The neurological and behavioral consequences of dystrophin deficiency in Duchenne muscular dystrophy: insights from mouse models

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CHAPTER 4

Behavioral characterization of the *mdx*^{5cv}, *mdx52* and *DMD-null* mouse models of Duchenne muscular dystrophy

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Abstract

Duchenne muscular dystrophy is a severe neuromuscular disorder, caused by mutations in the *DMD* gene. Normally, the *DMD* gene gives rise to multiple dystrophin isoforms, of which multiple are expressed in the brain. The location of the mutation determines the number of dystrophin isoforms affected, and the absence thereof leads to behavioral and cognitive impairments. Even though behavioral studies have thoroughly investigated the effects of the loss of Dp427, and to a lesser extend of Dp140, in mice, direct comparisons between models lacking multiple dystrophin isoforms are sparse. Furthermore, a behavioral characterization of the *DMD-null* mouse, which lacks all dystrophin isoforms, has never been undertaken. Using a wide variety of behavioral tests, we directly compared impairments between *mdx-^{5cv}*, *mdx52* and *DMD-null* mice. We confirmed the role of Dp427 in emotional reactivity. We did not find any added effects of loss of Dp140 on fear, but showed the involvement of Dp140 in spontaneous behavior, specifically in habituation and activity changes due to light/dark switches. Lastly, Dp71/Dp40 seems to play an important role in many behavioral domains, including anxiety and spontaneous behavior.

Keywords

Dystrophin, cognition, anxiety, learning, spontaneous behavior, social interaction

Summary statement

In mice, the number of dystrophin isoforms lacking in the brain correlates with the severity of behavioural impairments, where those lacking all dystrophin isoforms are most severely affected.

Introduction

Duchenne muscular dystrophy (DMD) is a progressive X-linked neuromuscular disorder affecting about 1 in 5,000 new-born males (Mendell et al. 2012). The disorder is characterized by severe muscle wasting, causing loss of muscle function and eventually cardiorespiratory failure resulting in death at about 30 to 40 years of age in the Western world (Walter and Reilich 2017). DMD is caused by mutations in the *DMD* gene, which encodes for the protein dystrophin. The gene contains multiple promotors, giving rise to several unique dystrophin isoforms which differentiate in their expression patterns and functionalities. In muscle, only Dp427m is expressed, while in the brain many dystrophin isoforms are expressed in different regions (Doorenweerd et al. 2017).

Many studies have investigated dystrophin expression in the mouse brain (reviewed in (Tetorou et al. 2024)). Dp427 is mostly expressed in GABAergic inhibitory synapses. More specifically, while Dp427c is found in the neurons of the cortex, CA regions of the hippocampus and the cerebellum, Dp427p is selectively expressed in the cerebellum (García-Cruz et al. 2023). Dp140 expression peaks during fetal development, while levels in adult animals are relatively low, but present along the walls of blood vessels, specifically the perivascular astrocyte end feet (García-Cruz et al. 2023). Dp71 is the main isoform expressed in astrocytes (Belmaati Cherkaoui et al. 2021, García-Cruz et al. 2023), although recent literature has specified different splice variants playing roles during embryonic and postnatal development (González-Reyes et al. 2024). Dp40 is found in the axon terminals of excitatory cells (García-Cruz et al. 2023).

The location of the mutation in the *DMD* gene determines the number of missing dystrophin isoforms in DMD patients; mutations at the 5'end of the gene likely only affect Dp427 expression, while mutations closer to the 3'end are expected to also affect shorter isoforms. Lack of dystrophin in the brain leads to cognitive and behavioral problems in a subset of patients. The severity of these impairments seems to correlate to the number of lacking dystrophin isoforms (Taylor et al. 2010, Chamova et al. 2013, Ricotti et al. 2016). Overall, DMD patients have an IQ which is 1 standard deviation below the population average (Hinton et al. 2007, Banihani et al. 2015), and a subset of patients suffer from attention-deficit hyperactivity disorder, autism spectrum disorder, obsessive-compulsive disorder, depression, anxiety, inattention, reading deficits and/or epilepsy (Hinton et al. 2006, Hendriksen and Vles 2008, Waite et al. 2012, Banihani et al. 2015, Ricotti et al. 2016). There is however still a lot unknown about how the lack of dystrophin affects the brain.

DMD mouse models have been used to investigate the consequences of dystrophinopathy on the brain. The *mdx* mouse, which lacks Dp427 due to a nonsense mutation in exon 23, has been most extensively studied (Sicinski et al. 1989). *Mdx*

mice have deficits in anxiety (Manning et al. 2014, Remmelink et al. 2016, Vaillend and Chaussenot 2017, Saoudi et al. 2021), fear response (Sekiguchi et al. 2009, Yamamoto et al. 2010, Vaillend and Chaussenot 2017, Razzoli et al. 2020, Lindsay et al. 2021, Saoudi et al. 2021, Lindsay and Russell 2023), social interaction (Miranda et al. 2015) and possibly long term memory (Vaillend et al. 1995, Sesay et al. 1996, Vaillend et al. 2004, Remmelink et al. 2016, Comim et al. 2019, Bagdatlioglu et al. 2020). Direct comparisons of the *mdx* mouse with strains lacking multiple dystrophin isoforms, to investigate correlations between the number of dystrophin isoforms lacking and disease severity, are hindered by differences in their genetic backgrounds (*mdx:* C57BL/10ScSnJ versus other models: C57BL/6J). The *mdx*^{5cv} mouse, which lacks Dp427 due to a point mutation in exon 10 (Im et al. 1996), could serve as a viable alternative as it is on a C57BL/6J genetic background. *Mdx*^{5cv} mice show abnormalities in social preferences, similar to *mdx* mice (Alexander et al. 2016) but otherwise, the deficits in *mdx*^{5cv} mice remain unclear.

Mdx52 mice, which lack Dp427, Dp260 and Dp140 due to a deletion of exon 52 (Araki et al. 1997), have been used to study the effects of loss of an additional isoform in the brain. This model shows deficits in anxiety (Saoudi et al. 2021, Hashimoto et al. 2022), social behavior (Hashimoto et al. 2022) and fear learning (Saoudi et al. 2021). Due to the lack of Dp260, which is normally expressed in the retina, *mdx52* mice also display altered visual processing (Barboni et al. 2021, Barboni et al. 2023).

The function and consequences of the lack of Dp71 and Dp40 have been studied in Dp71-null mice, showing deficits in social interactions, spatial learning, navigation, cognitive flexibility and retinal function (Dalloz et al. 2003, Helleringer et al. 2018, Chaussenot et al. 2019, Miranda et al. 2024). However, since these mice do express the longer dystrophin isoforms, their translational value is limited. The *DMD-null* mouse, which lacks all dystrophin isoforms via a Cre-loxP recombination technique, would be a better model to tackle this issue. They are characterized by restlessness and abnormal maternal behavior (Kudoh et al. 2005), but further knowledge on behavioral impairments is lacking. While it is unknown if the retina of *DMD-null* mice is affected, the lack of Dp260 and Dp71, which are both normally expressed in this tissue, are expected to influence visual processing, at least to a similar extend as in the *mdx52* and the Dp71-null mice (Howard et al. 1998, Dalloz et al. 2003, Barboni et al. 2021).

Our study aimed to unravel the consequences of the lack of one, multiple or all brain dystrophin isoforms on behavior in the *mdx*^{5cv}, *mdx52* and *DMD-null* models (Table 1). We used an elaborate test set-up to study anxiety, fear, social interaction, spontaneous behavior, learning, memory and learning flexibility. This study showed similar alterations in *mdx*^{5cv} mice as previously reported in *mdx* mice, including increased anxiety and fear. Furthermore, we found no additional deficits in *mdx52* mice when compared to *mdx*^{5cv} mice. Subtle abnormalities in spontaneous behavior

were found in *mdx52* mice. Lastly, our study provided the first extensive overview of the behavioral abnormalities in *DMD-null* mice, reporting increased anxiety, fear and deviations in spontaneous behavior compared to the other DMD models.

Mouse model	Mutation	Dp427	Dp260	Dp140	Dp116	Dp71/ Dp40
Mdx^{5cv}	Point mutation exon 10	-	+	+	+	+
Mdx52	Deletion of exon 52	-	-	-	+	+
DMD-null	Whole *Dmd* gene removed	-	-	-	-	-

Table 1: Overview of mouse models used in this study and their corresponding dystrophin isoforms.

Materials and methods

Mice

Male *mdx^{5cv}* (B6Ros.Cg-*Dmd$^{mdx\text{-}5Cv}$*/J) (Im et al. 1996), *mdx52* (Araki et al. 1997), *DMD-null* (Kudoh et al. 2005) and wildtype (WT) mice, all on a C57BL/6J genetic background, were bred at the animal facility of the Leiden University Medical Center (LUMC). For all strains, heterozygous females were paired with WT males to produce both DMD and WT male littermates. WTs from all three strains were pooled in one group, which consisted of 8 *mdx^{5cv}* WT, 8 *mdx52* WT and 16 *DMD-null* WT males. Other groups all consisted of 16 males. Mice were genotyped after birth and at the end of the study (Fig. S1). One animal was mislabeled at the start of the study, resulting in 9 *mdx52* WT mice and 15 *mdx52* mice. Mice were housed in groups of 2 to 4 animals in individually ventilated cages (Makrolon type II) filled with sawdust and enriched with nesting (Bed-r'Nest BRN8SR, Life Science Equipment, Oud-Turnhout, Belgium) and bedding (LIGNOCEL BK-8-15-00433, JNR, Holzmühle, Germany) materials and a cardboard tunnel (GLP fun tunnels mini 1022006). *Ad libitum* access to standard RM3 chow (SDS, Essex, United Kingdom) was provided to the mice, except during the food reward task in the PhenoTyper cages. *Ad libitum* access to water was provided to the mice during the whole study. Mice were exposed to a 12h:12h, dark-light cycle with lights being turned on between 7:00-19:00. All tests were performed in rooms dedicated to behavioral experiments during the light phase between 7.00-17.00h, with the exception of the continuous tracking in the PhenoTyper cages. Timing of each test was kept as consistent as possible for all the animals, meaning that while some tests were performed in the morning and others in the afternoon, tests started at roughly the same time of the day for all cohorts. Researchers were blinded to the genotypes while performing the tests. Tests were primarily performed by three female researchers. Animals were handled by their tail when placed into an experimental setting. The experiments were approved by

the Animal Ethics Committee of the LUMC (AVD 1160020171407, PE.17.246.037) and executed conform the Directive 2010/63/EU of the European Parliament.

Experimental setup

Mice entered the study at 8 weeks of age and underwent multiple behavioral tests, while body weight was recorded on a weekly basis (Fig. S2). Tests included: the dark-light box, open field test, 3 chamber social interaction test, Barnes maze, T-maze, novel object recognition, object placement, Morris water maze (MWM), restrained unconditioned fear test and housing in a PhenoTyper automated home cage in which spontaneous behavior and discrimination learning (DL) and reversal learning (RL) were assessed (Fig. 1). At least 1 day of rest was provided between each of the tests. Between trials, materials were cleaned with 70% ethanol. Behavior was tracked automatically using Ethovision XT, at a rate of 20 frames per second for the dark light box, open field, MWM, unconditioned fear and in the PhenoTyper cages. Interactions with objects in the 3 chamber social interaction test, Barnes maze, novel object recognition and object placement tasks were scored via automated tracking using DeepLabCut (Verhaeg et al. manuscript in preparation) (Mathis et al. 2018, Nath et al. 2019), instead of Ethovision, due to difficulties in tracking of the partially obscured animals caused by the camera angle and position of the objects. In short, DeepLabCut was trained on ±1000 labeled frames to detect the nose, head, ears, shoulders, back and tail of the mice. Coordinates of the locations of the body parts were extracted per frame and the location of the objects or target holes was determined with in-house image segmentation routines. Interaction was described as close proximity towards the object or hole. Scripts were developed for each test separately and validated on separate datasets with manually scored data from two independent scorers (correlation scores varied between 0.80 and 0.98 for the different behavioural tests, Verhaeg et al. manuscript in preparation). All testing equipment was made in-house with the exception of the PhenoTyper cages, which were acquired from Noldus. At 17 weeks of age, mice were sacrificed with CO_2.

Behavioral tests

Dark-light box

Anxiety was measured with the dark-light box, which consisted of 2 compartments (50x25 cm each) which were connected via a door (10x5 cm) (Saoudi et al. 2021). Animals were placed in the dark compartment and after 20 seconds the door was opened and animals were allowed to freely explore both compartments for 5 minutes. Behavior was only recorded in the light compartment.

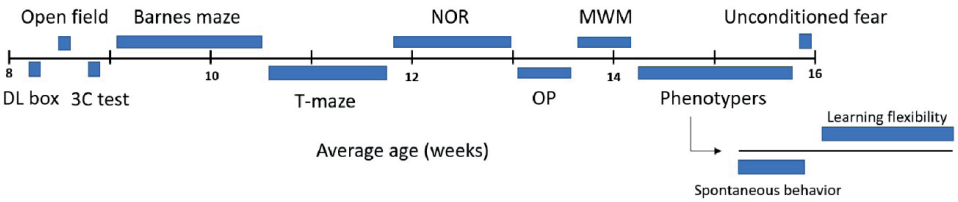

Figure 1: Study overview. *Mice were included at 8 weeks of age and underwent a variety of behavioral tests for 8 weeks. DL: dark-light box, 3C: 3 chamber social interaction test, NOR: novel object recognition, OP: object placement, MWM: Morris water maze.*

Open field test

To further assess anxiety, mice were exposed to the open field test. Animals were released in a white box (50x50x35 cm) for 30 minutes. The box was digitally divided in an inner (region of 40x40 cm in the middle of the box) and outer (the remaining area) zone during analyses. Location and movement of the mice was measured.

3 chamber social interaction test

The 3 chamber social interaction test was used to investigate social interaction in a controlled environment (Miranda et al. 2015). The setup consisted of 3 chambers (21x42x35 cm each) which were connected by doors (10x5 cm). The outer walls of the box were opaque while the inner walls dividing the chambers were transparent. A black tube (height: 20 cm, diameter: 8 cm, metal bars spaced 1 cm apart from each other) was placed in each lateral chamber, in such a way that the center of the tube was placed 10 cm from the outer walls. Mice used for the interaction (strangers) were all male WT mice. In total, 6 pairs of 2 strangers were used to keep age differences between strangers and experimental mice at a minimum (maximum age difference was 2 weeks). During the habituation trial, the experimental mouse was placed in the middle chamber and each lateral chamber contained an empty black tube. After 2 minutes, the doors to the lateral chambers were opened and the mouse was recorded for 10 minutes. In between trials, the experimental mouse was placed back in the home cage shortly to allow cleaning of the equipment. During the first trial, which was performed almost directly after habituation, an object was placed into one of the tubes. The other tube contained a novel mouse (stranger 1), which was unfamiliar to the experimental mouse. At the start of the trial, the experimental mouse was placed in the middle chamber again and doors were opened after 30 seconds. The mouse could walk around freely for 10 minutes. During the second trial, which was performed directly after the first trial, stranger 1 was placed in a tube in the same lateral chamber as in the previous trial. The other tube con-

tained a new mouse (stranger 2), which was again unfamiliar to the experimental mouse. The experimental mouse was placed in the middle chamber again. After 30 seconds, the doors were opened and the mouse could walk around freely for 10 minutes. Interaction with the objects and strangers was measured and quantified. Discrimination indexes (DIs) were calculated for sociability (trial 1; Interaction time stranger 1 / (interaction time stranger 1 + interaction time object)) and for social novelty seeking (trial 2; Interaction time stranger 2 / (interaction time stranger 1 + interaction time stranger 2)).

Barnes maze

Spatial learning and memory was assessed with the Barnes maze, which was a circular platform (120 cm diameter) made from wood covered with a water resistant top, which included 12 holes (10.5 cm diameter) which were equally spaced apart 12 cm from the circumference of the maze (Remmelink et al. 2016). A transparent platform beneath one of the holes (target hole) led to a hidden escape box. Visual cues were positioned around the maze for spatial orientation. During the first 5 days, two acquisition trials were held daily with a 5 minute interval between trials. During each trial, mice were placed in the middle of the maze and could try to locate the hidden platform for 5 minutes. If mice failed to reach the platform during this timeframe, they were manually placed into the correct hole. Mice could stay in the escape box for 30 seconds before being taken out. During the second trial of day 5, the platform was removed and behavior was recorded for 5 minutes. No trials were performed on day 6 and 7. On day 8, two more learning trials were held in the same manner as described before, with the escape box in place. On day 9 and 10, the platform and escape box were transferred to the opposite side of the maze. Three learning trials took place on day 9, with the same setup and interval as described before. On day 10, one learning trial similar to those of day 9, took place, but during the second trial, the platform was removed again. Distance to target hole and interaction with all the holes were measured as an indication of spatial learning and memory.

T-maze

Recognition memory was tested with the T-maze (Vaillend et al. 1995). The T-maze consisted of one start arm (35x10 cm), including a start box (13x10 cm) and two lateral arms (35x10 cm). Mice were placed in the start box at the start of each trial. On the first day (habituation trial), the start box and both lateral arms were open and the mice could explore the maze freely for 5 minutes. During day 2 and 3, mice were kept in the start box for 30 seconds before the latch was lifted. On day 2, two

consecutive learning trials were held, during which one lateral arm was closed off. The trial ended 30 seconds after the mice entered the open lateral arm. During this time, mice were not restricted to the lateral arm and allowed to walk back to the start arm. All mice entered the lateral arm freely within 5 minutes. In between the trials, mice were briefly placed in their home cage to allow cleaning of the apparatus. After a 24 hour delay (test trial), both arms were open again and the mice were allowed to freely explore the maze for 5 minutes. After 6 days of rest, learning and test trials were repeated with a delay of 6 hours between the last learning trial and the test trial. Choice of first entry (alternation) and total time spent in each arm were measured to assess recognition memory. DIs were calculated to analyze preference for the novel arm: time spent in novel arm / (time spent in novel + familiar arm).

Novel object recognition

Recognition memory was further investigated using the NOR test. The open field box was filled with bedding and objects of different shapes, colors and textures were selected for equal exploration. Objects were held in place by magnets on the exterior of the box and placed approximately 10 cm from the walls. Initially, 12 objects were tested for spontaneous interaction with 8 WT mice (mice were exclusively used to assess suitability of the objects); meaning the WT mice were subjected to sets of 3 random objects at a time for 10 minutes and interaction times were measured. Two objects that evoked significantly higher interaction compared to the other objects were excluded from further use.

Four days of habituation preceded the assessment of recognition memory (Vaillend et al. 2004). On the first day, two habituation sessions, spaced at least 4 hours apart, took place during which all mice from 1 cage were placed in the box together for 10 minutes. On day 2 and 3, the mice were individually placed in the box to explore for a single 10 minute session. This was also done on day 4, but now, 3 dedicated objects, which were not used for the remainder of the experiment were placed in the box. Learning trials were started 2 days after the last habituation session. Hereto, mice were individually placed in the box with 3 objects for three 5 minute trials with 5 minute intervals between them. After approximately 24 hours, a 5 minute test trial was performed in which one of the 3 objects was replaced by a novel object. After a maximum of 2 resting days, learning and test trials were repeated with a set of three novel objects in which a 10 minute delay instead of a 24 hour delay was used between learning and test trials. Objects were pseudo-randomly selected for each mouse, meaning object combinations and locations of the novel object (left, middle or right position) were similar between groups. Bedding was not changed for the duration of the experiment for one cohort (max 8 mice). The interaction time with

each object was measured and DIs were calculated during the test trials; interaction time novel object / (interaction time novel + old objects).

Novel object placement

The novel object placement task was performed to assess spatial memory. The test was also done in the open field box, utilizing the same bedding as for the novel object recognition test. Distinct black visual cues were added to the walls of the box for spatial orientation. Two identical objects were pseudo-randomly placed in the box; objects and relative locations (next to each other vs in opposite corners) were similar between groups.

The mice were placed in the middle of the box and three learning trials were conducted for 5 minutes with a 5 minute interval between them. During the test trial, 24 h later, the location of one of the two objects was pseudo-randomly changed. After a maximum of 2 days of rest, learning and test trials were repeated with a 10 minute interval between the last learning trial and the test trial. The interaction time with the objects was measured and DIs were calculated; interaction time novel object location / (interaction time novel object location + old object location).

Morris water maze

The Morris water maze was used to asses spatial memory further (Vaillend et al. 2004). A round bath (120 cm diameter) was filled with water (at 26°C) and white dye, making it opaque to prevent mice from visually navigating to a hidden platform (11 cm diameter) just beneath the water surface, located at approximately 27 cm from the edge of the pool. Visual cues were placed around the maze for spatial orientation. Four quadrants were determined in the maze: North-West (NW), South-West (SW), North-East (NE) and South-East (SE), with the platform positioned in the NE quadrant. Mice were habituated for one day during two sessions, with a minimum interval of 4 hours. Each session consisted of 4 consecutive trials, during which the mice were guided by hand through the water towards the platform, on which they could stay for 60 seconds. If mice jumped off the platform, they would swim around freely until the 60 seconds were over. On the second day, the platform location was acquired during 5 sessions, with a 15-20 minute interval between the sessions. Each session consisted of 5 consecutive trials. Mice were placed pseudo-randomly in one of the quadrants at the beginning of each trial (with the exception of the target quadrant that contained the platform). Trials lasted for 1 minute or till the mice found the platform, after which they could stay on the platform for 2 minutes. If the platform was not found after 1 minute, mice were put on the platform by hand.

Spatial memory was assessed 24 h later via a probe trial in which the platform was removed. Mice were pseudo-randomly placed in one of the quadrants (with the exception of the target quadrant that had previously contained the platform) and could freely swim for 1 minute. They were allowed to warm up under a heating lamp for approximately 15 minutes after each session. The protocol was stopped prematurely if the mice had trouble keeping their head above the water surface, which was the case for 2 *mdx52* and 5 *DMD-null* mice. Distance swum till finding the platform or entering the platform zone was measured during each trial. Additionally, swimming speed and time and distance in each quadrant was analyzed.

PhenoTyper automated home cages

PhenoTyper automated home-cages (model 3000, Noldus Information Technology, Wageningen, the Netherlands) consist of transparent Perspex walls (30x30x35 cm) and opaque Perspex floors and a infra-red camera in the top-unit. Drinking and feeding stations were included in the cage, as well as a rectangular shelter (10x10x5 cm) with two entrances (3 cm diameter). Cages were filled with sawdust bedding during all experiments.

Spontaneous behavior

To assess spontaneous behavior, mice were tracked continuously for 3 days with minimal interference (Loos et al. 2014). Mice were put in the PhenoTyper cages during the light phase (between 13h and 16h). Spontaneous behavior was analyzed starting at the beginning of the upcoming dark phase (19h local time). Mice were individually housed and had *ad libitum* access to standard RM3 chow and water. Spontaneous behavior was defined with 20 parameters described in Loos et al. 2014 for the dark and light phase of day 3 (Loos et al. 2014).

Discrimination and reversal learning task

The day after spontaneous behavior analyses were completed, cognitive flexibility was assessed via a food rewarded discrimination and reversal learning task. Standard chow was removed and a cognition wall (17 cm wide, 25 cm high) with three circular entrances (3 cm diameter) was introduced in the corner opposite to the shelter. A pellet dispenser, dispensing food pellets (Dustless Precision Pellets, 14 mg, Rodent Purified diet, Bio Serv, Frenchtown, NJ, USA), was placed such that the dispenser tube protruded behind the cognition wall. A pellet was released after each fifth correct entry through the target entrance. Correct entries did not have

to be consecutive. Discrimination learning lasted for 48 hours, during which the left hole was deemed the target hole. During the remaining five reversal days, the target entry was switched daily in the following pattern: right, left, middle, right, left.

Six *mdx*^{5cv}, one *mdx52* and one *DMD-null* mice were excluded from analysis due to technical issues during the test. Animals were weighted daily (Fig. S2B) and given extra food if weight loss was more than 2 g (approximately 7% weight loss) in 24 h or more than 5 g since the start of the task (approximately 15-20% weight loss). In total 1 WT, 1 *mdx*^{5cv}, and 5 *DMD-null* mice were taken out of the task prematurely (mostly between RL1 and RL2) due to extreme weight loss or signs of inactivity and distress. Learning abilities were assessed by calculating the amount of correct entries during a 50 entry window. After the test, animals were individually housed in individually ventilated cages, to prevent fighting after regrouping of adult males. They had *ad libitum* access to water and standard RM3 chow for the remainder of the study (2 days).

Restrained unconditioned fear test

To examine fear response without the interference of fear learning or association learning, freezing behavior was assessed after evoking an unconditioned fear response (Saoudi et al. 2021). Mice were held upside down for 15 seconds by scruffing them in the neck. Afterwards they were released in the open field box and freezing behavior was tracked for 10 minutes.

Genotyping

Ear cuts were made for identification and genotyping prior to weaning and at the end of the experiment after sacrifice to re-confirm the genotype of all mice. Ear pieces were suspended in a 50 µl buffer made of 100 mM Tris, 5 mM EDTA, 200 mM NaCl, 0.2% SDS to which 0.5 µl of Proteinase K was added and samples were incubated overnight at 55°C in a shaking water bath. Proteinase K was inactivated for 10 min at 97°C and samples were spun down 30 seconds at 13.000 rpm. Then, 1 µl of DNA was added to the PCR mix (0.4 µl dNTPs, 4 µl 5x Phire Reaction buffer (Thermo Scientific), 1 µl Forward primer (10 pmol), 1 µl Reverse primer (10 pmol), 0.2 µl Phire Hot Start II DNA Polymerase (Thermo Scientific) and 12.4 µl H_2O). The following PCR program was used: 30 seconds at 98°C followed by 30 to 35 cycles of: 5 seconds at 98°C, 5 seconds at a primer specific annealing temperature (Table S1), 10 seconds at 72°C. After the repeated cycles, the PCR was ended with 1 minute incubation at 72°C. For the *mdx*^{5cv} samples, a restriction endonuclease digest by the DraIII enzyme was performed. Hereto, 5 µl digestion mix (CutSmart Buffer 10x,

DraII-HF (20 U/µl), dH20) was added to 10 µl PCR product and incubated at 37°C for 45 minutes. Samples were run on a 2-3% agarose gel (Figure S1). PCRs were performed in duplo for each sample.

Data analysis

Statistical tests were executed in SPSS and RStudio. Data was assessed for normality and log10 transformed if needed to achieve normality (this was required for the Barnes maze distance walked in the reversal probe and the Morris water maze time in NW, SE and SW quadrants). If normality was confirmed, a one-way ANOVA test with Tukey *post-hoc* was done to compare group differences. Comparisons to chance level were done with a one-sample t-test. If data was not normal, group differences were tested with the Kruskal Wallis test, and if significance was found, repeated Mann-Whitney tests were performed to further analyze group differences. To compare chance levels in data that was not normally distributed, the one sample Wilcoxon signed rank test was performed per group. Changes in behavior over time were assessed using linear mixed models in Rstudio (version 4.3.1) via the LmerTest package (version 3.1.3). Group differences in terms of alterations in the T-maze were tested with a Chi-square test. Since differences between WT groups were minimal (Table S2), WTs were pooled into one group, except for the body-weight analysis. DMD models were tested for significant differences against this combined WT group and direct comparisons between DMD models were only done if at least one of the DMD models showed a significant difference compared to WTs. A *P*-value below 0.05 was considered significant. Spontaneous behavior data was corrected using false discovery rate via an online tool: https://tools.carbocation.com/FDR. Graphs were made via GraphPad, Rstudio and Adobe Illustrator. All data is shown as average ± standard error of mean (SEM). Additional statistical information can be found in table S2 and S3.

Results

Increased anxiety in all DMD models being most profound in DMD-null mice

To assess anxiety, the dark-light box and the open field tests were used. In the dark-light box, mice were allowed to move freely between the light and dark compartments for 5 minutes (Fig. 2A-C). *Mdx^{5cv}*, *mdx52* and *DMD-null* mice all visited the light compartment less often than the WT mice ($P = 0.009$, $P < 0.001$ and $P < 0.001$ respectively) and spent less time in the light compartment (all $P < 0.001$) (Fig. 2A-B).

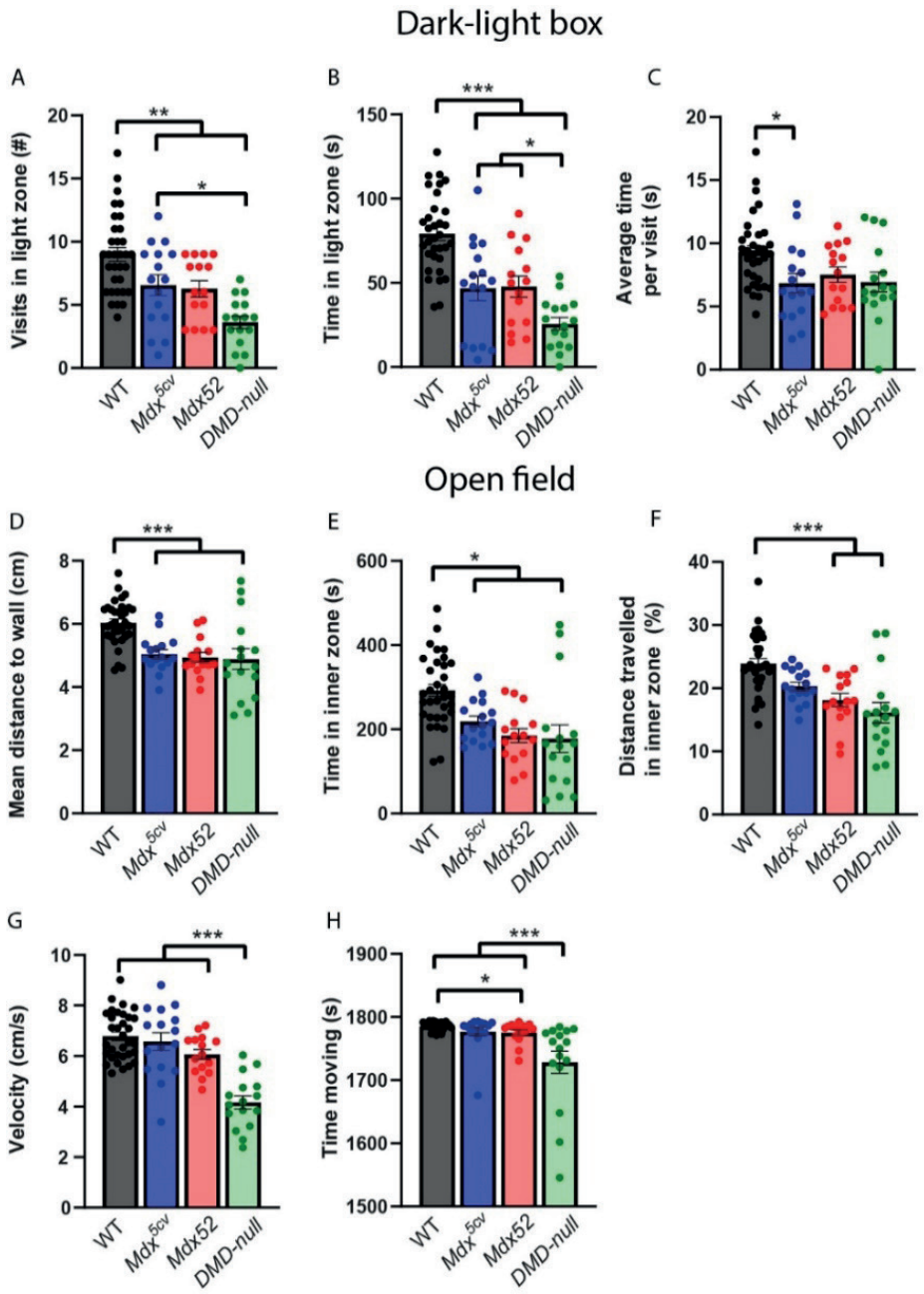

Figure 2: Anxiety in the dark-light box and open field. *Mdx^{5cv} (n = 16), mdx52 (n = 15), DMD-null (n = 16) and WT mice (n = 33). A) In the dark-light box, mdx^{5cv}, mdx52 and DMD-null mice all visited the light compartment less often compared to WTs (P = 0.009, P < 0.001 and P < 0.001 respectively). DMD-null mice visited the light compartment less often than mdx^{5cv} mice (P = 0.021). B) Mdx^{5cv}, mdx52 and DMD-null*

*mice all spent less time in the light compartment compared to WTs (all P < 0.001). DMD-null mice spent less time in the light compartment compared to mdx^{5cv} and mdx52 mice (P = 0.043 and P = 0.008 respectively) C) Visits to the light department were on average shorter for mdx^{5cv} mice compared to WTs (P = 0.022). D) In the open field test, all DMD models stayed closer to the walls compared to the WTs (all P < 0.001). E) Mdx^{5cv}, mdx52 and DMD-null mice spent less time in the inner zone of the box (P = 0.035, P < 0.001, and P < 0.001 respectively). F) Mdx52 and DMD-null mice walked less distance in the inner zone of the box (both P < 0.001). G) DMD-null mice walked slower compared to WT, mdx^{5cv} and mdx52 mice (all P < 0.001). H) DMD-null mice spent less time moving than WT, mdx^{5cv} and mdx52 mice (all P < 0.001). Mdx52 mice moved less compared to WTs (P = 0.017). *: P < 0.05, **: P < 0.01, ***: P < 0.001.*

Additionally, *DMD-null* mice visited the light compartment less often than *mdx^{5cv}* mice (*P* = 0.021) and spent less time in the light compartment than both *mdx^{5cv}* and *mdx52* mice (*P* = 0.043 and *P* = 0.008 respectively). Only *mdx^{5cv}* mice spent less time in the light compartment per visit compared to WTs (*P* = 0.022) (Fig. 2C).

The open field box was used to further assess anxiety (Fig. 2D-H). Both location and locomotion were tracked during this 30 minute test. Overall, *mdx^{5cv}*, *mdx52* and *DMD-null* mice stayed closer to the walls of the box (all *P* < 0.001) and spent less time in the inner zone of the box (*P* = 0.035, *P* < 0.001 and *P* < 0.001 respectively) (Fig. 2D-E). *Mdx52* and *DMD-null* mice walked relatively less distance in the inner zone of the box compared to WTs (both *P* < 0.001) (Fig. 2F). Additionally, *DMD-null* mice walked slower (all *P* < 0.001) and spent less time moving compared to WT, *mdx^{5cv}* and *mdx52* mice (all *P* < 0.001) (Fig. 2G-H). *Mdx52* mice also spent less time moving compared to WTs (*P* = 0.017). Additionally, locomotor behavior was analyzed for changes over time (Fig. S3). Apart from a lack of initial activity found in all DMD models in the first 5 minute time bin, no differences could be found between the groups over time. Overall, all DMD-models show increased anxiety, however the anxious behavior seems most aggravated in *DMD-null* mice.

The strong fear response found in mdx^{5cv} and mdx52 mice is even further aggravated in DMD-null mice

To assess unconditioned fear, mice were restrained and held upside down for 15 seconds before being put in the open field box. Walking velocity was severely decreased in all DMD models compared to WTs (all *P* < 0.001), while freezing time was drastically increased (*P* < 0.001) (Fig. 3A-B). Additionally, *DMD-null* mice walked slower than *mdx^{5cv}* mice (*P* = 0.012) and spent more time frozen compared to *mdx^{5cv}* mice (*P* = 0.019). A trend was also observed in the same direction when time spent

frozen was compared to *mdx52* mice ($P = 0.050$). To determine if freezing behavior was time dependent in the strains, the percentage of time frozen was calculated in time bins of 60 seconds. All DMD models behaved differently over time compared to WTs ($P < 0.001$), but no differences were found between DMD strains over time (Fig. 3C). *Mdx*^{5cv}, *mdx52* and *DMD-null* mice all exhibit a very strong increase in fear response, with *DMD-null* mice showing even more excessive freezing than the other models.

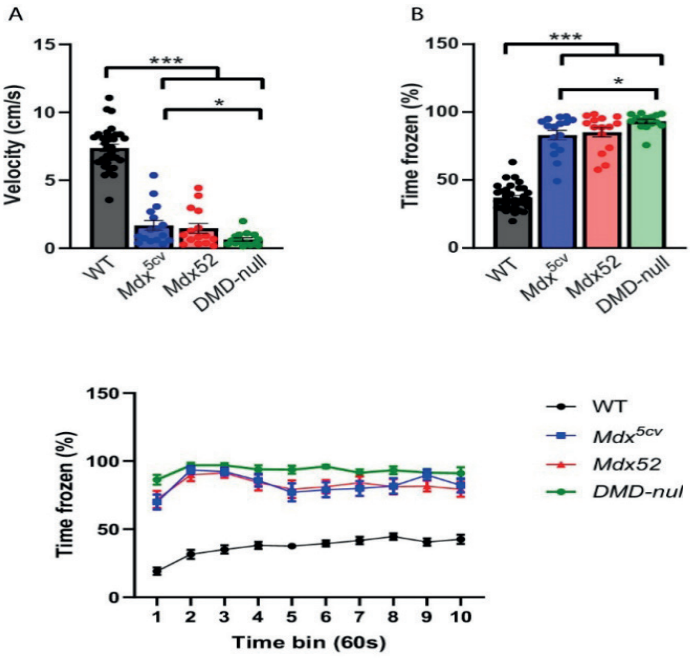

Figure 3: Unconditioned fear response after a short restrain. *Mdx*^{5cv} *(n = 16), mdx52 (n = 15), DMD-null (n = 16) and WT mice (n = 33). A) Walking velocity was decreased in all DMD models compared to WTs (all P < 0.001). DMD-null mice showed a further decrease compared to mdx*^{5cv} *mice (P = 0.012). B) Freezing time was increased in all DMD models (all P < 0.001). DMD-null mice spend more time frozen compared to mdx*^{5cv} *mice (P = 0.019). C) Freezing time per time bin of 60s. All DMD strains show a different pattern over time compared to WTs (P < 0.001), but no differences were found between the DMD strains. *: P < 0.05, ***: P < 0.001.*

Results on social interactions remain inconclusive due to lack of preference in WT mice

Social interaction was analyzed using the 3 chamber social interaction paradigm, where experimental mice could interact with other mice that were contained in a tube. After habituation, the animals could choose to investigate either an object or a novel mouse (Fig. S4A). Directly after this trial, the mice were given a new choice, this time between interaction with a novel mouse or a familiar mouse, which was used in the previous trial (Fig. S4B). Unfortunately, in both trials, the WT mice did not show any preference for one of the targets, meaning they spent an equal amount of time at both tubes in each trial (Fig. S4C). Furthermore, no differences were found between the groups. Notably, *mdx52* mice spent more time interacting with the mouse in the first trial, showing a preference for the investigation of the mouse vs the object which was significantly different from chance level ($P = 0.024$). *DMD-null* mice interacted more with the novel mouse than with the familiar one ($P = 0.008$). However, due to the lack of preferences observed in WTs, data on DMD mice is inconclusive in terms of deficits in socialization or social novelty seeking.

Spatial memory dependent on stress in mdx52 and DMD-null mice, small deviations found in reversal learning in DMD-null mice

Spatial navigation, memory and flexibility were assessed using the Barnes maze and the Morris water maze. Due to decreased walking and swimming velocities in the DMD mouse models (Fig. S5A-B), latency to reach the target was excluded as a parameter, instead distance traveled till reaching the target was used. The Barnes maze consists of a large wooden circular platform with 12 holes. An escape box could be placed under any of the holes and the mice needed to navigate to the correct hole using external cues. The protocol consisted of 5 learning days, a probe trial, 2 rest days, another learning day, 2 days of reversal learning and a reversal probe trial (Fig. 4A). No differences were found in the distance the mice walked to find the target location during either the learning or probe trials (Fig. 4B-C) nor in the relative distance walked in the target quadrant during the probe trial (Fig. 4D). All groups walked significantly more distance in the target quadrant when compared to chance level (all $P < 0.001$). No deviations were found in the time spent at the target hole during the probe test (Fig. S5C).

Surprisingly, when reversal learning was started, *DMD-null* mice walked a shorter distance to reach the new target hole ($P = 0.015$) compared to WTs (Fig. 4E). No differences were found in distance traveled to the target hole during the reversal

probe trial even though all groups walked relatively more distance in the target quadrant compared to chance level (all $P < 0.001$). *DMD-null* mice also walked relatively more distance in the new target quadrant compared to WTs ($P = 0.033$) (Fig. 4F-G). No differences were found between groups in terms of distance traveled to the old target location neither in the learning nor in the probe trials (Fig. 4H-I). Animals did not differ in relative walking distance travelled in the old target quadrant (Fig. 4J), nor in the interaction time with any of the holes (Fig. S5D), indicating no change in the interference of the earlier training on the behavior of the animals.

During the Morris water maze, spatial learning and memory were assessed in a more stressful environment due to the high demand of the test on muscle function (Fig. 4K). *DMD-null* mice in particular struggled with the protocol and eventually two *mdx52* and five *DMD-null* mice had to prematurely stop with the acquisition learning due to the inability to keep their head above the water. No differences were found in the distance the mice travelled until reaching the platform during the learning (Fig. 4L) and the probe trial (Fig. 4M). No differences were found between groups when comparing the relative distance travelled in the quadrant in which the platform was located (Fig. 4N). However, only WT and *mdx*^{5cv} mice showed a distance travelled which is significantly different from the chance level of 0.25 ($P < 0.001$ and $P = 0.002$ respectively). Furthermore, *mdx52* and *DMD-null* mice spent less time in the target quadrant compared to both *mdx*^{5cv} ($P = 0.005$ and $P = 0.009$ respectively) and WT mice ($P = 0.012$ and $P = 0.022$ respectively), but no differences were found in time spend in any of the other quadrants. Overall, no deficits in spatial navigation could be found, *DMD-null* mice did however seem to perform better than WTs during the reversal learning tests. Results on memory retention are conflicting between the Morris water maze and the Barnes maze, possibly due to the difference in demand in motor function.

Spatial and non-spatial recognition memory were not affected in any of the DMD models

To assess recognition memory for both short and long term delay, mice underwent the novel object recognition task, the object placement task and the T-maze. During the object recognition task, mice were exposed to three objects. After a delay of 10 minutes or 24h, one of the objects was replaced by a novel object. The DI was calculated for the amount of time mice spend exploring the new or the old objects. All mice showed a preference for the new object during both delays, indicated by a DI above chance level, but no differences were found in DI between any of the groups for either time delay (Fig. S6A).

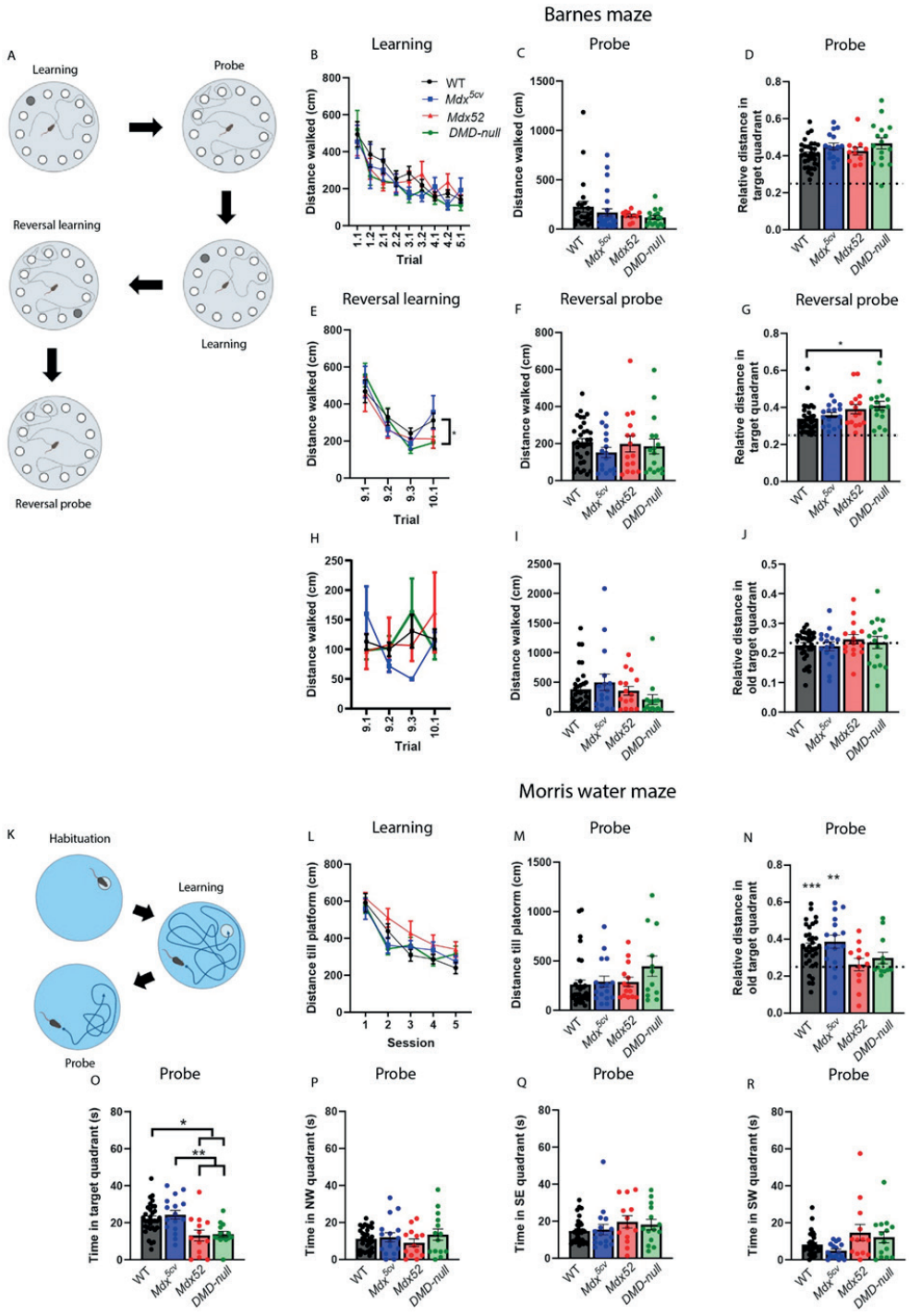
Barnes maze
A
Learning
Probe
Reversal learning
Learning
Reversal probe
B
Learning
Distance walked (cm)
Trial
WT
Mdx5cv
Mdx52
DMD-null
C
Probe
Distance walked (cm)
D
Probe
Relative distance in target quadrant
E
Reversal learning
Distance walked (cm)
Trial
F
Reversal probe
Distance walked (cm)
G
Reversal probe
Relative distance in target quadrant
H
Distance walked (cm)
Trial
I
Distance walked (cm)
J
Relative distance in old target quadrant
Morris water maze
K
Habituation
Learning
Probe
L
Learning
Distance till platform (cm)
Session
M
Probe
Distance till platorm (cm)
N
Probe
Relative distance in old target quadrant
O
Probe
Time in target quadrant (s)
P
Probe
Time in NW quadrant (s)
Q
Probe
Time in SE quadrant (s)
R
Probe
Time in SW quadrant (s)

Figure 4: Spatial learning and memory in the Barnes and Morris water maze. *A) Cartoon of the Barnes maze protocol. Mdx^{5cv} (n = 16), mdx52 (n = 15), DMD-null (n = 16) and WT mice (n = 33). B) Distance travelled to reach the platform during the acquisition learning. No differences were found between groups. C) All groups travelled approximately equal distances to reach the platform zone during the probe trial. D) No significant differences were found between groups in the relative distance travelled in the target quadrant. All groups travelled more distance in this quadrant compared to chance level (0.25, indicated by the dotted line) (all P < 0.001). E) DMD-null mice showed increased distance travelled to the new platform location during reversal learning compared to WTs (P = 0.015). F) No differences were found between groups in the distance traveled to reach the new platform zone during the reversal probe trial. G) DMD-null mice showed a higher relative distance travelled in the new target quadrant compared to WTs (P = 0.033). All groups performed above chance level (all P < 0.001, indicated by the dotted line). H-I) No differences were found between groups in distance travelled till reaching the old platform location during either the reversal learning or the reversal probe. J) No differences found between groups in the relative distance travelled in the old target quadrant. None of the groups differed from chance level. K) Cartoon of Morris water maze protocol. Mdx^{5cv} (n = 16), mdx52 (n = 13), DMD-null (n = 11) and WT mice (n = 33). L) No differences were found between groups in the distance they needed to swim to find the platform. M) No differences were found in the distance swam till reaching the platform during the probe trial. N) No differences were found between groups in the relative distance swum in the target quadrant. Only performance of WT and mdx^{5cv} mice differed from chance level, indicated by the dotted line at 0.25 (P < 0.001 and P =0.002). O) Mdx52 and DMD-null mice spent less time in the target quadrant compared to mdx^{5cv} (P = 0.005 and P = 0.009 respectively) and WT mice (P = 0.012 and P = 0.022 respectively). P-R) No difference found in the time spend in any of the other quadrants. *: P < 0.05, **: P < 0.01,***: P < 0.001. Cartoons were created with BioRender.*

During the object placement task, two identical objects were placed in the box, together with spatial visual cues. After a delay of 10 minutes or 24h, one of the objects was moved to a different corner and the DI was calculated for the new versus old placement. No differences were found in DI scores, however, even though all mice showed a preference for the displaced object after a 10 minute delay, only WT mice showed a significant difference compared to chance level after a 24 hour delay (P = 0.014) (Fig. S6B).

Mice were subjected to the T-maze test two times, once with a 6 hour delay and once with a 24 hour delay. When reintroduced into the maze after the delay, the portion of time spent in the target arm versus the total time in either arm was calculated (discrimination index). WT mice did not show any preference for the novel

arm in either delay (Fig. S6C). Furthermore, no differences were found in the DI for any of the groups at either the 6 hour or 24 hour delay and no differences were found in the percentage of mice which chose the target arm as a first entry (alternation) in any of the groups at either delay (Fig. S6C). Notably, *mdx52* and *DMD-null* mice did perform above chance level in terms of percentage of alternation ($P = 0.02$ and $P = 0.046$ respectively). Since WT mice did not show a significant alteration, the results of this test are inconclusive. However, based on the other tests, none of the dystrophin isoforms seems to have a substantial effect on short or long term recognition memory.

Initial reversal learning deficits found in mdx^{5cv} and mdx52 mice seem to be absent in DMD-null mice

To further assess flexibility of memory in an environment with minimal external influences, mice were individually housed in PhenoTyper cages. A cognition wall with three holes was placed in one corner with a pellet dispenser at the back, providing the only food available for the mice. A food pellet was given if the mouse went through the correct entrance for five times (which did not have to be consecutive). Weight loss was monitored closely (Fig. S2B) and mice were taken out of the experiment prematurely in case of more than 20% weight loss since the start of the task or tonic immobility. To adjust for differences in activity between the strains, learning curves were calculated per amount of entries rather than time. Fractions of correct entries, in bins of 50 entries, were calculated for the target hole (Fig. 5) and the perseverative errors (meaning the mouse entered the target from the previous day) and neutral errors (meaning the mice entered the 3rd hole, which was not the current target, nor the target of the previous day) (Fig. S7). As not all mice performed the same amount of entries, the amount of animals on which the curves are based, decreases as the curves progress. For visual purposes graphs are depicted only as long as the average curve is calculated from $n > 3$ mice per group.

During the discrimination learning phase in the first two days, the left entrance was deemed the target entrance, afterwards, the target entrance changed every 24 hours during the reversal learning phase. Both *mdx^{5cv}* and *mdx52* mice learned the correct target hole faster than WT ($P < 0.001$ and $P = 0.011$ respectively) and *DMD-null* mice ($P < 0.001$ and $P = 0.018$ respectively) (Fig. 5A). After the first change in target entrance, *mdx^{5cv}* and *mdx52* mice both showed a decreased learning curve compared to WTs, however this was only significant in *mdx^{5cv}* mice ($P = 0.017$) (Fig. 5B). Notably, *mdx52* and *DMD-null* mice were less active, only showing activity for 9 or 10 bins of 50 entries. Activity per hour was plotted to check for any abrupt

changes in activity, but no sudden changes in activity could be observed, only a continuous lower activity level of the mice throughout the day (Fig. S8). During the second switch, the target hole moved back to the original location. *Mdx52* showed increased performance compared to WT and *mdx*^{5cv} mice ($P < 0.001$ and $P = 0.013$ respectively) (Fig. 5C). This increase in performance is most likely due to the decreased activity on the previous day, preventing *mdx52* mice to learn the new location during the first reversal day. Even though *DMD-null* mice had similar levels of activity as the *mdx52* mice, this did not lead to increased performance for these mice. During the 3rd target switch, the target entrance changed to the middle entrance for the first time. Interestingly, *mdx52* mice performed better than the WT mice ($P = 0.018$) and *mdx*^{5cv} and *DMD-null* mice also showed a similar trend as the other DMD models ($P = 0.05$ and $P = 0.066$) (Fig. 5D). During reversal learning day 4 (target hole right) and day 5 (target hole left), no differences were found between the groups (Fig. 5E-F).

DMD-null mice showed several deviations in spontaneous behavior pointing to more restless type of activity while small alterations were also found in mdx52 mice

Spontaneous behavior was assessed during three days of continuous monitoring in the PhenoTyper cages with minimal external influences and no direct handling. Twenty parameters of spontaneous behavior concerning activity, changes in activity due to light switches, sheltering behavior and movement patterns were analysed as described in Loos et al (Loos et al. 2014) (Fig. 6). When possible, parameters were measured during day 3 to minimize the impact of the new environment.

Four parameters showed significant differences between WT groups (Fig. S9). These parameters included: change in activity in anticipation to the dark phase, activity duration during the light phase, dark/light activity index and long arrest threshold. In one of these parameters, change in activity in anticipation to the dark phase, results were similar between groups whether or not the DMD models were compared to the individual or the pooled WT group (Fig. S9A). Therefore, this parameter was not excluded from analysis. The other three parameters only showed significant differences between DMD models and WT mice when the WT group was pooled, but not when compared to their own WTs, and were therefore excluded from analysis to prevent false positives (Fig. S9B-D).

Mdx52 mice showed an increased habituation index compared to WT, *mdx*^{5cv} and *DMD-null* mice ($P < 0.001$, $P = 0.025$ and $P < 0.001$ respectively) (Fig. 6A). As the average habituation index of the *mdx52* mice is close to 1, this means that they had similar activity levels during the 1st and 3rd dark phase, while mice with a habituation

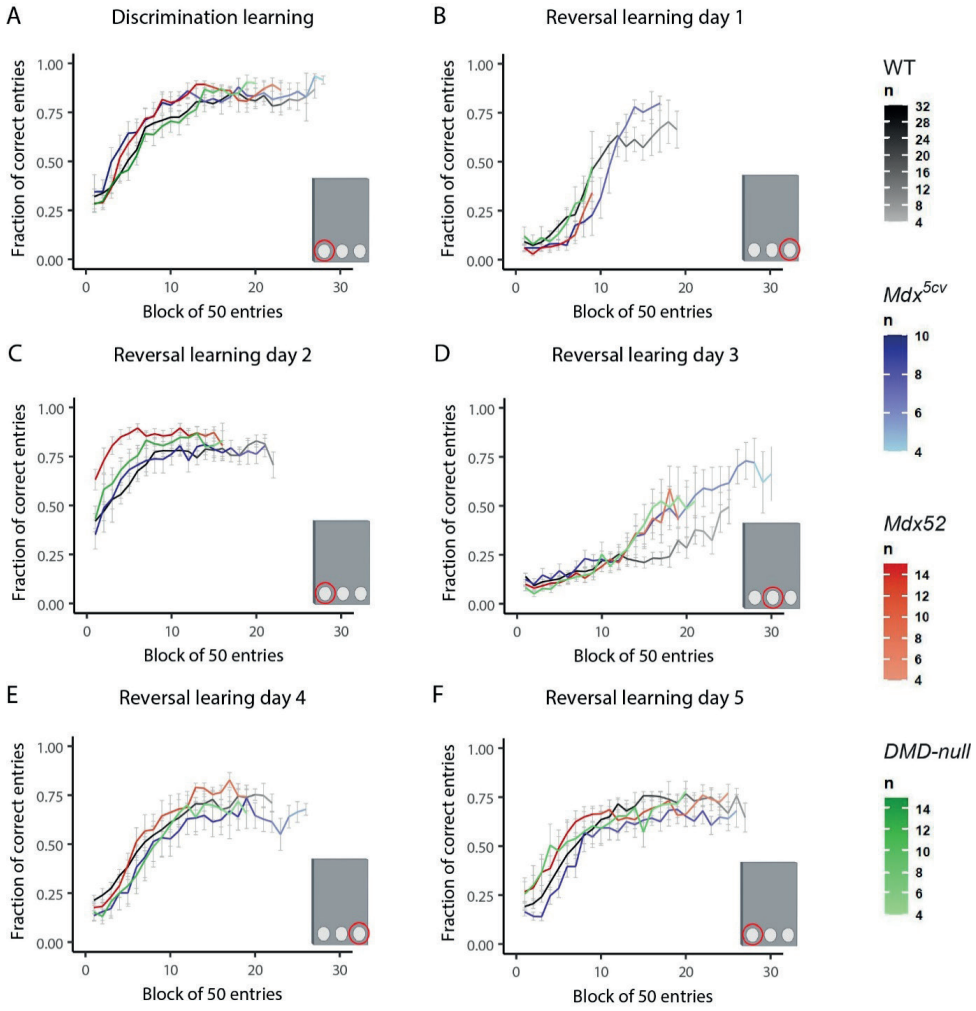

Figure 5: Serial reversal learning with food reward in PhenoTyper cages. *Mdx^{5cv} (n = 9-10), mdx52 (n = 14), DMD-null (n = 10-15) and WT mice (n = 32-33). A) Discrimination learning (left target). Both mdx^{5cv} and mdx52 mice showed steeper learning curves compared to WTs (P < 0.001 and P = 0.011 respectively) and DMD-null mice (P < 0.001 and P = 0.018 respectively). B) Reversal day 1 (right target). Mdx^{5cv} mice showed decreased learning compared to WTs (P = 0.017). C) Reversal day 2 (left target). Mdx52 mice show increased performance compared to WTs and mdx^{5cv} mice (P < 0.001 and P = 0.013 respectively). D) Reversal day 3 (middle target). Mdx52 showed increased learning compared to WTs (P = 0.018). E) Reversal day 4 (right target). No differences were found between groups. F) Reversal day 5 (left target). No differences were found between groups. All graphs were cut off at n = 3 for visual purposes. Cartoons were created with BioRender.*

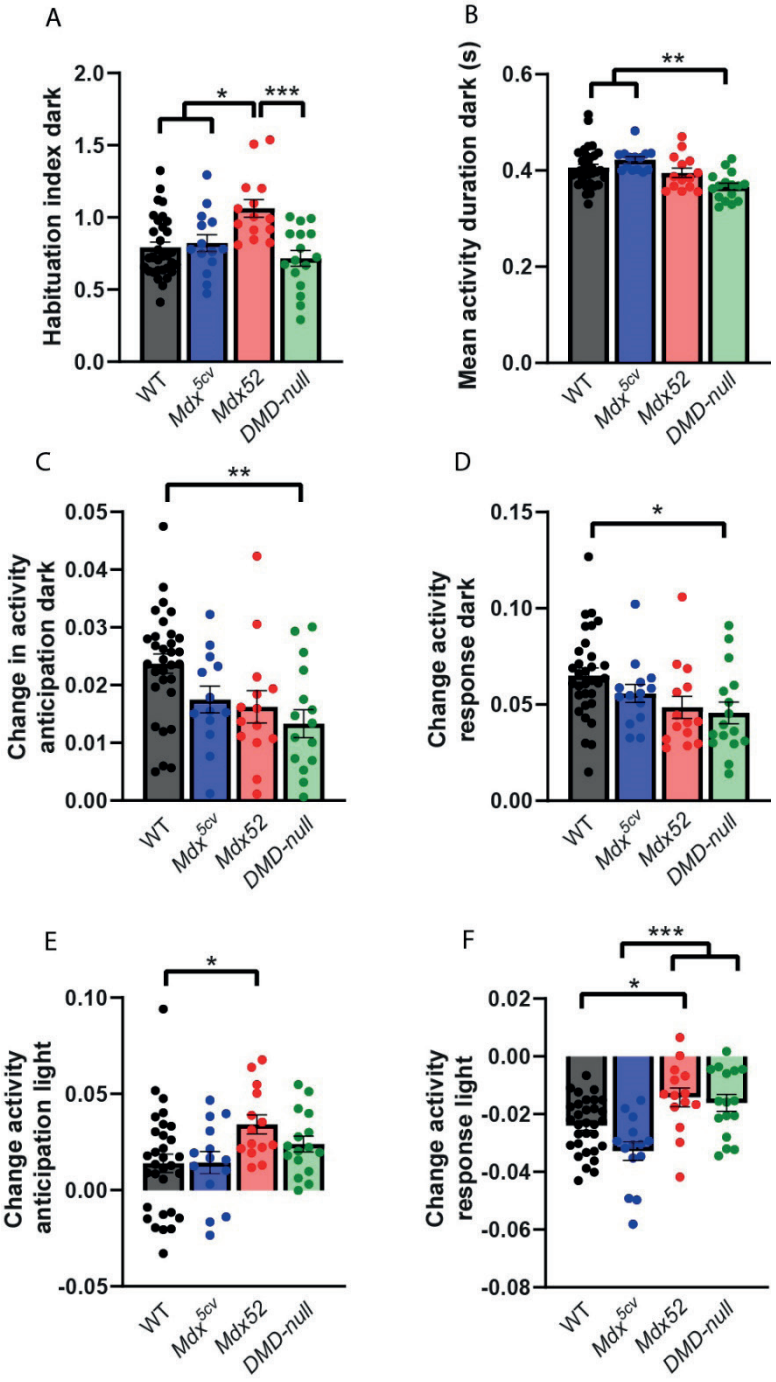
A
Habituation index dark
2.0
1.5
1.0
0.5
0.0
*
***
WT
Mdx5cv
Mdx52
DMD-null
B
Mean activity duration dark (s)
0.6
0.4
0.2
0.0
**
WT
Mdx5cv
Mdx52
DMD-null
C
Change in activity anticipation dark
0.05
0.04
0.03
0.02
0.01
0.00
**
WT
Mdx5cv
Mdx52
DMD-null
D
Change activity response dark
0.15
0.10
0.05
0.00
*
WT
Mdx5cv
Mdx52
DMD-null
E
Change activity anticipation light
0.10
0.05
0.00
-0.05
*
WT
Mdx5cv
Mdx52
DMD-null
F
Change activity response light
0.02
0.00
-0.02
-0.04
-0.06
-0.08
***
*
WT
Mdx5cv
Mdx52
DMD-null

Figure 6: Spontaneous behavior in the PhenoTyper cages; activity based behavior. *Mdx^{5cv} (n = 14), mdx52 (n = 14), DMD-null (n = 16) and WT mice (n = 33). A) Mdx52 mice showed a higher habitation index during the dark phase compared to WT, mdx-^{5cv} and DMD-null mice (P < 0.001, P = 0.025 and P < 0.001 respectively). B) Mean activity duration during the dark phase was lower in DMD-null mice compared to WT and mdx^{5cv} mice (P = 0.002 and P < 0.001 respectively). C) DMD-null mice's activity in anticipation of the dark phase was less changed compared to WT mice (P = 0.004). D) DMD-null mice showed less change in activity in response to the start of the dark phase compared to WTs (P = 0.023). E) Mdx52 mice showed a higher increase in activity in anticipation of the start of the light phase compared to WTs (P = 0.032). F) Mdx52 mice showed a lower decrease in activity change in response to the start of the light phase compared to WT and mdx^{5cv} mice (P = 0.037 and P < 0.001 respectively). DMD-null mice also showed less of a decrease in activity compared to mdx^{5cv} mice (P < 0.001). *: P < 0.05, **: P < 0.01, ***: P < 0.001.*

index below 1, had a lower activity on the 3rd compared to the 1st dark phase (Loos et al. 2014). During the 3rd dark phase, the average duration of activity was lower in *DMD-null* mice compared to WT and *mdx^{5cv}* mice (*P* = 0.002 and *P* < 0.001) (Fig. 6B). To assess differences in the anticipation or the response of a switch between dark and light phases, the average activity during the last two hours of the original phase (when in anticipation) or during the first two hours of the next phase (when in response) were calculated and the baseline, the average of the 8th, 9th and 10th hour of activity of the original phase, was subtracted. During both anticipation of the dark phase and in response to the start of the dark phase, *DMD-null* mice showed less of an increase in activity than WTs (*P* = 0.004 and *P* = 0.023 respectively) (Fig. 6C,D). It should be noted that for the change in activity in

anticipation of the dark phase, significant differences were found between WT groups, however, when they were split, *DMD-null* mice still showed a significant smaller change in activity compared to their own WT group (*P* = 0.020) (Fig. S9A). Change in activity in anticipation of the light phase was increased in *mdx52* mice compared to WTs (*P* = 0.032) (Fig. 6E). After the light phase started, *mdx52* mice showed decreased change in activity compared to both WT and *mdx^{5cv}* mice (*P* = 0.037 and *P* < 0.001 respectively) (Fig. 6F). In *DMD-null* mice, the decrease in activity was also less pronounced than in *mdx^{5cv}* mice (*P* < 0.001).

To assess more complex patterns of sheltering behavior, the duration of each shelter visit was measured, log2 transformed and plotted in a frequency plot per mouse so that three Gaussian curves could be fitted over the data. These curves were used to determine the short shelter visit threshold (90th percentile of the 1st curve) and the long shelter visit threshold (intersection between the 2nd and 3rd curve). Significant differences were found in the long shelter visit threshold of *mdx^{5cv}* mice compared

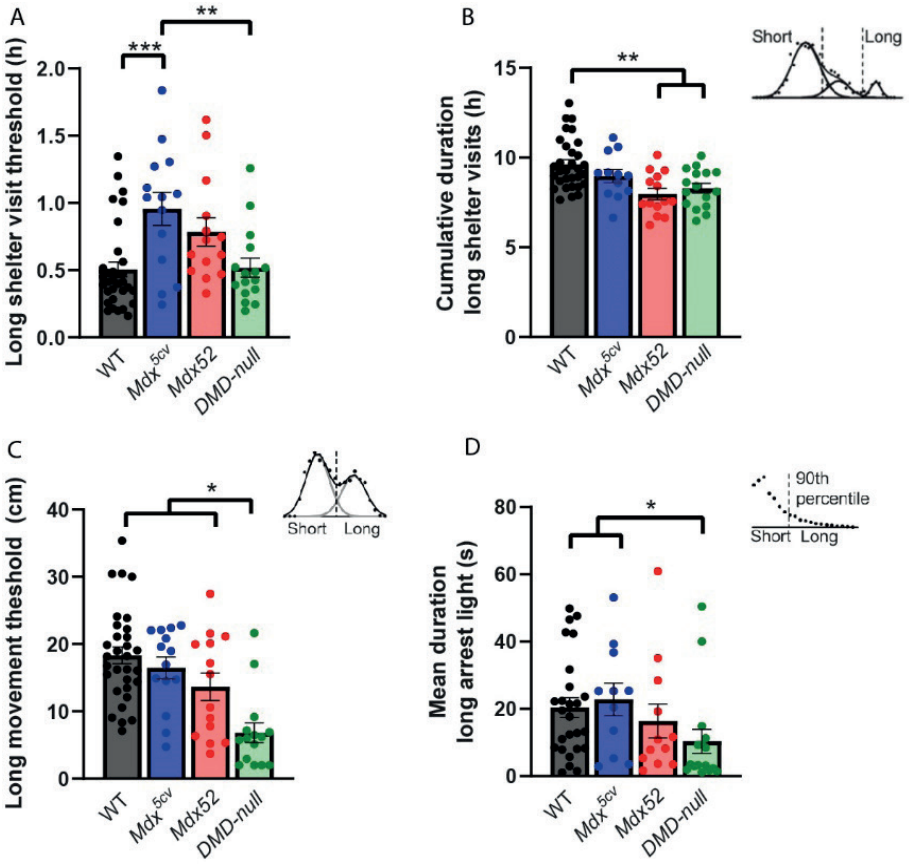

Figure 7: Spontaneous behavior in the PhenoTyper cages; sheltering behavior, movement and arrest. *Mdx^{5cv} (n = 14), mdx52 (n = 14), DMD-null (n = 16) and WT mice (n = 33). A) The long shelter visit threshold was increased in mdx^{5cv} mice compared to WT and DMD-null mice (P < 0.001 and P = 0.007 respectively). B) Both mdx52 and DMD-null mice showed a lower cumulative duration of shelter visits above the long shelter visit threshold compared to WTs (P < 0.001 and P = 0.009 respectively). C) The long movement threshold was lower in DMD-null mice compared to WT, mdx^{5v} and mdx52 mice (P < 0.001, P < 0.001 and P = 0.027 respectively). D) DMD-null mice had a lower duration of arrests above the threshold compared to WT and mdx^{5cv} mice (P = 0.014 and P = 0.009). *: P < 0.05, **: P < 0.01, ***: P < 0.001.*

to WT and *DMD-null* mice ($P < 0.001$ and $P = 0.007$ respectively) (Fig. 7A). However, when calculating the cumulative duration of long shelter visits, *mdx52* and *DMD-null,* but not *mdx^{5cv}* mice, showed a decrease in long shelter visit duration compared to WTs ($P < 0.001$ and $P = 0.009$ respectively) (Fig. 7B).

To assess movement patterns, the distance of each movement was measured, log2 transformed and plotted in a frequency plot to fit two Gaussian curves over the data. The intersection between the two curves determined the long movement threshold. *DMD-null* mice showed a lower long movement threshold compared to WT, *mdx^{5cv}* and *mdx52* mice ($P < 0.001$, $P < 0.001$ and $P = 0.027$ respectively) (Fig. 7C). Lastly, arrest patterns were analysed by calculating the duration of each arrest. A long arrest threshold was determined by the 90th percentile of data. The threshold did not differ significantly between strains, but when looking at the arrests that were above the threshold, the average duration of these arrests was lower in *DMD-null* mice compared to WT and *mdx^{5cv}* mice ($P = 0.014$ and $P = 0.009$ respectively) (Fig. 7D).Overall, mostly *DMD-null* mice showed changes in spontaneous activity, but small deviations could be found in all models.

Discussion

The consequences of the lack of Dp427 in the brain have been thoroughly investigated (Sekiguchi et al. 2009, Yamamoto et al. 2010, Manning et al. 2014, Remmelink et al. 2016, Vaillend and Chaussenot 2017, Razzoli et al. 2020, Saoudi et al. 2021, Lindsay and Russell 2023) and in recent years our knowledge on the consequences of a lack of Dp140 has expanded (Saoudi et al. 2021, Hashimoto et al. 2022). However, many studies focused on a single DMD mouse model only, making it difficult to directly compare the extent of behavioral alterations between strains lacking one or multiple dystrophin isoforms. Furthermore, behavioral characterization of *DMD-null* mice had not been undertaken before, limiting the knowledge on the consequences of the additional loss of Dp71 and Dp40. This study aimed to directly compare different behavioral aspects between three DMD mouse models to gain new insights in the cognitive deficits of these models.

Anxiety increases due to the lack of Dp427, Dp140, Dp71 and Dp40

One of the most studied behaviors in DMD mouse models is anxiety. Literature involving mice lacking Dp427 (*mdx* and *mdx^{5cv}*), suggested these models to have a subtle and somewhat borderline deficit in anxiety, while mice lacking Dp427 and Dp140 show a more profound anxiety response (Saoudi et al., 2021). Although dif-

ferences between our *mdx*^{5cv} and *mdx52* mice were subtle, multiple parameters did show a stepwise pattern in which the *mdx52* mice are consistently slightly more anxious than *mdx*^{5cv} mice. The ability to detect anxious behavior can be highly dependent on the protocol and surroundings, possibly variations of materials could affect the chance of finding subtle differences between the strains. Furthermore, the anxiety test which showed the largest effects in literature, the elevated plus maze, was not included in our study (Saoudi et al. 2021), due to inaccessibility of the equipment. Interestingly, *DMD-null* mice were more anxious than males of the other DMD strains, indicating a prominent role of Dp71 and/or Dp40 in anxiety, which has been indicated before in Dp71-null mice, as they are more anxious compared to WTs (Miranda et al. 2024).

Due to the lack of Dp260, *mdx52* mice have altered visual processing (Barboni et al. 2021). While this has not been investigated in *DMD-null* mice, similar deficits could be expected in this model. We cannot rule out that altered visual processing could have influenced the behavior of *mdx52* and/or *DMD-null* mice in these anxiety tests.

Overall, it seems that each of the brain dystrophin isoforms has, to a varying degree, a role in anxiety. Anxious behavior increases with the lack of each additional dystrophin isoform, however, this change in behavior can be very subtle and difficult to distinguish especially when only the longer dystrophin isoforms are lacking.

Freezing phenotype further exacerbated by the lack of Dp71 and Dp40

The most robust behavioral deficit found in mice lacking Dp427 is the profound freezing response after a short period of restraint (Sekiguchi et al. 2009, Yamamoto et al. 2010, Vaillend and Chaussenot 2017, Razzoli et al. 2020, Saoudi et al. 2021, Lindsay and Russell 2023). Our study confirmed that this deficit was present in the *mdx*^{5cv} mice and that the additional lack of Dp140 in the *mdx52* mice did not further exacerbate the phenotype. Surprisingly, we observed, for the first time, that *DMD-null* mice show a further increase in freezing compared to *mdx52* mice and a trend in the same direction was found compared to *mdx*^{5cv} mice. It should be noted that mice had lost a significant amount of weight in the days prior to the fear test due to the serial reversal learning task. While mice recovered quickly and were almost back to their previous bodyweight before execution of the fear test, a potential influence of this cannot be completely ruled out. Lack of Dp71 and Dp40 seems to have a significant negative effect on the already affected fear response seen in mice lacking Dp427.

Inconclusive observations for sociability and social novelty seeking

Social interaction has been investigated multiple times in *mdx* mice using the 3 chamber social paradigm, with varying results. While a lack of social preference was reported in 8 week old *mdx* mice (Alexander et al. 2016), these results could not be replicated in older mice (Miranda et al. 2015, Hashimoto et al. 2022). *Mdx52* mice have been reported before to have increased social preference compared to WT and *mdx* mice (Hashimoto et al. 2022). Unfortunately, our set up proved to be suboptimal as WT mice did not show a preference for social interaction or social novelty. Due to this lack of preference, results on the other groups are inconclusive. We would like to note that even in the suboptimal setting, *mdx52* mice still showed a small increase in social preference, as described in literature before. *DMD-null* mice showed a preference for social novelty in the second trial, but his warrants further investigations.

Spatial and reversal learning are not decreased by the lack of dystrophin isoforms

Different types of learning and memory were assessed during this study, including hippocampus dependent spatial learning in both relatively low (motor) stress conditions in the Barnes maze and during high (motor) stress conditions in the Morris water maze. Literature on *mdx* mice has reported deficits in spatial recall in older mice (6-, and 12-month-old) but not younger mice (4-month-old) (Sesay et al. 1996, Remmelink et al. 2016, Bagdatlioglu et al. 2020, Verhaeg et al. 2024). It should be noted that Vaillend *et al.* reported impairments in long term memory in younger *mdx* mice, however this conclusion was largely based on time spent in the target zone (Vaillend et al. 2004). Due to the decreased swimming speed of the DMD mouse models, this parameter might not purely represent spatial recall. Reversal learning in the Barnes maze has been reported to be affected in 4-month-old *mdx* mice (Remmelink et al. 2016). Our results in *mdx^{5cv}* mice partly match the literature, since we did not find any deficits in spatial learning and recall. We were unable to find the deficits in reversal learning in our *mdx^{5cv}* mice, that were previously found in *mdx* mice (Remmelink et al. 2016). Differences in the design of the Barnes maze, with 24 holes in the set up used by Remmelink et al, while ours only contained 12 holes, could underlie this difference. Furthermore, the difference in genetic background of the mouse models (*mdx* vs *mdx^{5cv}*) could also be an underlying factor.

The lack of deficits in *mdx52* mice in acquisition, recall and reversal learning in the Barnes maze is in line with our previous data from *mdx^{4cv}* mice, which also lack Dp427 and Dp140 (Verhaeg et al. 2024). Interestingly, the *DMD-null* mice showed

improved performance during reversal learning and recall in the Barnes maze. This could be caused by an increased motivation to find sheltering, due to higher anxiety. To our knowledge, no direct correlations have been previously found between anxiety and motivation for shelter seeking in a spatial learning task, however, we noted that WT mice sometimes, after locating the correct hole, did not enter the shelter box, highlighting their lower motivation to seek shelter.

During the higher stress conditions of the Morris water maze, a slight deviation in search pattern was observed in *mdx52* and *DMD-null* mice. These mice did not have trouble reaching the platform location at first, but after realizing that the platform was absent, they did not show a preference for searching in the quadrant in which the platform should be located, as indicated by the shorter relative distance travelled and time spent in the target quadrant. Possibly, this could be caused by a lower confidence in the platform location, causing them to search more randomly after not finding it. It should be noted that since this effect was only present during the Morris water maze, this is likely an (indirect) effect of the higher motor demand of the task for the animals. Many *DMD-null* mice and several *mdx52* mice had trouble completing the learning day, due to the numerous acquisition trials. The high motor stress is probably due to the decreased muscle function of these animals (Chesshyre et al. 2022) and their higher body weight.

Learning and memory tasks are highly reliant on the motivation and stress levels of the animal and this could possibly influence the outcome of the tasks. One should therefore always take into account the motivational drive and physical condition of the animals while performing these tests.

Recognition memory is not affected by the lack of dystrophin isoforms

In terms of recognition memory, the field has been unable to reach consensus about a possible deficit. While some studies found deficits for long term recognition in the NOR and T-maze in *mdx* mice (Vaillend et al. 1995, Vaillend et al. 2004, Vaillend and Chaussenot 2017, Comim et al. 2019), others did not (Sesay et al. 1996, Remmelink et al. 2016, Bagdatlioglu et al. 2020). Dp71-null mice have shown reduced alternation in the T-maze at multiple delays, but did not show any altered behavior in the NOR test (Daoud et al. 2009). Due to the inconsistencies in literature, we performed multiple tests, with both short and long term delays. We did not observe any deficits in the NOR, OP or T-maze tests in our current study, though it should be noted that we failed to see preferences in the WT animals in the T-maze and barely saw a preference during the 24 hour delay in the OP test. This could suggest that these protocols might not have been optimal to study recognition memory, as

normally WT mice show a clear preference in these tests (Deacon and Rawlins 2006, Vogel-Ciernia and Wood 2014). The lack of preferences in the T-maze, makes any conclusions regarding a deficit in the other models inconclusive. Overall, no deficits have been observed in recognition memory in the NOR and OP test. Results from the T-maze were inconclusive due to a suboptimal protocol and possible influences of anxiety on the motivation of the animals.

Challenges in the food-related reversal task could affect data interpretation

The capacity for flexibility of learning was further examined using a food-rewarded serial reversal task in the automated home cages. *Mdx^{5cv}* and *mdx52* mice showed increased performance during the initial discrimination learning. This behavior has been reported before in *mdx* mice, although inconsistently (Remmelink et al. 2016, Lewon et al. 2017, Engelbeen et al. 2021) and is most likely caused by an increased food drive leading to higher food seeking behavior (Lewon et al. 2017). Interestingly, *DMD-null* mice did not show this increase in learning. Whether this is due to the lack of an increased food drive, or a learning deficit remains unclear. It should however be noted that a preference for the left hole could not be ruled out as the target whole was not randomized between individuals. After the initial reversal learning switch, *mdx^{5cv}* mice showed a decrease in performance compared to WT mice, which was borderline in *mdx52* mice and absent in the *DMD-null* mice. The participation of *mdx52* and *DMD-null* mice was noticeably lower during the first reversal day indicative of a lower engagement overall which could result from anxiety (Fig. S8). It remains unclear why activity was most heavily affected during this specific day. Interestingly, the lack of activity in *mdx52* and *DMD-null* mice and the probable lack of establishment of this new location seemed to have a bigger impact on *mdx52* mice, as only these mice show improved performance during the second reversal trial, when the target location goes back to the initially learned location. This was confirmed by the perseverative error curve where *mdx52* mice already start at a much lower fraction, showing less interest in the previous target than other groups. A potential preference for the left hole in the discrimination trial in some strains could also be an underlying cause for the altered performance in the first and second reversal trials.

During the next reversal day, in which the middle hole was introduced as a target, all DMD models outperformed the WT mice which had a lower affinity for the target hole compared to both lateral holes. We hypothesize that the WT mice learned a stronger correlation or pattern during the first days in the PhenoTyper cages where they specifically correlated both lateral entrances to food rewards, whereas the different DMD mice did not recognize this left-right-left pattern. Therefore, it would

be easier for the DMD mouse models to adjust their behavior, since their switches would not favor the lateral sides necessarily. Taken together, these results suggest that *DMD-null* mice behave differently to the other DMD models in this food motivated reversal tasks, possibly due to lack of increased discrimination learning, and lack of decreased initial reversal learning. Additionally, problems with pattern recognition in all DMD models could be present and should be investigated further. However, as many factors can influence the behavior in the reversal learning trials, including food motivation, progressive impact of the prolonged food restriction, preference of a specific hole or confusion induced by previous trials and overall activity, conclusions should be made with care.

Lack of Dp140 results in lack of habituation, loss of Dp71 and Dp40 lead to restlessness

One of the great advantages of the tasks performed in the PhenoTyper cages, is that there is minimal disruption from unintentional external stimuli, which is especially important when doing deep phenotyping analysis of spontaneous behavior. It should however be noted that the single housing conditions could impose stress, especially in DMD mice which are known to be sensitive to stress, thereby potentially influencing results. Overall, we saw many subtle differences in activity based behavior in the *mdx52* and *DMD-null* mice. The deviations in *mdx52* behavior were more inconsistent and subtle, and mostly related to changes from the dark to light phase. Notably, *mdx52* mice also showed a lack of habituation, which could have an impact on the execution of other behavioral tests, such as learning tasks, that rely on habituation to decrease anxiety effects. It would be beneficial in future studies to prolong the experiment to investigate how long this lack of habituation remains present. Previous experiments in *mdx* and *mdx*^{4cv} mice, lacking Dp427 and Dp427+Dp140 respectively, have shown deviations in spontaneous behavior in similar directions in most, but not all, parameters (Verhaeg et al. 2024). It should be noted that the duration of the experiment differed between the studies (2 days in Verhaeg et al. vs 3 days in this study). Since habituation can play an important role in behavioral changes, this could have contributed to the differences between the studies. Also the use of different strains (*mdxbl6* versus *mdx*^{5cv} and *mdx*^{4cv} vs *mdx52*), could have played a role. Lastly, the strain of the subsequent testing could have influenced behavior in this study, especially in *mdx52* and *DMD-null* mice, as they showed difficulties in completing the Morris water maze test, which was performed 24h before the start of the PhenoTyper analysis.

The *DMD-null* mice showed overall shorter activity bouts, less reactivity to the dark and light changes, decreased duration of long shelter visits and shorter movement and arrest bouts. This suggests that the activity of the *DMD-null* mice is more errat-

ic, having more alternations between activity and rest, without necessarily affecting the total amount of activity. This restless type of behavior has been observed before (Kudoh et al. 2005), but has not been quantified until now. Furthermore, the decreased time of long shelter visits could indicate a disruptive sleep pattern, as these long shelter visits usually are multiple hours long. Taken together, the *DMD-null* mice appear to be more restless in their spontaneous behavior, which is illustrated by multiple outcome measures.

Translational value

Although the severity and abundance of alterations, such as anxiety, fear and reversal learning seen in *DMD-null* mice are partly in line with those made in patients, it should be noted that the differences found between mice lacking Dp427 and Dp140 vs mice also lacking Dp71 and Dp40 seem to be less substantial and abundant than the differences seen in corresponding patients, where the additional lack of Dp71 and Dp40 leads to a very strong phenotype (Lenk et al. 1993, Moizard et al. 2000, Daoud et al. 2009, Taylor et al. 2010). Further analysis of this model and it's translational value is warranted. Possibly, the differences observed between clinical and preclinical data could be influenced by the lack of corticosteroid administration in our mouse models. Corticosteroids are part of the standard of care in DMD and used by the vast majority of patients to slow down the muscle degeneration (Fenichel et al. 1991, Khan 1993). Corticosteroids are known to have negative effects on behavior in healthy humans and mice (Schmidt et al. 1999, Kajiyama et al. 2010, Ciriaco et al. 2013, Dos Santos et al. 2019, Prado and Crowe 2019). The effects of corticosteroids on behavior in DMD patients and mouse models remains largely unclear, however it is known that the treatment regime of corticosteroids is linked to the brain pathology, gray matter volume specifically, in DMD patients (Geuens et al. 2023) and depressive-like behavior in *mdx* mice (Liu et al. 2024). We can therefore not rule out that the lack of corticosteroid treatment in this study might have contributed to the differences seen between our mouse models and DMD patients.

Limitations

While this study has provided new insights into the deficits of DMD models, the study design used could have (partly) influenced outcomes. Firstly, mice were housed in groups of 2 to 4 mice in individually ventilated cages. Both the variation in social environment and the use of ventilated cages could have influenced behavior such as depression and anxiety and makes comparisons to literature more complicated (York et al. 2012, Horii et al. 2017). Other types of behavior, like recognition memory and social interaction seem unaltered by housing in individually ventilated

cages.

Secondly, to minimize the amount of animals required for the study, we chose an elaborate setup of subsequent behavioral testing, meaning that mice were subjected to several tests in a short time period. While the order of testing was chosen such to minimize stress of the cumulative testing, we cannot rule out that results were influenced by this study design. To minimize this risk, tests inducing high stress levels such as the Morris water maze were conducted at the end of the test battery, and data analysis of the subsequent Phenotyper cage test was restricted to the 3rd night which they spend there. It cannot be ruled out that spontaneous behaviour was affected by this approach.

Conclusion

This study aimed for a broad generic behavioral description and deep phenotyping of mice lacking one, multiple or all brain dystrophin isoforms. We confirmed already established deficits of anxiety and fear in *mdx*^{5cv} and *mdx52* mice and showed that this was further exacerbated in *DMD-null* mice, indicating a specific role of Dp71/Dp40 in the anxiety and the fear responses. We showed that *mdx52* and *DMD-null* mice might suffer from subtle deficits in spatial memory only when under high motor stress.-Lastly, we showed subtle changes in spontaneous behavior especially in *mdx52* and *DMD-null* mice. Altogether, this study provides the field with an extensive overview of behavioral deficits in different DMD models, giving new insights in the behaviors in which dystrophin isoforms are involved, that could be used in future preclinical research.

Ethical statement

The animal study was reviewed and approved by Central Authority for Scientific Procedures on Animals and performed according to Dutch regulation for animal experimentation, and in accordance with EU Directive 2010/63/EU.

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Competing interests

None related to this work.

Author contributions

M.A.T. Verhaeg: Methodology, Investigation, Software, Formal analysis, writing – original draft, writing- review and editing, visualization. E.M. van der Pijl: Investigation. D. van de Vijver: Investigation. C.L. Tanganyika-de Winter: Investigation. T.L. Stan: Investigation. A. van Uffelen: Investigation. L. Censoni: Software. M. van Putten: Conceptualization, Methodology, writing- review and editing, supervision, funding acquisition

Supplementary data

Table S1: Genotyping information. *Primer sequences and primer specific PCR information used for genotyping of the mouse models.*

Number	Strain	Primer sequences (3' to 5'): Forward primer Reverse primer	Annealing temperature	Number of PCR cycles	Product size
1	*Mdx^{5cv}*	TGGAGACGGAAGTAAATCTGG	58°C	35	147 bp
		CCTCATGAGCATGAAACTGTTC			
2	*Mdx^{5cv}*	Primer pair as describe above + digestion mix	58°C	35	93 bp & 54 bp
3	*Mdx52* (WT)	AGGCAACACTGCAAGATTTGGAAC	60°C	30	383 bp
		AACTCAAATAGATGATTGGTAAGAGGC			
4	*Mdx52* (Mutant)	AGGATCTCCTGTCATCTCACCTTGCTCCTG	60°C	30	493 bp
		AAGAACTCGTCAAGAAGGCGATAGAAGGCG			
5	*DMD-null* (WT)	TGGGCAAGAGTGAATTTTCC	65°C	32	437 bp
		ACCACCCACTTCAGGTTGAG			
6	*DMD-null* (Mutant)	GAATTCAGCGAGAGCCTGAC	65°C	32	453 bp
		GATGTTGGCGACCTCGTATT			

Table S2: Overview of statistical tests performed and P-values of WT comparisons. *Differences between WT groups were found for bodyweight (WT mdx^{5cv} vs WT DMD-null: P = 0.008, WT mdx52 vs WT DMD-null: P = 0.001) and during spontaneous behavior for change in activity in anticipation of the dark phase (WT mdx^{5cv} vs WT mdx52: P = 0.003, WT mdx^{5cv} vs WT DMD-null: P = 0.001). Significant differences are indicated in bold. DI: discrimination index, DL: discrimination learning, RL: reversal learning, MWM: Morris water maze, NOR: novel object placement, OP: object placement.*

Test	Parameter	Statistical test	*P* value
Dark light box	Visits in light zone	One-way ANOVA	0.992
	Time in light zone	Kruskal-Wallis test	0.446
	Average time per visit	One-way ANOVA	0.174
Open field	Mean distance to wall	One-way ANOVA	0.110
	Time in inner zone	One-way ANOVA	0.339
	Distance travelled in inner zone	One-way ANOVA	0.338
	Velocity	One-way ANOVA	0.122
	Time moving	Kruskal-Wallis test	0.068
Unconditioned fear	Velocity	Kruskal-Wallis test	0.200
	Time frozen	Kruskal-Wallis test	0.208
3 chamber	DI – Trial 2	One-way ANOVA	0.575
	DI – Trial 3	One-way ANOVA	0.381
Barnes maze	Distance walked – Learning	Linear mixed models	0.674
	Distance walked – Probe	Kruskal-Wallis test	0.092
	Relative distance walked in target quadrant – Probe	One-way ANOVA	0.096
	Distance walked – Reversal learning	Linear mixed models	0.329
	Distance walked – Reversal probe	One-way ANOVA (log10)	0.618
	Relative distance walked in target quadrant – Reversal probe	Kruskal-Wallis test	0.763

	Distance walked old target – Reversal learning	Linear mixed models	0.948
	Distance walked old target– Reversal probe	Kruskal-Wallis test	0.675
	Relative distance walked in old target quadrant – Reversal probe	One-way ANOVA	0.299
	Velocity	Linear mixed models	0.534
	Interaction time target hole – Probe	One-way ANOVA	0.254
	Interaction time target hole – reversal probe	One-way ANOVA	0.639
	Interaction time old target hole – reversal probe	One-way ANOVA	0.844
MWM	Velocity	Linear mixed models	0.578
	Distance till platform – acquisition	Linear mixed models	0.661
	Distance till platform – probe	Kruskal-Wallis test	0.395
	Relative distance in target quadrant	One-way ANOVA	0.439
	Time in NE quadrant (target)	One-way ANOVA	0.794
	Time in NW quadrant	One-way ANOVA (log10)	0.508
	Time in SE quadrant	One-way ANOVA (log10)	0.723
	Time in SW quadrant	One-way ANOVA (log10)	0.296
NOR	DI – 10 min	One-way ANOVA	0.646
	DI – 24h	One-way ANOVA	0.920
OP	DI – 10 min	One-way ANOVA	0.714
	DI – 24h	One-way ANOVA	0.453
T-maze	DI – 6h	One-way ANOVA	0.351
	Alternation - 6h	One-way ANOVA	0.093
	DI – 24h	One-way ANOVA	0.875
	Alternation – 24h	One-way ANOVA	0.630
Serial reversal	DL	Linear mixed models	0.890
	RL1	Linear mixed models	0.900
	RL2	Linear mixed models	0.084
	RL3	Linear mixed models	0.290
	RL4	Linear mixed models	0.460
	RL5	Linear mixed models	0.110
Spontaneous behavior	Mean activity duration dark	One-way ANOVA	0.933
	Change in activity anticipation dark	One-way ANOVA	**0.001**
	Change in activity response dark	One-way ANOVA	0.791
	Change in activity anticipation light	One-way ANOVA	0.090
	Change in activity response light	One-way ANOVA	0.548
	Habituation index dark	One-way ANOVA	0.388
	Long shelter visit threshold	One-way ANOVA	0.506
	Cumulative duration long shelter visits	One-way ANOVA	0.772
	Long movement threshold	One-way ANOVA	0.989
	Mean long arrest duration	One-way ANOVA	0.834
Bodyweight	8-15 wks	Linear mixed models	**0.001**

Table S3: Overview of statistical parameters. *Additional statistical parameters reported in case of significant differences (P < 0.05). Note, P-values reported in this table for spontaneous behavior are before false discovery rate correction. DI: discrimination index, DL: discrimination learning, MWM: Morris water maze, NOR: novel object recognition, OP: object placement, RL: reversal learning.*

Test	Parameter	Statistical test	Comparison	*P*-value	Additional statistical values
Dark light box	Visits in light zone	One-way ANOVA	Overall strain comparison	0.001	F(3,75)=16.45
	Time in light zone	Mann-Whitney repeated tests	*Mdx*^{5cv} vs WT	0.001	Z=-3.54
			Mdx52 vs WT	0.001	Z=-3.53
			DMD-null vs WT	0.001	Z=-5.40
			Mdx^{5cv} vs *DMD-null*	0.043	Z=-2.03
			Mdx52 vs *DMD-null*	0.008	Z=-2.61
	Average time per visit	One-way ANOVA	Strain comparison	0.014	F(3,75)=3.80
Open field	Mean distance to wall	One-way ANOVA	Overall strain comparison	0.001	F(3,76)=11.28
	Time in inner zone	One-way ANOVA	Overall strain comparison	0.001	F(3,76)=8.92
	Distance travelled in inner zone	One-way ANOVA	Overall strain comparison	0.001	F(3,76)=11.91
	Velocity	One-way ANOVA	Overall strain comparison	0.001	F(3,76)=24.44
	Time moving	Mann-Whitney repeated tests	*Mdx52* vs WT	0.017	Z=-2.39
			DMD-null vs WT	0.001	Z=-4.88
			Mdx^{5cv} vs *DMD-null*	0.001	Z=-3.51
			Mdx52 vs *DMD-null*	0.001	Z=-2.91
Unconditioned fear	Velocity	Mann-Whitney repeated tests	*Mdx*^{5cv} vs WT	0.001	Z=-5.59
			Mdx52 vs WT	0.001	Z=-5.46
			DMD-null vs WT	0.001	Z=-5.51
			Mdx^{5cv} vs *DMD-null*	0.012	Z=-2.49
	Time frozen	Mann-Whitney repeated tests	*Mdx*^{5cv} vs WT	0.001	Z=-5.54
			Mdx52 vs WT	0.001	Z=-5.46
			DMD-null vs WT	0.001	Z=-5.51
			Mdx^{5cv} vs *DMD-null*	0.019	Z=-2.33
3 chamber	DI vs chance level – Trial 2	One sample t-test	*Mdx52*	0.024	t=-2.56
	DI vs chance level– Trial 3	One sample t-test	*DMD-null*	0.008	t=3.06
Barnes maze	Relative distance walked in target quadrant - Probe	One sample t-test	WT	0.001	t=12.56
			Mdx^{5cv}	0.001	t=11.05
			Mdx52	0.001	t=8.35
			DMD-null	0.001	t=7.32
	Distance walked till target – Reversal learning	Linear mixed models	*DMD-null* vs WT	0.015	F(1,215)=6.00
	Relative distance walked in target quadrant – Reversal probe	One way ANOVA	Overall strain comparison	0.029	F(3,76)=3.16
		One-sample t-test	WT	0.001	t=6.96
			Mdx^{5cv}	0.001	t=7.36
			Mdx52	0.001	t=5.64
			DMD-null	0.001	t=6.67
	Relative distance walked in old target quadrant – Reversal probe	One-sample t-test	*WT*	0.008	t=-2.837
	Velocity	Linear mixed models	*Mdx52* vs WT	0.045	F(1,150)=4.09
			DMD-null vs WT	0.001	
			Mdx^{5cv} vs *DMD-null*	0.010	F(1,150)=6.78

MWM	Velocity	Linear mixed models	*Mdx^{5cv}* vs WT	0.001	F(1,72)=11.74
			Mdx52 vs WT	0.001	F(1,72)=71.99
			DMD-null vs WT	0.001	F(1,72)=58.30
			Mdx^{5cv} vs *DMD-null*	0.001	F(1,72)=15.21
			Mdx52 vs *DMD-null*	0.017	F(1,72)=5.877
	Relative distance in target quadrant	One-sample t-test	WT	0.001	Z=-4.60
			Mdx^{5cv}	0.002	Z=-2.63
	Time in NE quadrant (Target)	One-way ANOVA	Overall strain comparison	0.001	F(3,73)=6.922
NOR	DI vs chance level– 10 min	One-sample t-test	WT	0.001	t=5.31
			Mdx^{5cv}	0.036	t=2.00
			Mdx52	0.004	t= 3.58
			DMD-null	0.001	t=3.76
	DI vs chance level– 24h	One-sample t-test	WT	0.001	t=4.45
			Mdx^{5cv}	0.044	t=1.39
			Mdx52	0.0015	t=2.13
			DMD-null	0.011	t=2.44
OP	DI – 10 min	One-sample t-test	WT	0.001	t=4.82
			Mdx^{5cv}	0.001	t=4.76
			Mdx52	0.003	t=3.30
			DMD-null	0.001	t=5.62
	DI – 24h	One-sample t-test	WT	0.014	t=2.32
T-maze	Alternation vs chance level – 6h	One-sample t-test	*Mdx52*	0.020	t=2.81
			DMD-null	0.046	t=2.24
Serial reversal	DL	Linear mixed models	*Mdx^{5cv}* vs WT	0.001	F(1,76)=16.23
			Mdx52 vs WT	0.011	F(1,76)=6.72
			Mdx^{5cv} vs *DMD-null*	0.001	F(1,76)=31.68
			Mdx52 vs *DMD-null*	0.018	F(1,76)=5.76
	RL1	Linear mixed models	*Mdx^{5cv}* vs WT	0.017	F(1,85)=5.87
	RL2	Linear mixed models	*Mdx52* vs WT	0.001	F(1,76)=22.13
			Mdx^{5cv} vs *Mdx52*	0.013	F(1,76)=6.39
	RL3	Linear mixed models	*Mdx52* vs WT	0.018	F(1,86)=5.84
Spontaneous behavior	Mean activity duration dark	One-way ANOVA	Overall strain comparison	0.001	F(3,71)=7.22
	Change in activity anticipation dark	One-way ANOVA	Overall strain comparison	0.004	F(3,71)=4.941
	Change in activity response dark	One-way ANOVA	Overall strain comparison	0.016	F(3,71)=3.65
	Change in activity anticipation light	One-way ANOVA	Overall strain comparison	0.032	F(3,71)=3.09
	Change in activity response light	One-way ANOVA	Overall strain comparison	0.001	F(3,71)=8.70
	Habituation index dark	One-way ANOVA	Overall strain comparison	0.001	F(3,71)=6.98
	Long shelter visit threshold	One-way ANOVA	Overall strain comparison	0.001	F(3,71)=6.50
	Cumulative duration long shelter visits	One-way ANOVA	Overall strain comparison	0.001	F(3,71)=6.51
	Long movement threshold	One-way ANOVA	Overall strain comparison	0.001	F(3,71)=10.32
	Mean long arrest duration	Mann-Whitney repeated tests	*DMD-null* vs WT	0.014	Z=-2.43
			Mdx^{5cv} vs *DMD-null*	0.009	Z=-2.60

Bodyweight	8-15 wks	Linear mixed models	*Mdx52* vs WT *mdx52*	0.017	F(1,22)=6.64
			DMD-null vs WT *DMD-null*	0.001	F(1,29)=36.88

Figure S1: Genotyping blots of DMD mouse models. *Genotyping performed after birth. Numbers correspond to primer pairs as described in Table S1. Product size per primer pair; primer 1: 147 bp (present in mdx^{5cv} and corresponding WT samples), primer 2: 93 bp & 54 bp (present in mdx^{5cv} samples), primer 2: 383 bp (present in corresponding WT samples), primer 4: 493 bp (present in mdx52 samples), primer 5: 437 bp (present in corresponding WT samples), primer 6: 453 bp (present in DMD-null samples).*

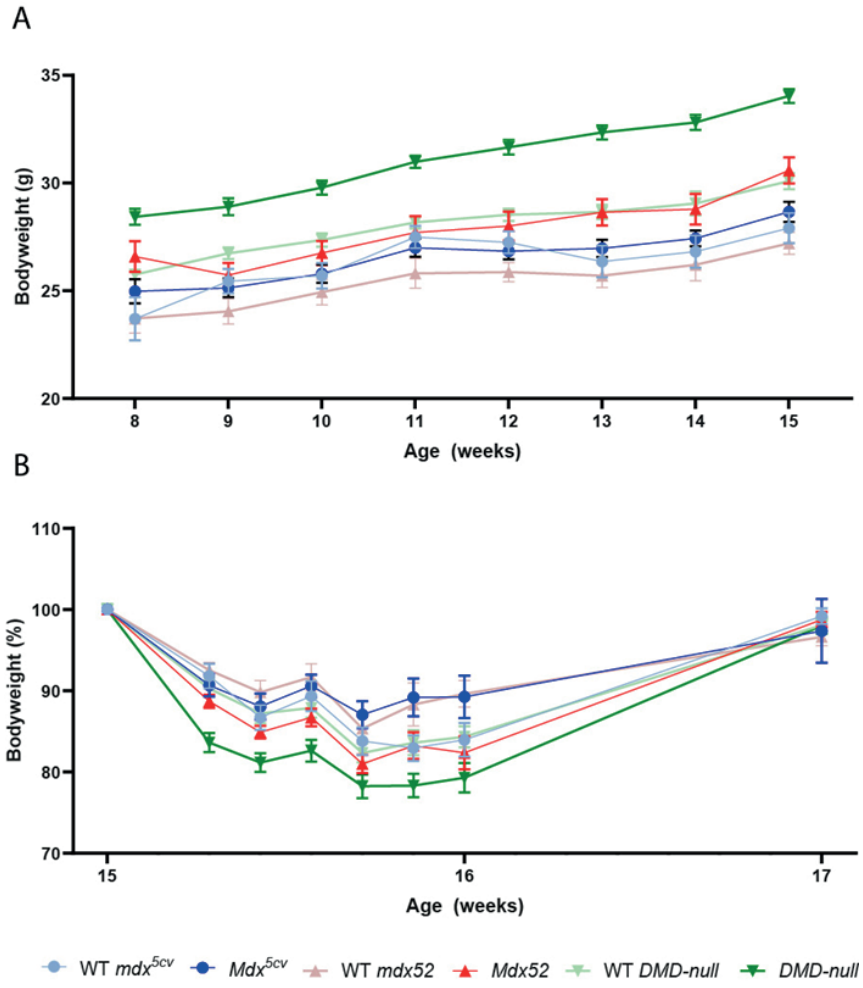

Figure S2: Bodyweight through the study. *Mdx^{5cv} (n = 16), mdx52 (n = 15), DMD-null (n = 16) and WT mice (n = 33). A) Bodyweight of mdx52 and DMD-null mice was increased compared to their respective WT groups (P = 0.017 and P < 0.001). No direct comparisons were made between DMD models due to differences between WT groups (P < 0.001). B) Bodyweight during spontaneous behavior and serial reversal learning in the PhenoTyper cages, as a percentage of the weight measured before starting the discrimination task. No statistical tests were performed.*

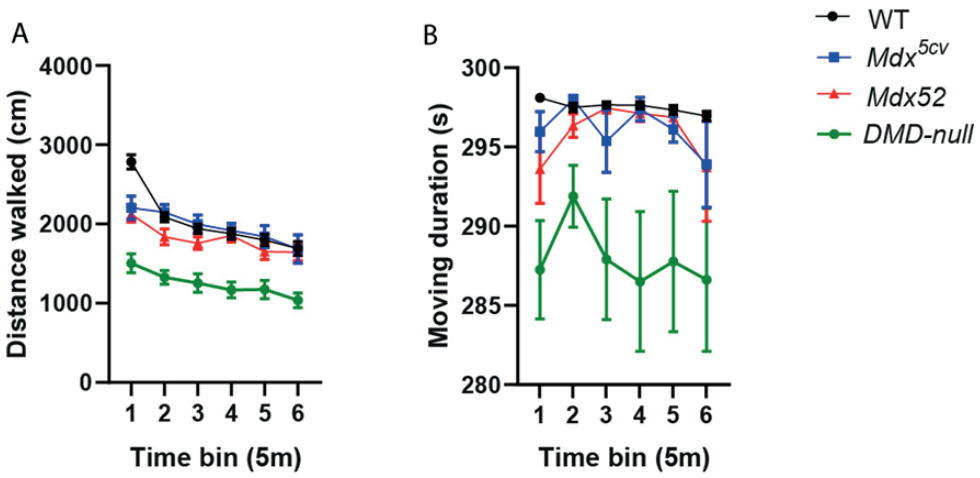

Figure S3: Locomotion in the open field task. *Mdx^{5cv} (n = 16), mdx52 (n = 15), DMD-null (n = 16) and WT mice (n = 33). A) The initial longer distance walked in the first time bin for WT mice was not seen in mdx^{5cv}, mdx52 or DMD-null mice (P = 0.04, P = 0.005 and P = 0.002 respectively). Overall, DMD-null mice walked a shorter distance compared to the other strains but no differences in distance walked over time could be found between the DMD strains. B) While DMD-null mice spent less time moving overall, no difference in moving pattern could be detected between the different strains*

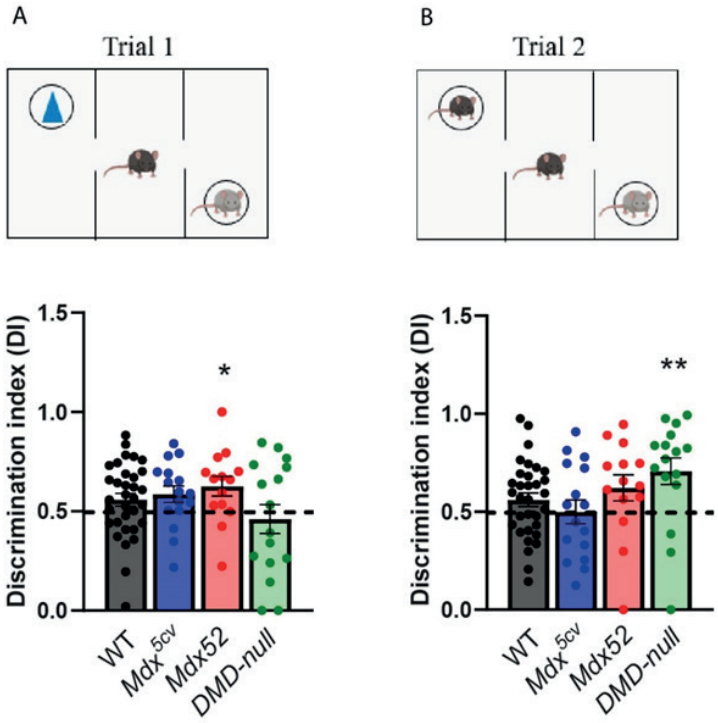

Figure S4: 3 chamber social interaction test. *Mdx^{5cv} (n = 16), mdx52 (n = 15), DMD-null (n = 16) and WT mice (n = 33). A) WT mice did not show a significant discrimination index (above chance level). No differences were found between groups in social preference. Mdx52 mice showed a preference towards social interaction over object interaction when compared to chance level (P = 0.024), but no conclusions can be drawn from this. B) Again, no performance above chance level was observed for the WT mice. No differences were found between groups in terms of social novelty seeking. DMD-null mice did show a preference towards the novel social interaction compared to familiar social interaction, when compared to chance level (P = 0.008). Dashed lines represent the chance level. Cartoons were created with BioRender. *: P < 0.05, **: P < 0.01.*

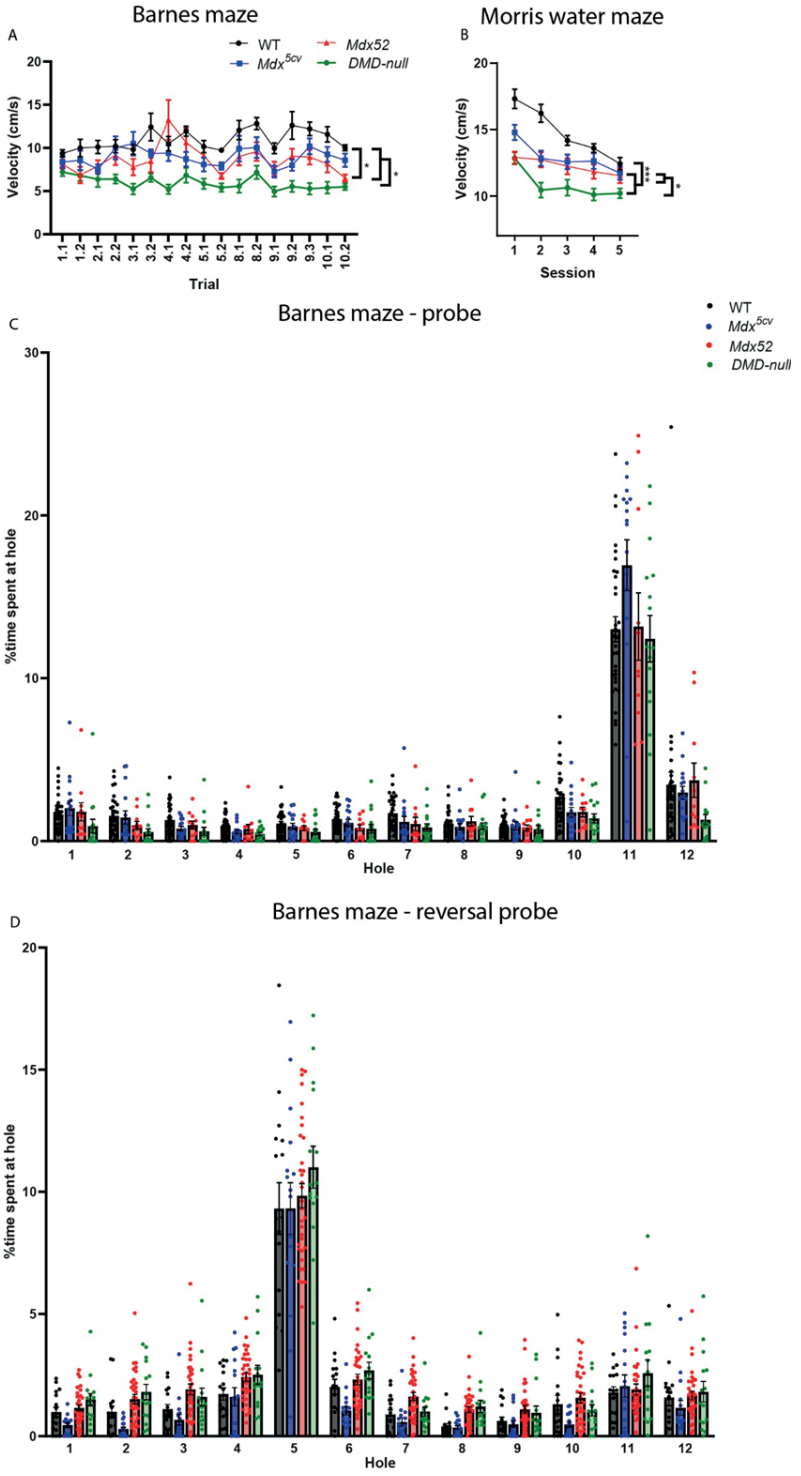
Barnes maze
A
WT
Mdx5cv
Mdx52
DMD-null
Velocity (cm/s)
Trial
Morris water maze
B
Session
Barnes maze - probe
C
%time spent at hole
Hole
Barnes maze - reversal probe
D

Figure S5: Morris water maze and Barnes maze velocity and interaction time. *Mdx^{5cv} (n = 16), mdx52 (n = 13-15), DMD-null (n = 11-16) and WT mice (n = 33). A) While navigating the Barnes maze, DMD-null mice walked slower compared to WT, mdx^{5cv} and mdx52 mice (P < 0.001, P = 0.010 & P = 0.019 respectively). Mdx52 mice displayed a lower walking velocity compared to WTs (P = 0.045). B) In the Morris water maze, mdx^{5cv}, mdx52 and DMD-null mice all swam slower compared to WT mice (all P < 0.001). DMD-null mice also swam slower than mdx^{5cv} and mdx52 mice (P < 0.001 & P = 0.017 respectively). C-D) Percentage of time spent at each hole was calculated for probe (C) and reversal probe trials (D). No differences were found between groups in interaction times with the target hole during the probe (hole 11) and reversal probe (hole 5) trial of the Barnes maze test. *: P < 0.05, ***: P < 0.001.*

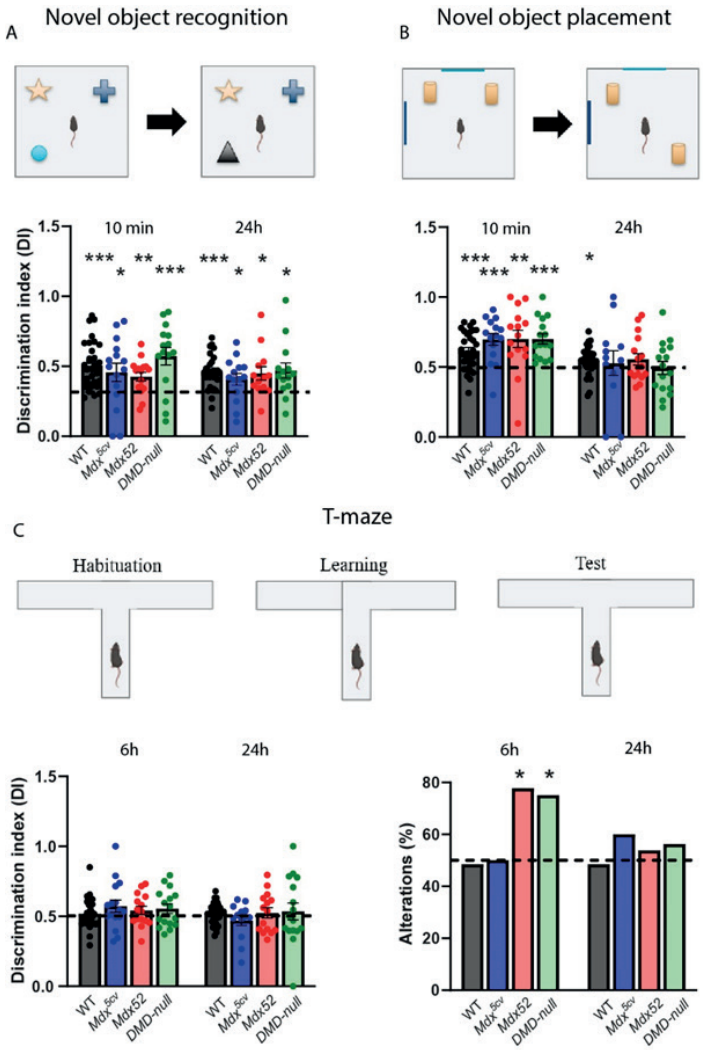

Figure S6: Recognition memory during the novel object recognition task, the object placement task and the T-maze. *Mdx^{5cv} (n = 16), mdx52 (n = 15), DMD-null (n = 16) and WT mice (n = 33). A) Discrimination index of the novel object recognition task did not differ between groups at either the 10 minute or 24h delay. Almost all groups performed above chance level (0.33) during the 10 minute (WT: P < 0.001, mdx^{5cv}: P = 0.036, mdx52: P = 0.004, DMD-null: P < 0.001) and 24h delays (WT: P < 0.001, mdx^{5cv}: P = 0.044, mdx52: P = 0.015, DMD-null: P = 0.011). B) No differences found between groups for the DI during the object placement task at either the 10 minute or 24h delay. All mice performed above chance level (0.5) after the 10 minute delay (WT: P < 0.001, mdx^{5cv}: P < 0.001, mdx52: P = 0.003, DMD-null: P < 0.001). Only WT mice performed above chance level after the 24h delay (P = 0.014). C) No differences were found between the groups at either the 6h or 24h delay in the T-maze in terms of DI values or alternations. None of the groups differed from chance level (0.5). Mdx52 and DMD-null mice alternation levels were above chance level (P = 0.02 & P = 0.046 respectively). Dashed lines depict the chance level. Cartoons were created with BioRender. *: P < 0.05, **: P < 0.01 ***: P < 0.001.*

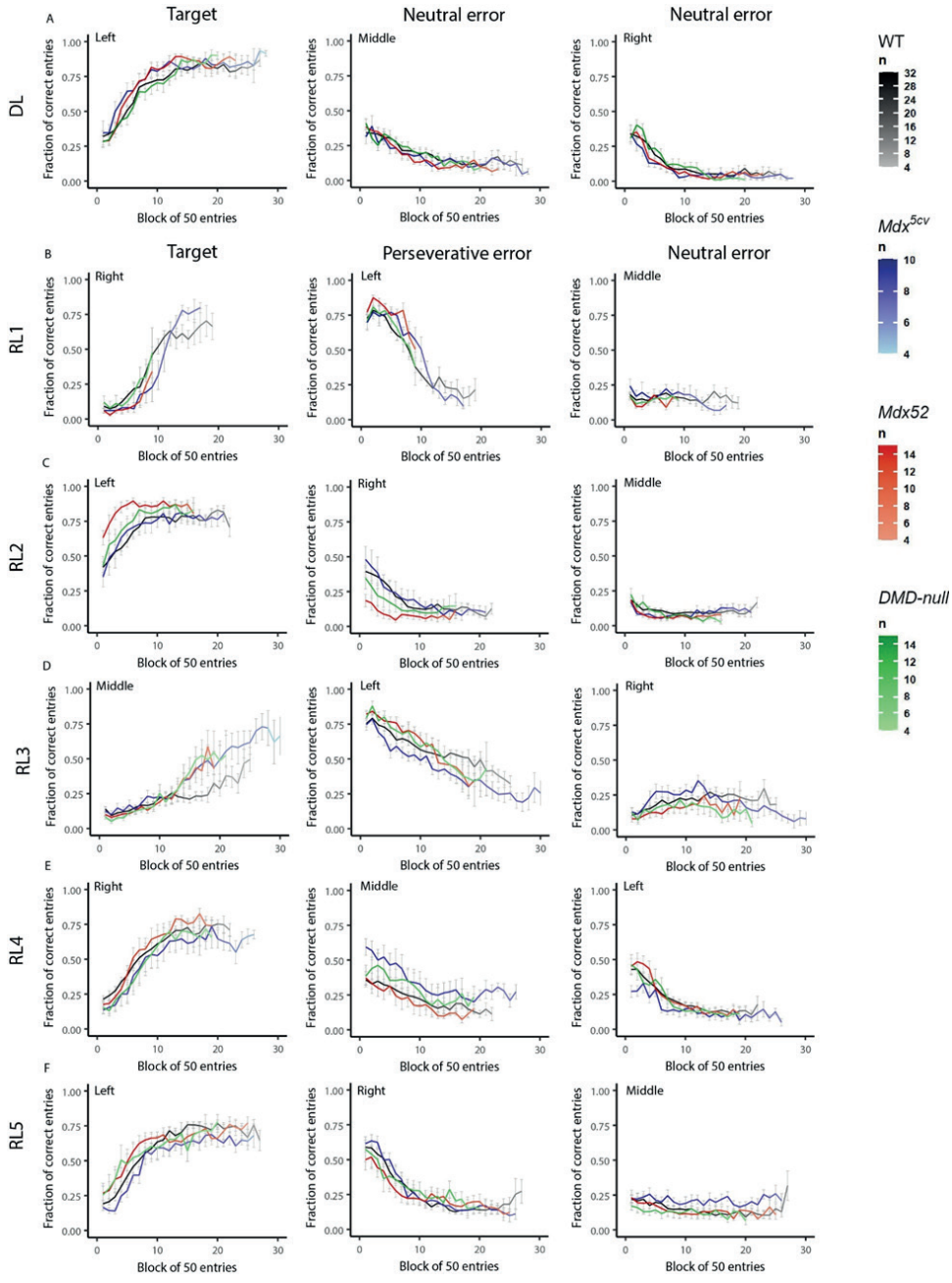

Figure S7: Perseverative and neutral error curves during serial reversal tasks. *Mdx^{5cv} (n = 9-10), mdx52 (n = 14), DMD-null (n = 10-15) and WT mice (n = 32-33). Statistical tests were only performed for strains that showed deviations in learning curve of the target hole. A) Fraction of correct entries per hole. Mdx^{5cv} and mdx52*

mice showed increased performance during initial discrimination learning compared to WT mice (P < 0.001 and P = 0.011 respectively) and DMD-null mice (P < 0.001 and P = 0.018 respectively). Mdx^{5cv} mice showed less preference for both the middle and right holes compared to WT (P = 0.002 and P < 0.001 respectively) and DMD-null mice (both P < 0.001). Mdx52 mice showed less preference for the right hole compared to DMD-null mice (P = 0.007). B) Mdx^{5cv} mice had a delayed learning curve for the initial reversal learning of the target. No differences were found in perseverative or neutral errors. C) Mdx52 mice started the second reversal day with a higher success rate compared to WT and mdx^{5cv} mice (P < 0.001 and P = 0013 respectively). Additionally, mdx52 mice showed a decrease in the perseverative error curve compared to WT and mdx^{5cv} mice (P < 0.001 and P = 0.009 respectively) and a decrease in neutral errors compared to WTs (P = 0.021). D) Mdx52 mice showed increased performance compared to WT mice (P = 0.018). Mdx52 mice showed decreased perseverative errors compared to WT mice (P = 0.003), while mdx^{5cv} mice showed decreased neutral errors compared to WT mice (P = 0.016). E) No significant differences were found between strains on reversal day 4. F) No significant differences were found between strains on reversal day 5.

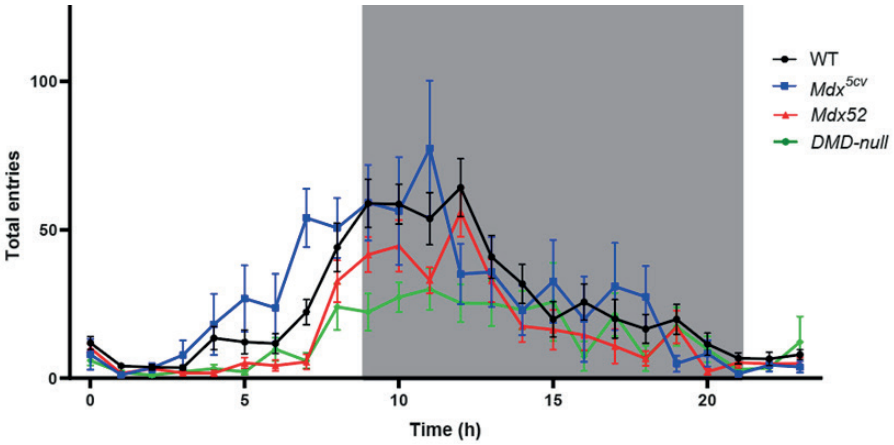

Figure S8: Amount of entries per hour during RL1. *Mdx^{5cv} (n = 10), mdx52 (n = 14), DMD-null (n = 10) and WT mice (n = 32). No statistics were performed. None of the groups showed any abrupt changes in activity over the 24h time period. Shaded area represents the dark phase.*

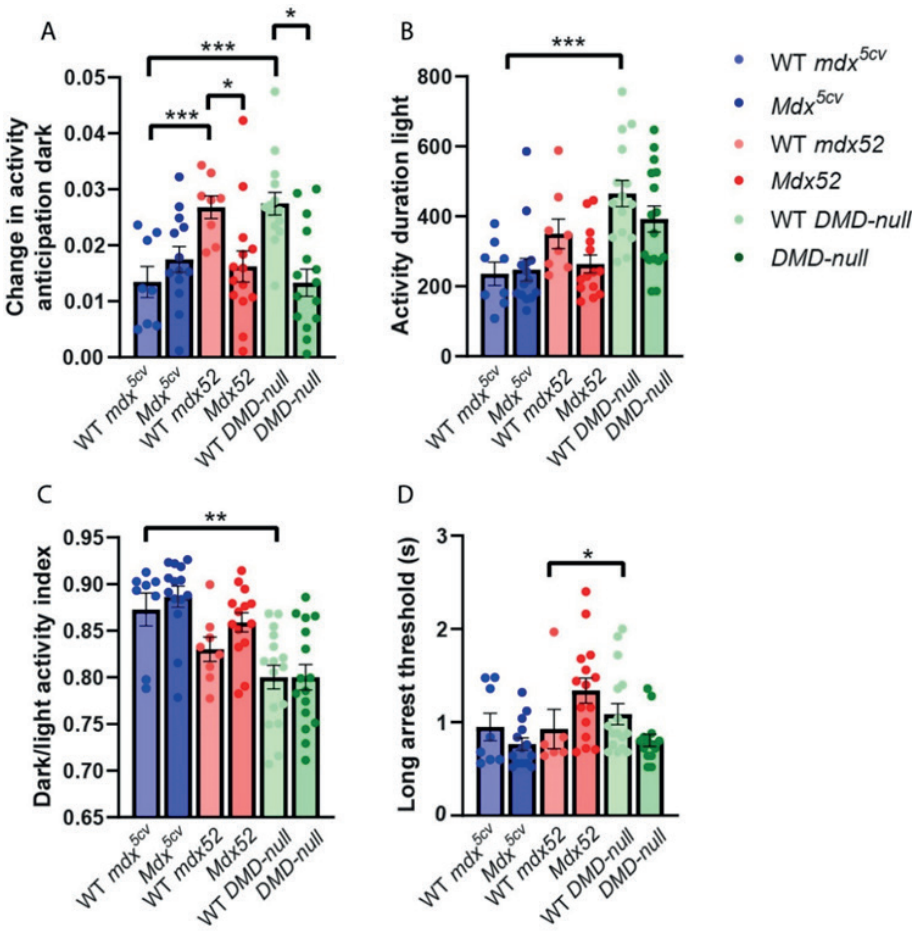

Figure S9: Spontaneous behavior parameters in which the WT groups differed from each other. *Mdx^{5cv} (n = 14), mdx52 (n = 14), DMD-null (n = 16), mdx^{5cv} WT (n = 8), mdx52 WT (n=9) and DMD-null WT mice (n=16) . A) DMD-null WTs and mdx52 WTs showed less change in activity in anticipation of the dark phase compared to mdx^{5cv} WTs (both P < 0.001). Mdx52 and DMD-null mice both showed less change in activity in anticipation of the dark phase compared to their corresponding WT groups (P = 0.040 & P = 0.020 respectively). B) DMD-null WTs were less active compared to mdx^{5cv} WTs (P < 0.001). C) The dark/light index was significantly lower in DMD-null WTs compared to mdx^{5cv} WTs (P = 0.004). D) The long arrest threshold was increased in DMD-null WTs compared to mdx52 WTs (P = 0.021). *: P < 0.05, **: P < 0.01, ***: P < 0.001.*