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Research paper

Social stress is lethal in the *mdx* model of Duchenne muscular dystrophy

Maria Razzoli[a,1], Angus Lindsay[b,1], Michelle L. Law[a,1], Christopher M. Chamberlain[b,1], William M. Southern[b], Madeleine Berg[a], John Osborn[a], William C. Engeland[c], Joseph M. Metzger[a], James M. Ervasti[b,*], Alessandro Bartolomucci[a,*]

[a] Department of Integrative Biology and Physiology, University of Minnesota Medical School, Minneapolis, MN, United States
[b] Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota Medical School, Minneapolis, MN, United States
[c] Department of Neuroscience, University of Minnesota Medical School, Minneapolis, MN, United States

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ABSTRACT

Background: Duchenne muscular dystrophy (DMD) is caused by the loss of dystrophin. Severe and ultimately lethal, DMD progresses relatively slowly in that patients become wheelchair bound only around age twelve with a survival expectancy reaching the third decade of life.
Methods: The mildly-affected *mdx* mouse model of DMD, and transgenic DysΔMTB-*mdx* and Fiona-*mdx* mice expressing dystrophin or utrophin, respectively, were exposed to either mild (scruffing) or severe (subordination stress) stress paradigms and profiled for their behavioral and physiological responses. A subgroup of *mdx* mice exposed to subordination stress were pretreated with the beta-blocker metoprolol.
Findings: Subordination stress caused lethality in ~30% of *mdx* mice within 24 h and ~70% lethality within 48 h, which was not rescued by metoprolol. Lethality was associated with heart damage, waddling gait and hypo-locomotion, as well as marked up-regulation of the hypothalamus-pituitary-adrenocortical axis. A novel cardiovascular phenotype emerged in *mdx* mice, in that scruffing caused a transient drop in arterial pressure, while subordination stress caused severe and sustained hypotension with concurrent tachycardia. Transgenic expression of dystrophin or utrophin in skeletal muscle protected *mdx* mice from scruffing and social stress-induced responses including mortality.
Interpretation: We have identified a robust new stress phenotype in the otherwise mildly affected *mdx* mouse that suggests relatively benign handling may impact the outcome of behavioural experiments, but which should also expedite the knowledge-based therapy development for DMD.
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1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked, progressive and lethal neuromuscular disease affecting 1 in 5000 boys [1] caused by mutations in the gene encoding the protein dystrophin [2]. DMD patients experience progressive muscle weakness, and typically become wheelchair bound by age 12 with eventual death in the third decade of their life due to respiratory or cardiac failure. DMD patients also suffer significant cardiomyopathy generally manifesting clinically in the early teenage years and becoming highly prevalent in the second decade [3–5]. Dilated cardiomyopathy and fibrosis become more severe with age, and these symptoms also develop in X-linked dilated cardiomyopathy patients who lack dystrophin expression only in cardiac tissues [4,6]. Furthermore, DMD patients exhibit aberrant autonomic signaling such as tachycardia, atrial and ventricular arrhythmias, sweating, and reduced heart rate variability even in the absence of overt cardiomyopathy [7]. The combination of dysautonomia and cardiomyopathy could play a critical role in acute episodes of heart failure observed in DMD patients [8].

The *mdx* mouse also lacks dystrophin expression, and is the most widely employed animal model of DMD. The *mdx* mouse recapitulates skeletal muscle, cardiac, and behavioural features of DMD [9–16], but presents with a lifespan that is only ~4 months shorter compared to wild type [17]. Like DMD patients, the *mdx* model also develops cardiomyopathy, but only after ~24 months of age, although acute heart pump failure can be unmasked earlier by inducing cardiac pharmacological stress with β-Adrenergic Receptor (AR) agonists [3,18–20]. The slow progression of cardiovascular disease in

* Corresponding authors.
E-mail addresses: jervasti@umn.edu (J.M. Ervasti), abartolo@umn.edu (A. Bartolomucci).
[1] Equal contribution.

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Research in context

Evidence before this study

Duchenne muscular dystrophy (DMD) is a fatal muscle disease caused by mutations in the dystrophin gene. Although significant progress has been made in understanding the molecular determinants of its pathogenesis, effective therapies for DMD are lacking. This limitation is at least in part due to the mild phenotype manifested by animal models where mechanistic studies are possible. Specifically, the slow progression of cardiovascular disease in the *mdx* mouse model of DMD slows preclinical testing of innovative and promising therapeutic approaches.

Added value of this study

We report the identification of an extreme vulnerability of the *mdx* mouse to stress resulting in exacerbation of the pathology, that could accelerate the pace of preclinical, proof of concept studies. The application of a severe social stress model to the *mdx* mouse induced ~70% lethality within 48 h. Social stress-induced lethality was associated with heart damage, waddling gait, reduced ambulation, and marked up-regulation of the stress endocrine axes. Furthermore, we showed that therapeutically relevant transgenic expression of dystrophin or utrophin in skeletal muscle protected *mdx* mice from stress-induced physiological responses including mortality.

Implications of all the available evidence

The implications of the extreme stress-vulnerability phenotype of the *mdx* mouse highlighted in our study is two-fold: while it raises concern that relatively benign handling of *mdx* mice may affect the outcome/interpretation of experiments, it also facilitates and accelerates therapy development for DMD by catalysing the disease progression in a preclinical and translationally relevant model. Additionally, it highlights a cardiovascular instability and hypotension phenotype associated with dystrophin deficiency in mice, which is paralleled by recent clinical observations in DMD.

mdx mice models slows preclinical testing of innovative and promising therapeutic approaches for DMD.

Here we report the identification of an extreme vulnerability of the *mdx* mouse to stress. We show that subordination stress [21–23] causes lethality in ~30% of *mdx* mice within 24 h and ~70% lethality within 48 h. The robust stress phenotype of *mdx* mice was replicated in two genetic backgrounds, was associated with heart damage, waddling gait and hypo-locomotion, and marked upregulation of stress endocrine axes. A novel cardiovascular phenotype emerged in *mdx* mice, in that even a mild stress (scruffing) caused a transient drop in arterial pressure, while subordination stress caused severe and sustained hypotension with concurrent tachycardia. Towards its potential value in preclinical testing, we show that transgenic expression of dystrophin or utrophin in skeletal muscle protected *mdx* mice from stress-induced physiological responses including mortality.

2. Methods

2.1. Animals

We used transgenic DysΔMTB-*mdx* mice [24], Fiona-*mdx* mice [25] and non-transgenic *mdx* littermates of each line. Because the DysΔMTB-*mdx* are on a mixed C57BL/10:C57BL/6J background, we used C57BL/6J as wild type controls (Jackson stock: 000664). The DysΔMTB-*mdx* line expresses a full-length dystrophin/utrophin chimera in which microtubule-binding spectrin repeats 20–24 of dystrophin are replaced by non-binding repeats 18–22 of utrophin [24] while Fiona-*mdx* mice express full-length utrophin [25]. In separate experiments we also used *mdx* mice on a C57BL/10 background (C57BL/10ScSn-Dmd^{mdx}/J; Jackson stock number: 001801) and C57BL/10ScSnJ as controls (Jackson stock: 000666). All mice were housed in groups of littermates (up to 4 mice per cage) maintained following standard specific pathogen free (SPF) procedures, and tested in a dedicated room when 4–5 months of age. Mice were given food (Teklad 2018, Harlan) and water ad libitum and were housed in static cages (186 mm x 298 mm x 128 mm) provided with corn cob bedding and Enviro-dri® for nesting material under a 12 h light : 12 h dark schedule and at 21 ± 2 °C. For all the general experimental and maintenance procedures, mice were handled by being lifted by the base of their tail.

CD1 male mice that served in the subordination stress model as aggressive residents were purchased from Charles River labs and individually housed in static cages to be used for the subordination stress protocol (257 mm x 483 mm x 152 mm) provided with food, water and bedding and under the same general environmental conditions as described above. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota.

2.2. Scruffing-induced inactivity

Activity was measured using an AccuScan system by Columbus Instruments, Inc. Activity was determined by measuring vertical beam breaks. Mice were placed in the open-field apparatus for 30 min prior to scruffing and activity was measured. Mice were then removed from the open-field apparatus and "scruffed" for 30 s. A "scruff" hold consisted of grasping the mouse by the nape of the neck between the index finger and thumb and then placing the tail between the palm of the hand and the 5th digit. Immediately following scruffing, mice were returned to the open-field apparatus and activity was recorded for another 30 min. Data are presented as post-scruff:pre-scruff vertical beam breaks.

2.3. Chronic subordination stress and 24 h subordination stress protocols

The protocol used is a modified version of our standard chronic subordination stress procedure [21–23,26]. The experimental mice were exposed to daily 10-min social defeat exposure by a CD1 mouse and continuous sensory contact (allowing auditory, olfactory and visual stimuli but preventing physical interaction) for 14 days. A separate group of mice was exposed to a shortened protocol consisting of one 10-min social defeat and 24 h sensory contact and sacrificed at the experimentally determined time point. Moribund mice were identified based on an independent assessment made by two investigators for conditions including prolonged respiratory distress, inability to eat or drink, or ataxia that prevents normal functions as approved by the IACUC, University of Minnesota. In the 14 days subordination stress protocol, organs were collected from all surviving mice, and from moribund mice when the tissues were in a good state of preservation (2 out of 5 *mdx*, 3 out of 4 Fiona-*mdx*, and 1 DysΔMTB-*mdx*) (See Fig. 1A,B).

In one group of mice tail vein blood was sampled immediately before the exposure to the social defeat (within 3 min of entering the animal room) and then at 30 min as well as 6 h after the 10-min social defeat. Blood samples were used for corticosterone analysis.

In one group of mice locomotor activity was determined throughout the experiments, except during the aggressive interaction, by means of an automated system that used small passive infrared sensors positioned on the top of each cage (ActiMeter, TecnoSmart SpA, Rome, Italy).

In a separate experiment, C57BL/10 *mdx* mice were pretreated for one week prior to 24 h of the subordination stress exposure with a β-AR antagonist (metoprolol, 2 mg/mL, Sigma) in the drinking water. This dose was chosen based on previously published data [27,28].

2.4. Radiotelemetry for hemodynamic monitoring

A dedicated group of mice underwent surgery for the implantation of a pressure catheter in the femoral artery attached to a telemetry device (PA-C10; Data Sciences International, Saint Paul, MN). Briefly, mice were be prepped for surgery under aseptic conditions, and anaesthetised with 2% isofluorane. A small incision was made in the flank to hold the body of the transmitter (DSI, St. Paul MN) and another incision was made in the groin to expose the femoral artery. The transmitter was inserted in a small nick made in the femoral and advanced until in the abdominal aorta, suture was placed around the femoral artery and the catheter to hold it in place. Subsequently, the body of the transmitter was tunneled to the flank and each incision was closed with 5-0 silk suture. Mice were housed individually, and after a 7-d recovery period, blood pressure was measured for 24 h before applying the scruffing assay as described above. Blood pressure, heart rate and activity were then continuously monitored for 2 days and in the following 24 h, mice were subjected to 24 h social subordination stress. Moribund mice were identified as defined above.

2.5. Hormonal analyses

For urinary corticosterone measurements, all urine was collected from mice over a 24 h period. Corticosterone was then measured by ELISA (Arbor Assays: K014-HI). To control for differences in overall urine concentration, urinary creatinine was measured by ELISA (Enzo Life Sciences: ADI-907-030A). A ratio of corticosterone:creatinine was then generated.

For plasma corticosterone measurements, tail blood or trunk blood following mouse decapitation was collected and immediately placed at 4 °C. Blood was centrifuged at 20,000 rcf at 4 °C and plasma was frozen at −20 °C. Plasma corticosterone was determined by I-125 radioimmunoassay (MP Biomedical, Solon, OH, USA) [26]. The intra-assay and inter-assay CVs for plasma corticosterone were 7.6% and 13.3%, respectively. Serum cardiac troponin I (cTnI) was measured by ELISA (Life Diagnostics: CTNI-1-HS).

2.6. Western blot analysis

Tissue was pulverised with mortar and pestle in liquid nitrogen. Tissue was then lysed with 1% SDS solution with added protease inhibitors [100 nM Aprotinin, 10 mg/mL E-64, 100 μM Leupeptin, 1 mM PMSF, 1 μg/mL Pepstatin] proportional to mass of the tissue pellet. Protein concentration was measured by A280 absorbance. Equal concentrations of lysate were then separated by electrophoresis at 150 V for 1 h, then transferred to PVDF membrane at 100 V/0.7Amp for 1 h. Membranes were blocked in either 5% non-fat milk in phosphate buffered saline or 0.1% Tween solution for 1 h. Primary antibodies used were anti-Dys1 (Leica) at 1:100 and anti-GAPDH (Sigma-G9545) at 1:10,000. Immunoreactive band intensity was quantified on a LI-COR Odyssey imaging system.

2.7. Histology

Tibialis anterior muscle and heart were dissected and cryopreserved in liquid nitrogen cooled isopentane before obtaining 10 μm cryosections at the mid-belly. Sections were fixed in 4% paraformaldehyde (PFA) for 15 min, washed in phosphate buffered saline (PBS), blocked for 1 h at room temperature with 3% BSA/PBS, and stained with laminin (1:500 diluted; Sigma-Aldrich L9393) and Ly6G (1:80; Proteintech 24633-1-AP) or laminin and CD68 (1:100; Proteintech 25747-1-AP) overnight at 4°C in a humidified chamber. Sections were then washed in PBS and incubated with anti-Rabbit Alexa Fluor 488 (1:750; ThermoFisher Scientific) or anti-Rat Alexa Fluor 494 (1:750; ThermoFisher Scientific) for 1 h at room temperature. Sections were washed a final time in PBS and mounted in ProLong Golf Antifade with DAPI (ThermoFisher Scientific). Images were acquired on a Leica DM5500 B microscope equipped with a Leica HC PLAN APO 10x objective and stitched together with LASX software (Leica) to allow visualization of the entire TA. All nuclei stained positive for either CD68 or Ly6G as well as DAPI were counted and combined to determine total neutrophil and macrophage-associated nuclei. Hearts were also stained with hematoxylin and eosin (H&E) and Masson's Trichrome by the Lillehei Heart Institute Histology Core (University of Minnesota, Minneapolis, MN). Images were obtained with the Zeiss Axio Imager M1 Upright Microscope. All histological images were quantified by an investigator blind to the experimental treatments.

2.8. Statistical analysis

Statistical significance was determined by one or three way ANOVA for repeated measures with Tukey's post hoc test, *t*-test (using Bonferroni correction of α level), or Kruskall−Wallis followed by Mann-Whitney U Test (using Bonferroni correction of α level) where appropriate. The survival analyses were implemented in R Studio from scratch using the methodological references given below. Survival probability was assessed through the use of the Log-Rank test to compare the differences in Kaplan−Meier survival curves using the "survival" package of the R language. The same survival package was used to fit the Cox proportional hazard model to actual lifespans to calculate the hazard ratio (HR). Multiple binary comparisons were conducted using Bonferroni corrected Log-Rank tests or Fisher exact probability tests performed between two groups of interest at a time following overall statistical significance. Where appropriate, data were sqrt transformed to better satisfy the assumptions of the ANOVA. Statistica (TIBCO Software Inc.) and R were used for analyses. Experimental mice were randomly allocated to the different experimental treatments by flipping a coin.

3. Results

3.1. Behavioural and neuroendocrine changes and lethality in response to graded levels of stress in mdx mice

One robust behavioural phenotype of *mdx* mice is decreased activity following a brief restraint by scruffing [15,16,29] or mild exercise [24,30,31], that has been attributed to exercise-induced muscle damage or an enhanced fear-induced freezing response. We replicated the reduced physical activity after a 30-s scruff-hold in *mdx* compared to wild type mice (Fig. 1A) while also reporting increased corticosterone as a functional measure of hypothalamic-pituitary-adrenal (HPA)-axis activation either in the urine (collected 24 h before and 24 h after scruffing) or in the plasma (before, 30 min and 6 h after scruffing) (Supplementary Fig. 1A-B) suggesting that *mdx* mice manifest an exaggerated neuroendocrine sensitivity to even a mild stressor.

The inactivity response of *mdx* mice to scruffing prompted us to expose them to a model of subordination stress, one of the most severe and translationally relevant protocols of environmental and psychosocial stress in rodents [26]. Our protocol consisted of brief social defeat episodes with an aggressive CD1 male followed by chronic sensory housing [21−23]. In this protocol, non-dystrophic mice manifest sustained tachycardia, hypercorticosteronemia and persistent HPA axis activation [26,32−34]. A single episode of social defeat followed by sensory housing caused a prolonged increase in plasma corticosterone 6 h after the occurrence of the actual social defeat in *mdx* mice, a time point when the response of the wild type subjects had already plateaued following the expected rapid increase measured at 30 min (Supplementary Fig. 1C).

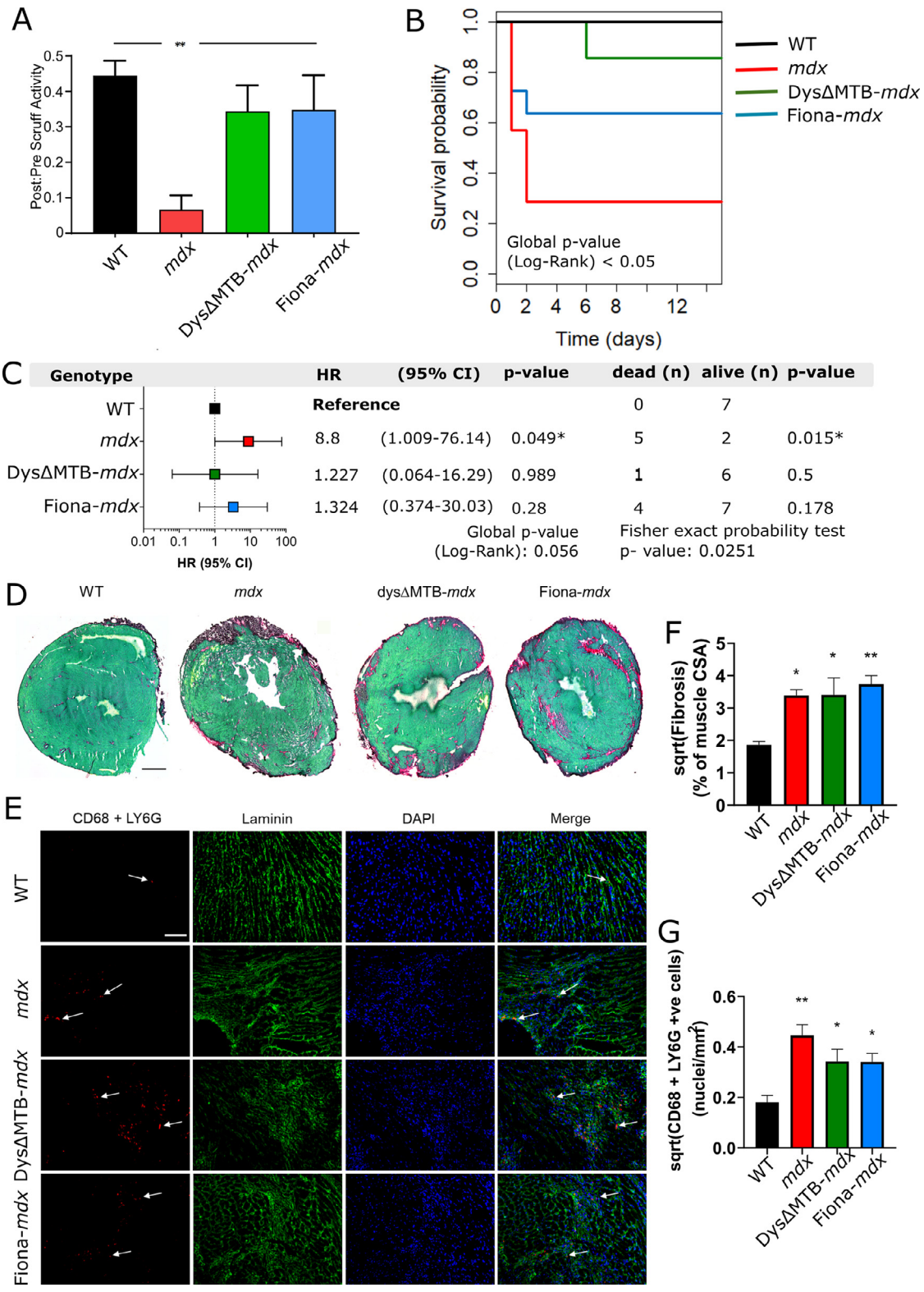

Fig. 1. Chronic Subordination Stress induces mortality in *mdx* mice that can be rescued by skeletal muscle expression of either dystrophin or utrophin, but that can't be explained by acute cardiac damage. (A) *mdx* mice display reduced physical activity following scruffing. This phenotype is rescued by skeletal muscle-specific expression of dystrophin or utrophin (DysΔMTB-*mdx* ($F(4,24) = 13.47$, $p < 0.0001$, $N = 3–11$). (B) 14-day chronic subordination stress impact on WT, *mdx*, DysΔMTB-*mdx*, and Fiona-*mdx* mice survival, as measured by survival probability (B), hazard ratio and number of mice surviving (C). (D) Representative images of fibrosis present in the myocardium of WT, *mdx*, DysΔMTB-*mdx*, and Fiona-*mdx* mice subjected to chronic subordination stress. Whole hearts harvested from surviving mice, and from moribund mice with good tissue preservation, were stained with Fast Green (myocardium) and Sirius Red (fibrosis). Scale bar = 500 μm. (E) Representative images of fluorescent microscopy of cardiac tissue for LY6G (immune cells; red), laminin (cell membrane; green), and DAPI (nuclei; blue). Arrows in LY6G and Merged panels indicate several examples of immune cells and their corresponding location. Scale bar = 50 μm. (F) Quantification of fibrosis present in the myocardium expressed as a percent of total cross-sectional area (CSA) ($F(3,24) = 6.70$, $p < 0.01$, $N = 4–7$). (G) Quantification of immune cells present in the myocardium of WT, *mdx*, DysΔMTB-*mdx*, and Fiona-*mdx* mice expressed as a percent of total DAPI-stained nuclei ($F(3,23) = 6.75$, $p < 0.01$, $N = 4–7$). Different letters mean statistical differences as highlighted by HSD Tukey's post-hoc test following significant ANOVAs.

We next subjected wild type and *mdx* mice to a 14-day chronic subordination stress protocol. Surprisingly, *mdx* mice did not tolerate this protocol with 72% of the *mdx* mice found dead or moribund within the first 48 h (Fig. 1B-C). Visual observations showed that stressed *mdx* manifested waddling gait (Supplementary Videos 1 and 2). Hearts harvested from moribund and surviving *mdx* mice exhibited increased immune cell infiltration and fibrosis compared to wild-type mice (Fig. 1D–G). Due to the study design we cannot discern if these alterations were preceding the exposure to the subordination stress, but published data support a notion that similar levels of cTnI, heart fibrosis and immune cell infiltration can be expected for *mdx* and wild type mice at baseline [35]. Since a large proportion of *mdx* mice died in the first 24 h (Fig. 1A), we used a shortened 24 h subordination stress protocol aimed at an early assessment of tissue damage allowing an early evaluation of myocardial and skeletal muscle damage and plasma cTnI. Consistent with the chronic subordination stress study, ~30% of *mdx* mice were found dead or moribund within 24 h of exposure to subordination stress (Supplementary Table 1). In contrast, no wild-type mice died despite receiving an equivalent amount of aggression (average number of attacks: wild-type = 4.3+/−0.2; *mdx* = 3.8 +/- 0.08). Following 24 h subordination stress, *mdx* mice presented with immune cell infiltration in both the tibialis anterior as well as the heart (Fig. 2A–D), in parallel with elevated plasma cTnI (Fig. 2E). The dead/moribund *mdx* mice also showed a marked inactivity, while non-moribund *mdx* mice showed only a transient decline that fully recovered afterwards (Supplemental Fig. 2). The same decreased survival, immune cell infiltration and plasma cTnI elevation outcome was replicated in *mdx* on the C57BL/10 background (Supplemental Fig. 3).

Based on previous data showing that βAR agonists exacerbate and unmask cardiovascular dysfunction in *mdx* mice [20], and the known involvement of adrenergic signaling in the stress response [36,37], we pre-treated *mdx* mice with the βAR blocker metoprolol for one week

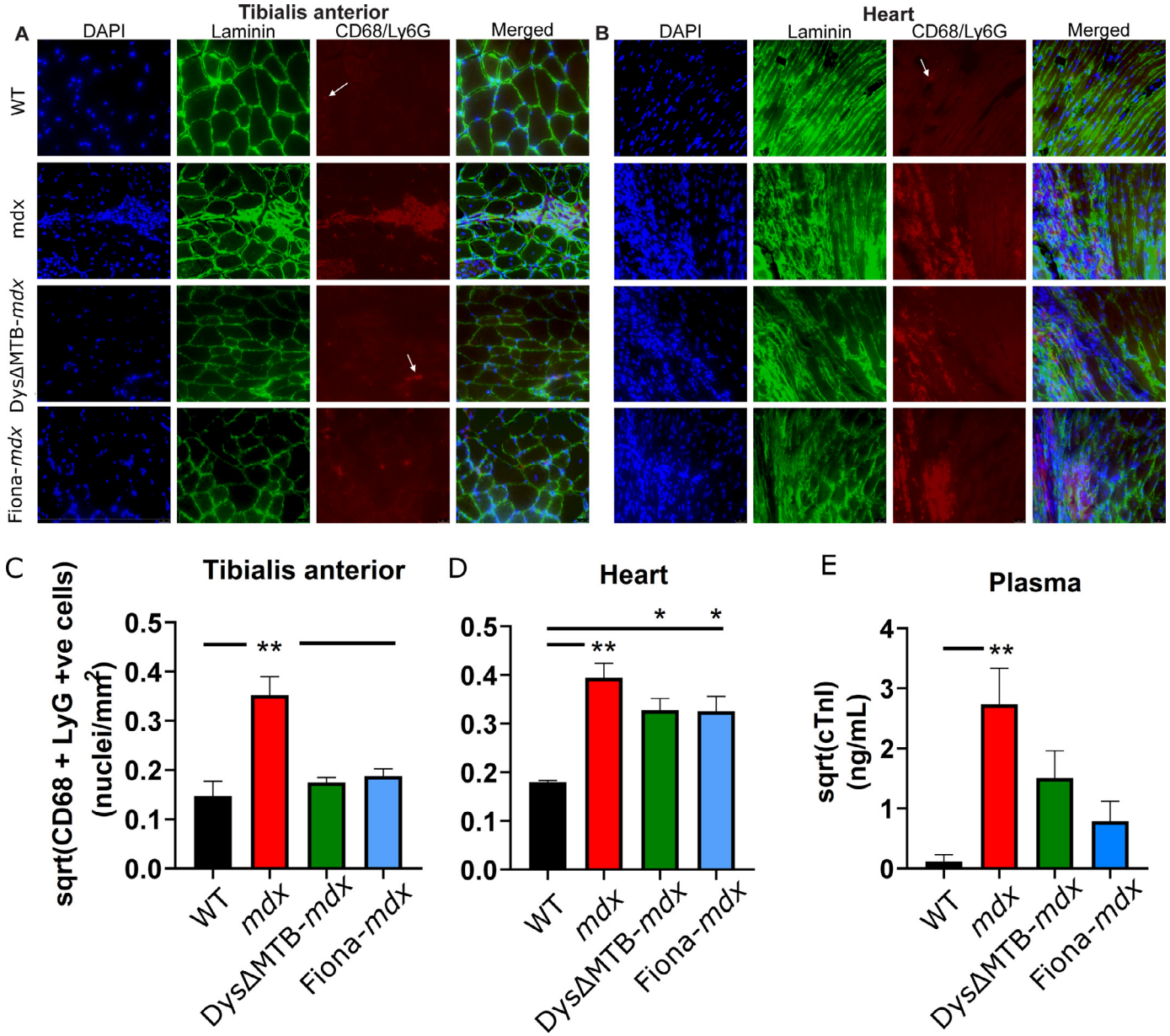

Fig. 2. Skeletal muscle-specific dystrophin reduces skeletal muscle inflammation but does not affect cardiac muscle inflammation following 24 h subordination stress. (A) Representative tibialis anterior and (B) heart cross-sections positive for CD68 (macrophages) and Ly6G (neutrophils) following 24 h of subordination stress. (C) Skeletal muscle-specific dystrophin lowers tibialis anterior CD68 + Ly6G positive cells following 24 h of CSS (ANOVA, $F(3, 19) = 2.946$, $p = 0.01$; $N = 4–7$). (D) Skeletal muscle-specific dystrophin does not affect heart CD68 + Ly6G positive cells following 24 h of CSS (ANOVA, $F(3, 19) = 2.731$, $p = 0.013$; $N = 4–7$). (E) Serum levels of cTnI were significantly elevated in moribund *mdx* mice following 24 h of CSS (ANOVA, $F(3,59) = 4.65$, $p<0.01$; $N = 9–24$). * indicates $p < 0.05$ and ** indicates $p < 0.01$ vs WT at the level of Tukey HSD post hoc test.

before exposure to the 24 h subordination stress protocol. Contrary to the hypothesis that lethality was caused by exaggerated adrenergic activation, metoprolol failed to prevent stress-induced effects in *mdx* mice: i) Frequency of mice found dead/moribund was not different (*mdx* vehicle = 4/10 moribund; *mdx* metoprolol = 2/9 moribund. Chi-square test, NS); ii) cTnI was not normalised by pretreatment with metoprolol (*mdx* stress vehicle moribund = 4.7 ± 0.7 ng/mL, *mdx* stress vehicle non moribund = 0.1 ± 0.6 ng/mL; *mdx* stress metoprolol moribund = 4.3 ± 0.9 ng/mL; *mdx* stress metoprolol non moribund = 0.2 ± 0.6 ng/mL. ANOVA $F(3,16) = 23.6$, $p < 0.001$. Post hoc, moribund groups different from non-moribund ($p < 0.01$). Overall, our results identify a behavioural, neuroendocrine and cardiac phenotype in *mdx* mice as a function of graded levels of stress. Interestingly, we identified an extreme stress-vulnerability phenotype in *mdx* mice wherein acute death in response to subordination stress could not be attributed to aberrant adrenergic regulation. This phenotype contrasts with the general notion that *mdx* are only mildly affected by dystrophin deficiency, and offers a preclinical platform to test and develop innovative therapies for DMD.

3.2. *Dystrophin deficiency causes hypotension in response to graded levels of stress in* mdx *mice*

To determine whether altered hemodynamic responses might underlie subordination stress-induced lethality, we used radiotelemetry to measure mean arterial pressure (MAP) and heart rate (HR) in freely

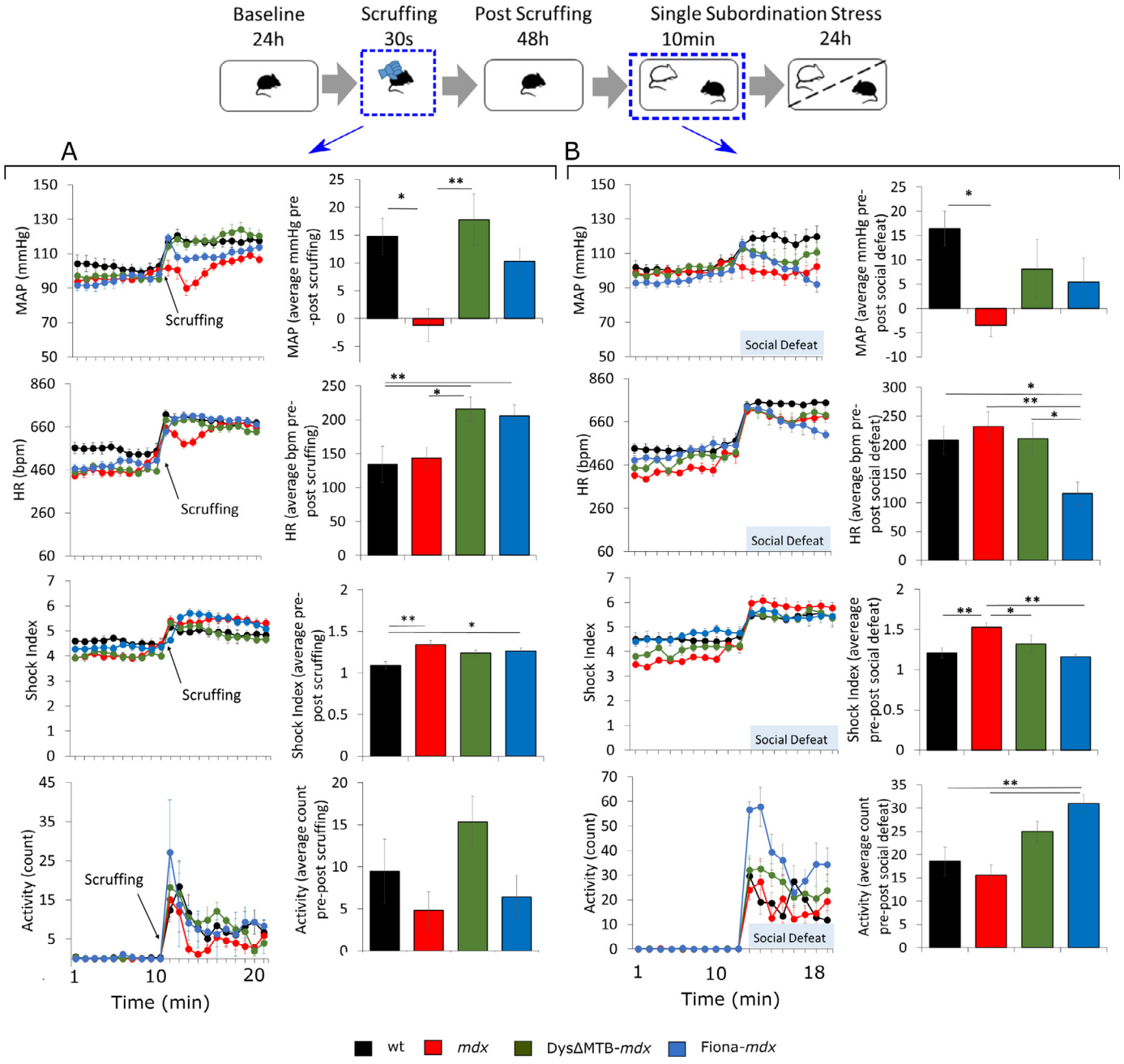

Fig. 3. Skeletal muscle-specific dystrophin expression protects *mdx* mice against hemodynamic instability triggered by scruffing and subordination stress. Mean arterial pressure, heart rate, shock index (heart rate/systolic blood pressure) and activity levels time-course as well as ratio over baseline values as a function of either scruffing (A, MAP Genotype $F(3,30) = 7.27$, $p < 0.001$; HR Genotype: $F(3,30) = 6.35$, $p < 0.01$; Shock Index Genotype: $F(3,29) = 6.37$, $p<0.01$) or social subordination (B, MAP Genotype: $F(3,29) = 3.25$, $p < 0.05$; Shock Index Genotype: $F(3,29) = 11.40$, $p < 0.001$; Activity Genotype: $F(3,28) = 8.23$, $p < 0.001$). * indicates $p < 0.05$ and ** indicates $p < 0.01$ at the level of Tukey HSD's post hoc test. ($N = 8–9$).

moving mice. While stress-induced tachycardia is well established in mice [34,38,39], the effect of stress on blood pressure has received limited study so far [40]. We first established that 14 days of chronic subordination stress induced a marked and sustained tachycardia and hypertension in wild type mice (Supplementary Fig. 4). Next, wild type and *mdx* mice were exposed to scruffing, followed 48 h later by a 24 h subordination stress protocol. *Mdx* mice showed an unexpected transient drop in MAP resulting in an overall lower delta MAP in the 10 min after scruffing when compared to wild type, in the presence of a normal tachycardia (Fig. 3A) and a significant increase in the clinically-relevant shock index (SI) [41] (Fig. 3A). Nevertheless, *mdx* mice fully recovered after this transient hypotensive response to scruffing (Supplementary Fig. 5). Conversely, when exposed to a 24 h subordination stress protocol, *mdx* mice showed, again, normal stress-induced tachycardia in the presence of an overall lower delta MAP and increased SI when compared to wild type mice (Fig. 3B). The tachycardia and the SI elevation in response to social defeat were more pronounced when compared to scruffing (Fig. 3). By analysing changes in MAP based on the time of the first attack by the CD1 aggressor, *mdx* mice showed a significant MAP drop of ~20 mmHg which lasted a few minutes (Supplementary Fig. 6) and which was of greater magnitude than the MAP drop induced by scruffing (Fig. 4). At variance with the full recovery in cardiovascular functions observed after the scruffing (Supplementary Fig. 5), *mdx* mice did not return to a normal and stable MAP during the 24 h subordination stress protocol. MAP remained lower and the SI higher when compared to wild type mice despite sustained tachycardia (Fig. 3B). Hypotension developed over the next 24 h, and was followed by a decrease in HR, upon which 5/9 *mdx* mice were found to be dead/moribund (Fig. 5A–C, E-F). Overall, these data suggest that death in *mdx* mice occurred due to a mismatch between demand (HR) and supply (MAP) throughout the 24 h period of subordination stress, thus proving fatal in the affected subpopulation. Acute hypovolemia and hypotension are strong signals for activation of the HPA axis [42], and likely explain the elevated corticosterone levels that were observed after scruffing and social defeat in *mdx* mice.

3.3. *Dystrophin or utrophin transgenic expression in skeletal muscle rescue the stress-induced lethality and cardiovascular phenotype but not the myocardial damage seen in* mdx *mice*

Skeletal muscle-specific expression of dystrophin or utrophin rescue all or nearly all known phenotypes described in the *mdx* mouse [24,30,31]. Therefore, we compared the stress-vulnerability phenotype(s) of *mdx* to DysΔMTB-*mdx* [24], which express dystrophin driven by the skeletal muscle-specific HSA promoter, and Fiona-*mdx* [25] mice, which has a transgenic over-expression of utrophin in the skeletal muscle driven by the HSA promoter (Supplementary Fig. 7). Both transgenic models rescue physical inactivity observed after scruffing (Fig. 1A). Furthermore, both models rescued to wild type level skeletal muscle fibrosis (Fig. 2A,C), home cage activity (Supplementary Fig. 2) and survival (Fig. 1B–D, Supplementary Table 1) in response to either 14 days chronic subordination stress or the 24 h subordination stress protocols. Conversely, fibrosis and immune cell infiltration in the heart as well as plasma cTnI were similarly and significantly elevated in DysΔMTB-*mdx* and Fiona-*mdx* mice compared to wild type, independently of the duration of the subordination stress protocol (Figs. 1 and 2). Next, we analysed the cardiovascular response to scruffing and 24 h subordination stress of DysΔMTB-*mdx* and Fiona-*mdx* mice. DysΔMTB-*mdx* mice were rescued to wild type level for the scruffing- and subordination stress-induced decrease in MAP and did not present with the elevation of SI seen in *mdx* mice (Figs. 3–5). Consistent with the partial lethality, transgenic expression of utrophin in Fiona-*mdx* mice corresponded to an intermediate phenotype characterized by cardiovascular instability (Figs. 3 and 5). Overall, we showed that targeted skeletal muscle expression of dystrophin or utrophin is sufficient to rescue to wild type level the behavioural and cardiovascular abnormalities manifested by *mdx* mice in response to stress.

4. Discussion

Chronic stress has been associated with numerous cardiovascular effects both in animal models and in humans [39,43]. Here we report significant lethality following exposure to acute subordination stress as a robust phenotype emerging in *mdx* mice of different cohorts, and two genetic backgrounds. Specifically, the application of a subordination stress protocol led to a mortality higher than 70% for the *mdx* population within 48 h of exposure with only a few animals surviving up to 14 days. The heart damage and elevation of circulating cTnI in *mdx* mice is consistent with significant cardiac injury, although the evidence that DysΔMTB-*mdx* and Fiona-*mdx* have similar cardiac damage but survived the chronic subordination stress protocol led us to disregard acute cardiac damage as the possible direct mechanism related to the *mdx* death. It is important to point out that the

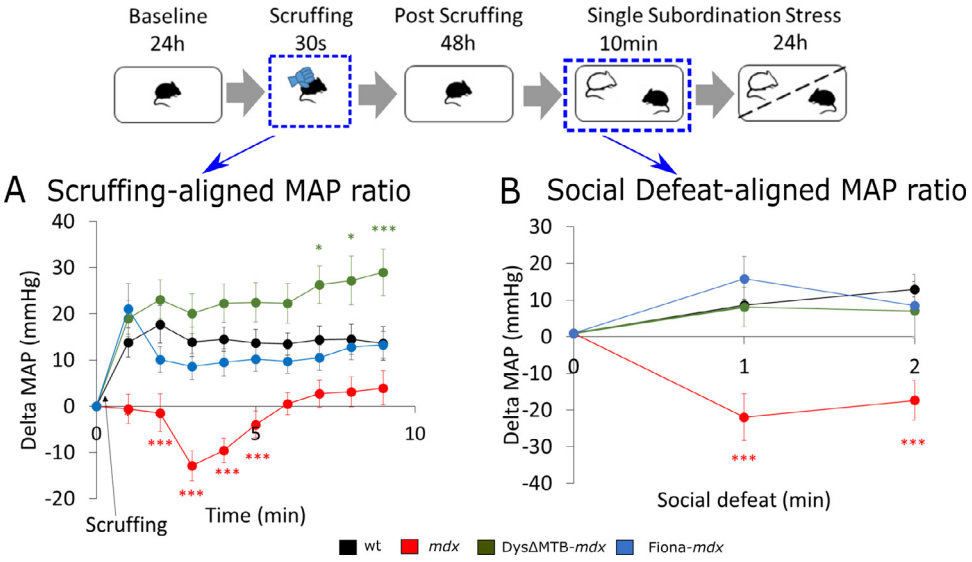

Fig. 4. Subordination stress induces a more profound hypotensive response than scruffing. Mean arterial pressure as a ratio over baseline values of the data in correspondence of scruffing (A, genotype: $F(3,30) = 10.15$, $p < 0.0001$; genotype x time: $F(24,240) = 2.74$, $p < 0.001$) that elicits a blood pressure drop of a lesser magnitude than the one triggered at the time of the physical attack (B, genotype: $F(3,29) = 7.80$, $p < 0.001$; genotype x time: $F(3,29) = 2.79$, $p = 0.06$). * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$ at the level of Tukey HSD's post hoc test of the respective color group vs wt mice. ($N = 8–9$).

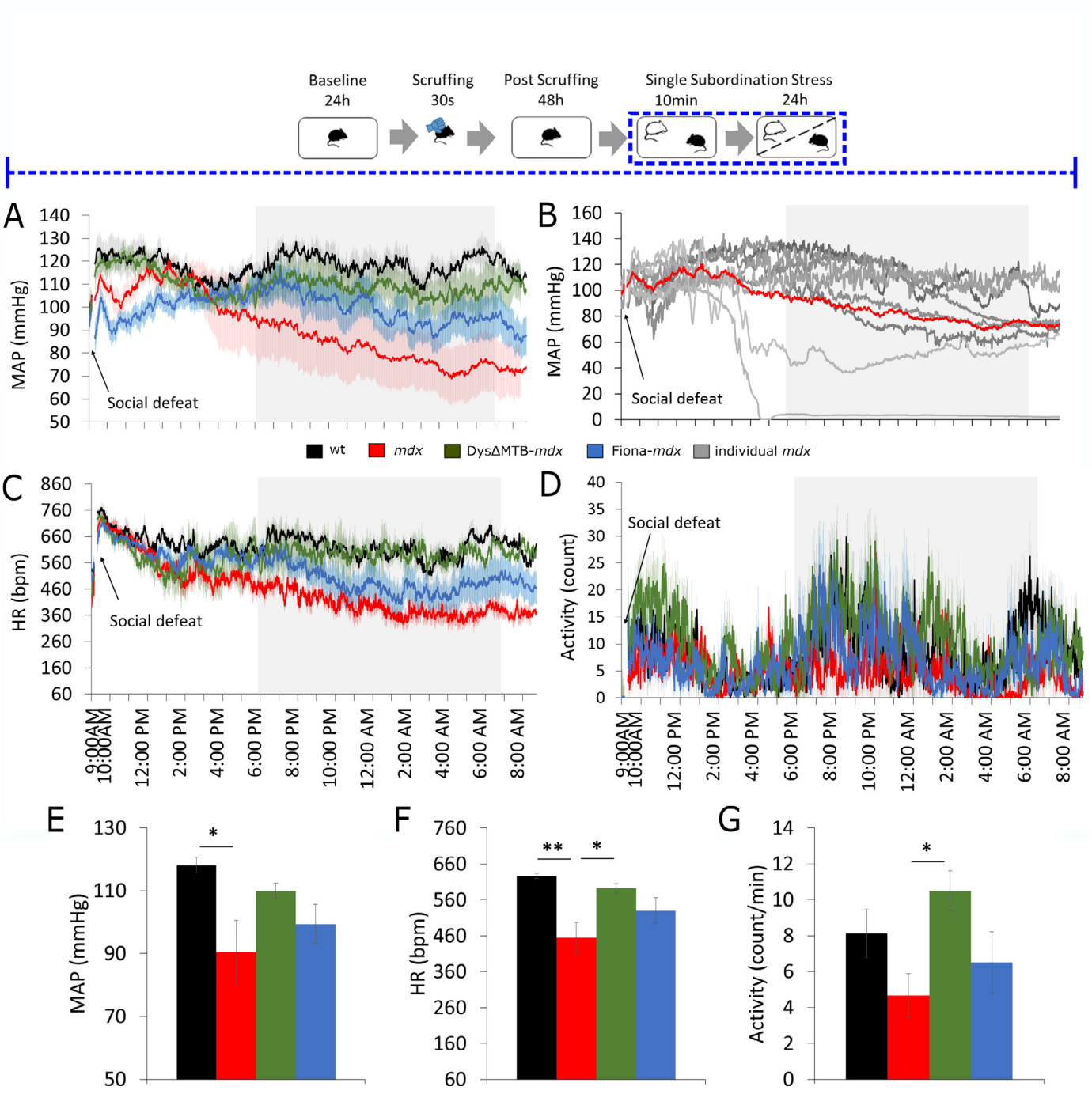

Fig. 5. Skeletal muscle-specific dystrophin expression protects against hemodynamic instability triggered by a single subordination stress in *mdx* mice. Time course of the 24 h sensory contact phase following a single social defeat: A) mean arterial pressure (MAP) time-course; B) individual *mdx* mice MAP traces; C) heart rate (HR) time-course; D) activity time-course; E) average MAP ($F(3,29) = 3.7$, $p < 0.05$); F) average HR ($F(3,29) = 6.448$, $p < 0.01$); G) average activity ($f(3,29) = 3.082$, $p < 0.05$). Unless otherwise specified, data are represented as group averages ± SEM. * indicates $p < 0.05$; **indicates $p < 0.01$ at the level of HSD Tuckey's post hoc test ($N = 8–9$).

physiological impact of the subordination stress models used in this study is not equivalent to the effect of low social rank in groups of sibling male mice [22,44]. This model was designed to elicit profound physiological responses which can be understood by taking into account the remarkable territorial behaviour and intolerance toward unfamiliar intruders manifested by male mice [22,44,45]. Yet, these data suggest that social and aggressive behaviour, a major source of stress in social mammals [36], should be carefully considered when investigating *mdx* mice.

Increased heart rate and concomitant decreased blood pressure response indicate a hemodynamic instability in response to stress that could be linked to organ damage and be lethal. In this light, comparable levels of elevated shock index (>40%) as observed in our study have been associated to myocardial and microvascular damage in humans [46]. Recent clinical studies have called attention to a critical role for low blood pressure in DMD [47–50]. Specifically, low blood pressure in an ambulatory setting emerged as a distinctive feature of DMD patients [47,49,50]. In a retrospective study of 43 DMD patients [48], Cheeran and coworkers identified low systolic pressure as one of the prognostic factors that predicts a worse outcome and death.

Our results demonstrate that *mdx* mice manifest enhanced stress sensitivity phenotypes along a gradient, distributed from inactivity,

to exaggerated HPA-axis activation, to hypotension, and ultimately to death. Inactivity phenotypes have been previously reported in *mdx* mice following brief downhill treadmill running [31], scruffing [15,16,29], electrical foot shock [15], and measurement of whole limb force [51], and have been ascribed to an exaggerated fatigue response [31], fear-induced freezing [15,16,29], or avoidance of antalgic stimuli [51]. Here, we show that *mdx* mice manifest an inactivity phenotype associated with a drop in blood pressure in response to both mild scruff stress and more severe subordination stress. Both scruff- and subordination stress-induced inactivity are corrected by skeletal muscle-specific expression of either dystrophin or utrophin. Collectively, ours and others' [15,16,29,31,51] data raise caution that even relatively benign handling (ie., scruffing) of the *mdx* mouse model can significantly impact measured pathophysiology and complicate interpretation of experimental outcome.

The dystrophin glycoprotein complex functions to mechanically link the cortical cytoskeleton and the extracellular matrix [52]. The loss of sarcolemmal dystrophin is known to cause membrane fragility in *mdx* mice and DMD patients, and is rescued in DysΔMTB-*mdx* [24] and Fiona-*mdx* mice [30]. Here, we proved that skeletal muscle-specific expression of dystrophin or utrophin was sufficient to protect *mdx* mice against subordination stress-induced inactivity, hemodynamic imbalances and lethality. These results suggest an additional potential therapeutic benefit of restoring dystrophin and/or utrophin in young DMD patients using gene therapy approaches to target muscle tissue [53–56]. Because dystrophin is absent from both the brain, heart and smooth muscle of DysΔMTB-*mdx* and Fiona-*mdx* mice (Supplementary Fig. 7), our data support two different scenarios, i) a skeletal muscle>brain>vasculature pathway mediated by sensory nerves innervating the skeletal muscle, or ii) a direct skeletal muscle vasculature loop. In both scenarios our data are consistent with the existence of yet unidentified signal(s) secreted/released from dystrophin-null skeletal muscle. Dystrophin-null skeletal muscle cells are notoriously unstable and subject to frequent damage [57], potentially associated with the secretion/release of metabolites and/or signaling molecules [58–62] that might trigger a rapid vasodilation and HPA-axis activation, to precipitate cardiovascular and/or cardiorespiratory failure during an intense stress response.

Although transgenic expression of full-length dystrophin or utrophin was sufficient to rescue most of the stress-induced phenotypes in *mdx* mice, the heart was still damaged by subordination stress. This result is in keeping with the susceptibility to heart failure manifested by dystrophin deficient patients [4] and mouse models [10,63] and suggests that DysΔMTB-*mdx* and Fiona-*mdx* can manifest latent cardiac damage and lifespan shortening in the presence of chronic subordination stress [18,45]. This result is coherent with the findings from X-linked dilated cardiomyopathy patients, where the loss of dystrophin expression specifically in cardiac tissues causes a severe cardiac phenotype [64,65]. While the direct mechanistic relationship between dystrophic muscle and cardiomyopathy remains unclear, chronic dystrophin loss in the heart clearly has an impact on cardiac health that warrants further investigation.

Stress-induced hypotension was a robust and potentially lethal phenotype in *mdx* mice. However, identifying the cause of death in a mouse is a very difficult endeavor [45,66,67]. Death could have been caused by one or more conditions associated with hypotension, such as stroke, cardiac arrest, or respiratory failure. These conditions are among the most common causes of death in DMD [48,68]. Thus, one limitation of the present study is the inability to precisely ascertain the cause of death of the experimental subjects.

5. Conclusion

In summary, we have identified a robust stress-vulnerability phenotype of the otherwise mildly affected *mdx* mouse and we developed a protocol that can be easily adopted and will arguably speed the screening and development of new therapies for DMD. This finding suggests that even the relatively benign handling of *mdx* mice may affect the outcome/interpretation of experiments with this strain of mice. Additionally, our data suggest that dystrophin loss in skeletal muscle mediates a failure of the adaptive autonomic/cardiovascular response to stress [36,43,69], which can be lethal in mice.

Declaration of Competing Interest

The authors who have taken part in this study declare that they do not have anything to disclose regarding conflicts of interest concerning this manuscript.

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Author contributions

MR, AL, MLL, CMC, WMS, MB, and WCE performed experiments and analysed data; MR, AL, MLL, CMC, WCE, JO, JMM, JME and AB, conceived the experiments; MR, AB and JME wrote the paper with input from all authors.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2020.102700.

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