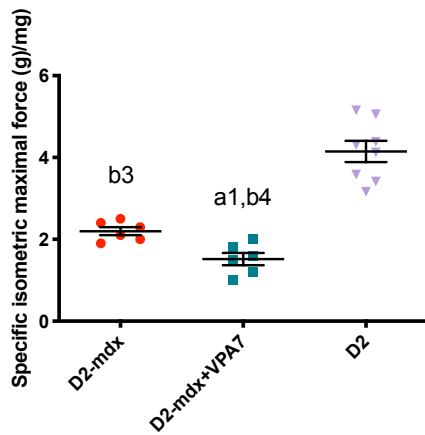
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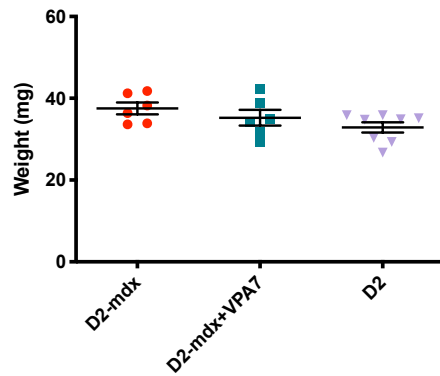
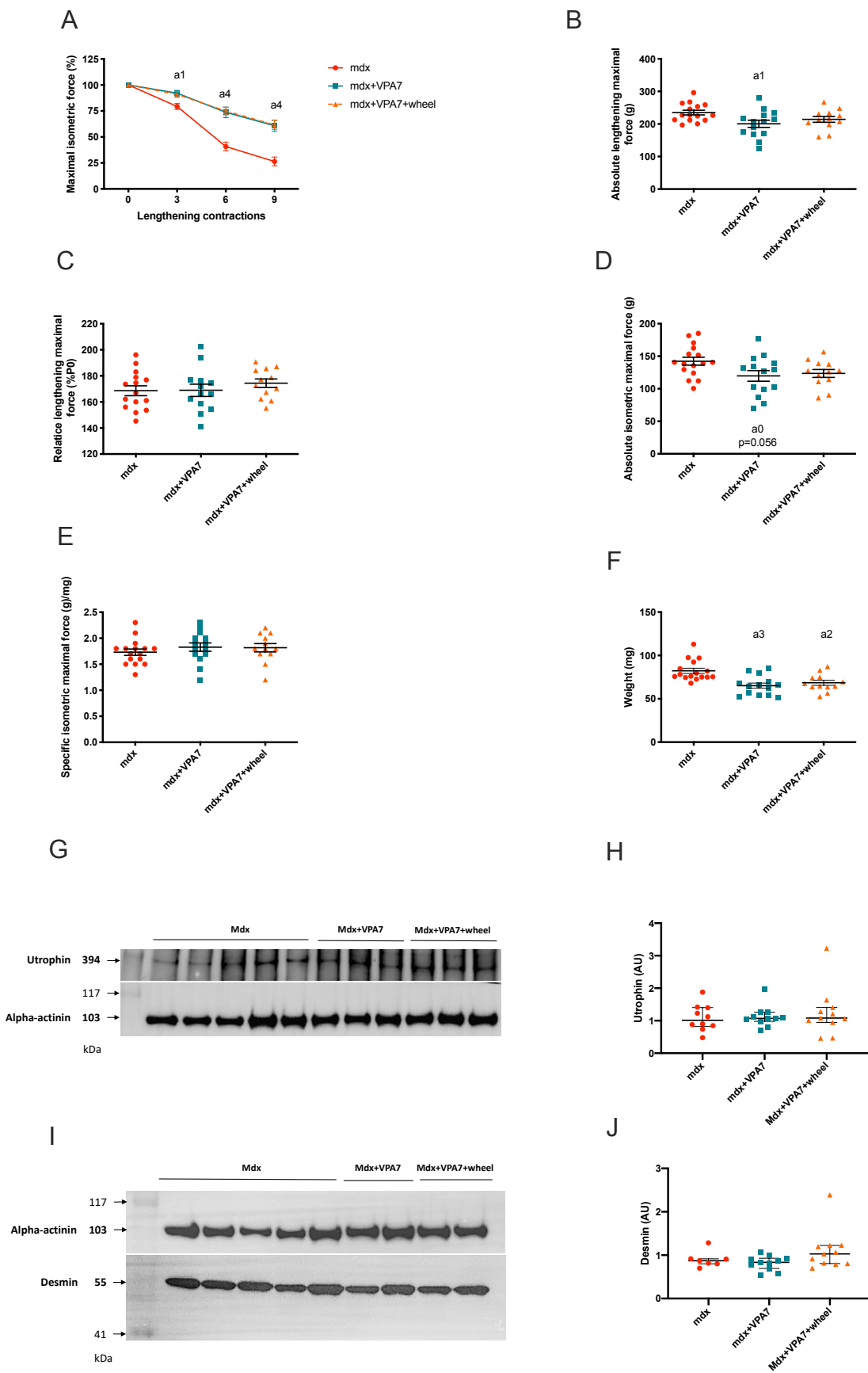
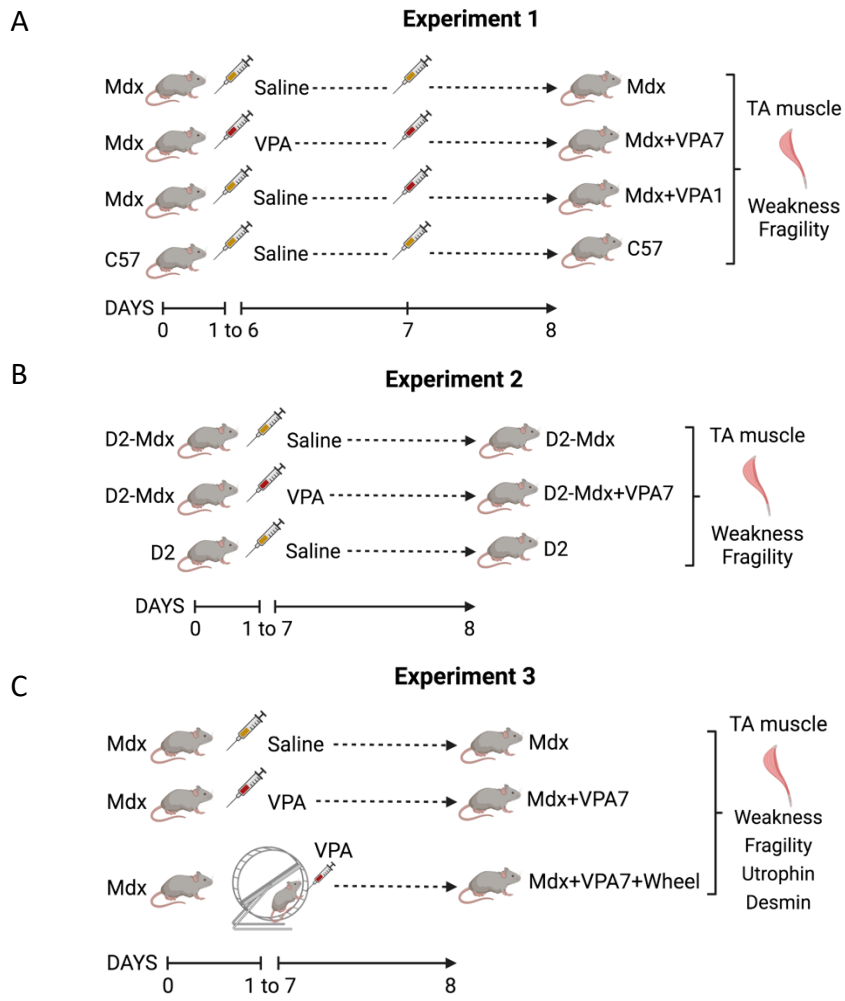


Figure 3



# Supplementary Figure 1



Supplementary Figure 2

