



Startle responses in Duchenne muscular dystrophy: a novel biomarker of brain dystrophin deficiency

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Duchenne muscular dystrophy (DMD) is characterized by loss of dystrophin in muscle, however patients also have variable degree of intellectual disability and neurobehavioural co-morbidities. In contrast to muscle, in which a single full-length dystrophin isoform (Dp427) is produced, multiple isoforms are produced in the brain, and their deficiency accounts for the variability of CNS manifestations, with increased risk of comorbidities in patients carrying mutations affecting the 3' end of the gene, which disrupt expression of shorter Dp140 and Dp71 isoforms. A mouse model (*mdx* mouse) lacks Dp427 in muscle and CNS and exhibits exaggerated startle responses to threat, linked to the deficiency of dystrophin in limbic structures such as the amygdala, which normalize with postnatal brain dystrophin-restoration therapies. A pathological startle response is not a recognized feature of DMD, and its characterization has implications for improved clinical management and translational research.

To investigate startle responses in DMD, we used a novel fear-conditioning task in an observational study of 56 males aged 7–12 years (31 affected boys, mean age 9.7 ± 1.8 years; 25 controls, mean age 9.6 ± 1.4 years). Trials of two neutral visual stimuli were presented to participants: one 'safe' cue presented alone; one 'threat' cue paired with an aversive noise to enable conditioning of physiological startle responses (skin conductance response and heart rate). Retention of conditioned physiological responses was subsequently tested by presenting both cues without the aversive noise in an 'Extinction' phase. Primary outcomes were the initial unconditioned skin conductance and change in heart rate responses to the aversive 'threat' and acquisition and retention of conditioned responses after conditioning. Secondary and exploratory outcomes were neuropsychological measures and genotype associations.

The mean unconditioned skin conductance response was greater in the DMD group than controls [mean difference $3.0 \mu\text{S}$ (1.0, 5.1); $P = 0.004$], associated with a significant threat-induced bradycardia only in the patient group [mean difference -8.7 bpm ($-16.9, -0.51$); $P = 0.04$]. Participants with DMD found the task more aversive than controls, with increased early termination rates during the Extinction phase (26% of DMD group versus 0% of controls; $P = 0.007$). This study provides the first evidence that boys with DMD show similar increased unconditioned startle responses to threat to the *mdx* mouse, which in the mouse respond to brain dystrophin restoration. Our study provides new insights into the neurobiology underlying the complex neuropsychiatric co-morbidities in DMD and defines an objective measure of this CNS phenotype, which will be valuable for future CNS-targeted dystrophin-restoration studies.

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Introduction

Duchenne muscular dystrophy (DMD) is an X-linked, life-limiting, progressive neuromuscular disorder with onset in early childhood, caused by mutations in the *DMD* gene encoding the protein dystrophin.¹ The *DMD* gene is large (2.2 Mb) and comprises 79 exons. It contains seven promoters for different dystrophin isoforms whose expression starts at different exons in the gene: three full-length 427 kD isoforms (muscle, Dp427m; cerebral, Dp427c; Purkinje Dp427p, where 427 represents the molecular weight of the protein) and five shorter isoforms (Dp260, Dp140, Dp116 and Dp71, with its splice variant isoform Dp40), that exhibit developmental, regional and cell-type specificity within different tissues (Fig. 1).² In the human brain, the full-length isoforms Dp427m and Dp427c are present throughout the cortex and basal ganglia with the highest expression in the hippocampus and amygdala and lowest in the cerebellum, whereas Dp427p is expressed at lower levels compared to the remaining full length isoforms.^{3,4} The shorter Dp140, Dp71 and Dp40 isoforms are also expressed in the brain, whilst Dp260 and Dp116 isoforms are not.³ Dp140 is expressed at higher levels during development, with lower expression throughout adulthood, and Dp71 and Dp40 are alternative spliced products of the same promoter, with Dp71 being ubiquitously expressed.^{3,5}

In all people with DMD, loss-of-function *DMD* gene mutations cause deficiency of full-length Dp427 dystrophin, leading to progressive skeletal muscle and cardiac dysfunction. The location of the mutation determines whether expression of shorter dystrophin isoforms is preserved or lost (Fig. 1). Mutations upstream of exon 45 (towards the 5' end of the gene) do not affect expression of the Dp140 isoform, whilst those downstream of exon 50 cause loss of Dp140 expression. Mutations within exons 45–50 have an indeterminate effect on Dp140 expression, due to a long untranslated region (UTR) encompassing these exons. Distal *DMD* mutations, from exon 63 to the 3' C-terminal end, affect expression of the Dp71 isoform as well as Dp140.

Approximately half of individuals with DMD are affected by a complex neuropsychiatric phenotype, which can include intellectual disability, language delay, autism spectrum disorder (ASD), attention-deficit-hyperactivity disorder (ADHD), emotional disorders and obsessive-compulsive disorder.^{6,7} Intellectual disability is strongly related to mutations disrupting expression of Dp140 and Dp71 isoforms. Cumulative loss of these isoforms is associated with increased intellectual impairment: boys deficient only in Dp427 have a mean IQ of 96; those also lacking Dp140 have a mean IQ of <75; the mean IQ for those lacking Dp140 and Dp71 is <50.^{8–10} Some previous studies have suggested an increased risk

of emotional, behavioural and neurodevelopmental disorders in individuals lacking the shorter isoforms, including an increased risk of internalizing problems (encompassing anxiety and depressive symptoms), but these findings vary across studies.^{11–13} These isoform-related differences, along with the heterogeneous nature of the neuropsychiatric phenotype in DMD, suggest that several pathophysiological mechanisms are involved.

A mouse model of DMD, the *mdx* mouse, lacks full-length Dp427 but retains shorter isoforms due to a nonsense mutation in exon 23 of the *Dmd* gene.¹⁴ The CNS phenotype of the *mdx* mouse includes impairments in long-term spatial and recognition memory, learning, cognitive flexibility and social communication,^{15–18} as well as increased fearfulness and stress reactivity, such as enhanced defensive 'freezing' startle responses during mild behavioural stress¹⁹ or foot shock.²⁰ Recent work comparing the *mdx* mouse with a mouse model lacking both Dp427 and Dp140 (*mdx52*) found no differences in startle responses between the *mdx* and *mdx52* mice, although *mdx52* mice showed increased anxiety behaviours.²¹ The parallels between the CNS phenotype in the *mdx* mouse and humans with DMD suggest that much of this enhanced defensive behaviour is due to loss of full-length Dp427 dystrophin and that Dp140 deficiency may enhance this pathological phenotype.

In wild-type mice, Dp427 dystrophin co-localizes with a subset of GABA_A-receptors in the cerebral cortex, amygdala, hippocampus and cerebellum.²⁰ GABA_A-receptor distribution is decreased in *mdx* mouse amygdalae and a recent human SPECT study also demonstrated a reduction of GABA_A-receptors in the prefrontal cortex of adults with DMD.²² The Dp140 isoform is closely co-expressed with genes involved in early neurodevelopmental processes in humans.³ Murine studies demonstrate the role of Dp71 in glutamatergic transmission and glial-dependent extracellular ion homeostasis, and Dp40 might play a crucial role in presynaptic functions in the brain.²³

The reduced GABA_A-receptor density in the hippocampus and amygdala is considered central to the pathological fear responses of the *mdx* mouse.^{24,25} Postnatal cerebral dystrophin restoration in *mdx* mice can be achieved using antisense oligonucleotides (AON), which induce the skipping of the mutation-carrying exon 23 to cause an in-frame deletion that allows a shortened but functional protein to be produced. Several studies have shown that this technique can restore both dystrophin expression in the amygdala and postsynaptic GABA_A-receptor density and normalize the pathological fear response.^{20,26} Systemic AON therapies that induce exon-'skipping' to convert out-of-frame *DMD* mutations into 'in-frame' mutations that allow the production of shortened but

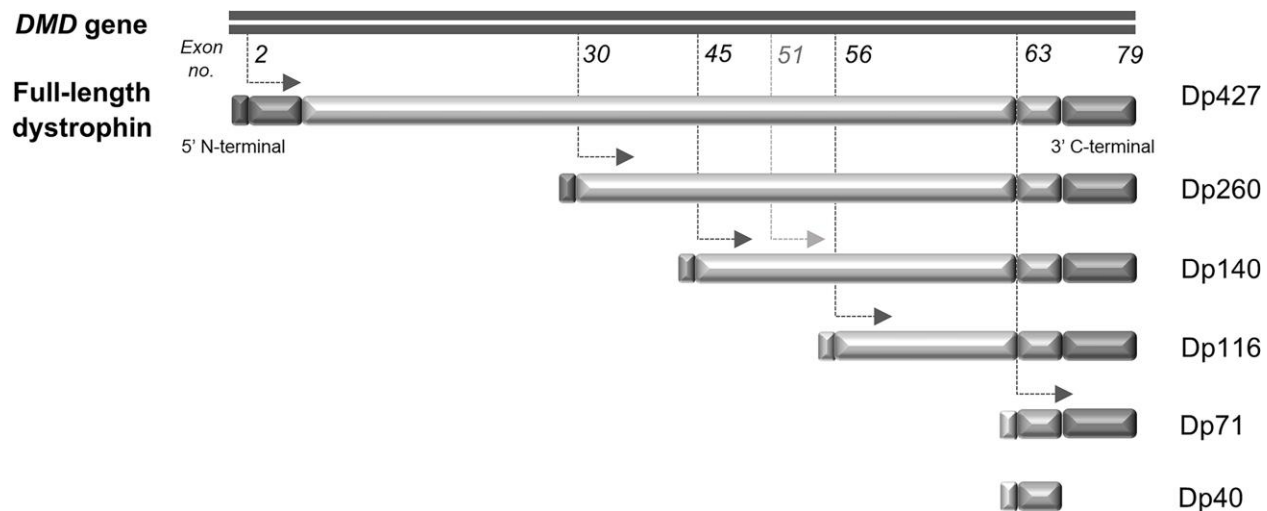


Figure 1 Schematic representation of DMD genomic organization. The DMD gene, which encodes the protein dystrophin, is located on Xp21.2 and comprises 79 exons and seven promoters linked to unique first exons. Dotted arrows indicate the splice sites of these different internal promoters, which splice into the indicated exons (or preceding introns in the case of Dp140) to generate multiple dystrophin isoforms, shown in succession below the full-length dystrophin protein. The isoforms are named by their size in kDa. The three 427 kDa isoforms are Dp427m (muscle), Dp427c (cerebral) and Dp427p (Purkinje); shorter isoforms are Dp260, Dp140, Dp116 and Dp71, the latter of which can be further alternatively spliced to form a 40 kDa Dp40 isoform. Each isoform has a different 5' N-terminal domain, and all retain the same 3' C-terminal domain. Numbers in italics indicate the exon number. In the case of the Dp140 isoform, transcription starts at intron 44 (just upstream of exon 45) but translation of the protein does not start until exon 51 (light grey arrow), leading to a long UTR from intron 44–exon 50. It is difficult to predict the effect on Dp140 expression of mutations in this untranslated region. Adapted from figure in Muntoni et al.²

functional proteins are in current clinical trials or approved therapies in some countries.²⁷ These AONs do not cross the blood–brain barrier, although intrathecal administration would potentially allow brain dystrophin restoration.

These findings raise the intriguing possibility that AON therapies directed to restoring expression of brain dystrophin could also address some of the CNS comorbidities in DMD. Given the increasing life-expectancy in DMD with current standards of care and emerging therapies, improving neuropsychiatric symptoms could significantly benefit quality of life. In order for any therapy to be evaluated, outcome measures are needed to evaluate the effectiveness of dystrophin-restoration. The reversibility of the startle response in the *mdx* mouse after dystrophin-restoration therapy led us to propose that a similar phenotype may be present in boys with DMD. However, startle responses have not previously been investigated in DMD.

Anxiety disorders, particularly the fear-based disorders (such as social and separation anxiety, panic disorder and specific phobias) are associated with increased startle responses to unpredictable threat, which is thought to relate to hyperarousal and exaggerated anticipatory anxiety.^{28,29} Heightened reactivity to unpredictable threat has been implicated in the onset, maintenance and treatment response in fear-based anxiety disorders.^{30,31} Threat-induced anxiety symptoms can impact on executive skills, such as processing efficiency and cognitive flexibility, thus potentially affecting daily adaptive functioning.^{32,33} Anxiety symptoms are reported in 24–33% of people with DMD,^{12,34,35} and work from our group found increased fear-based anxiety symptoms (social and separation anxiety) in DMD compared to the typical population.³⁶ Therefore, assessment of behavioural startle responses can provide an objective measure of anxiety.

Behavioural startle responses can be investigated using experimental fear-conditioning paradigms, where exposure to a threat stimulus causes an 'innate' or unconditioned physiological

response. If a neutral stimulus is paired with the 'threat' stimulus, the physiological response becomes conditioned to occur with subsequent presentations of the neutral stimulus.³⁷ We hypothesized that when tested using a fear-conditioning task, individuals with DMD would have heightened startle responses compared to control participants. We used a novel discrimination fear-conditioning task, which paired an aversive loud noise 'threat' stimulus with a neutral visual stimulus to elicit physiological startle responses that became conditioned to the neutral stimulus. These conditioned responses subsequently extinguished when the 'threat' was removed. Whilst this method of assessing psychophysiological responses is more suited to experimental than clinical settings, it provides both insight into the underlying neurobiological circuits involved in emotional disorders and the potential to measure the effects of therapeutic interventions.^{31,38} As an objective measure the startle response also has advantages over instruments more prone to subjectivity and bias, such as neuropsychological assessments and self-/informant-report neuropsychiatric symptom scales.^{39,40}

Our aims were to determine whether group differences existed between DMD and control groups for the magnitude of unconditioned startle responses to threat, conditioned response acquisition and the degree of retention and extinction of conditioned responses. We also investigated associations of startle responses with anxiety and other neuropsychiatric symptoms and conducted an exploratory analysis of the influence of the Dp140 isoform on these outcomes.

Materials and methods

Study design and setting

This observational cross-sectional study compared startle responses in young males with DMD with an age-matched male

control group using a fear-conditioning task. The study was conducted from February–November 2019 at Great Ormond Street Hospital for Children (GOSH) and UCL Great Ormond Street Institute of Child Health.

Participants

DMD and Control participants were recruited via: GOSH outpatient clinics (patients or siblings); a research database; advocacy groups; and advertising to UCL staff and students. Universal eligibility criteria were male gender, age 7–12 years and no significant visual or hearing impairment or noise-sensitivity. DMD-specific criteria were a genetic diagnosis of DMD, not receiving ataluren (dystrophin-modulating therapy that may cross the blood–brain barrier); control-specific criteria were no neurological or psychiatric diagnosis. Informed written consent (parents) and assent (participants) was obtained according to the Declaration of Helsinki. All participants were allocated unique identifiers at recruitment. Ethical approval was granted by the Health Research Authority, London Bridge Research Ethics Committee (18.LO.1575).

Demographic and medical information was documented using a proforma: co-morbidities, medication, motor function, genotype (DMD), pubertal status and socio-economic status estimation.^{41,42}

Task parameters

To determine physiological startle responses, we used a novel fear-conditioning task (Fig. 2) detailed in our previous work.⁴³ Neutral visual conditioned stimuli (CS–, ‘safe’ cue and CS+, ‘threat’ cue) were coloured squares presented on a computer screen for 6 s. An aversive auditory unconditioned stimulus (UCS; white noise,

approximately 85 dB) was presented binaurally through headphones with onset 5 s after CS+, duration 1 s and co-terminating with CS+. Four phases comprised: pre-task calibration; familiarization (CS+ and CS– presented with no aversive UCS; eight trials); acquisition (CS+ paired with UCS and CS– alone; three blocks of eight trials); and extinction (CS+ and CS– alone; five blocks of eight trials; occurring at least 1 h after acquisition). CS+/CS– order was pseudo-randomized, CS colour was randomly counterbalanced amongst participants and 10/12 CS+ trials were reinforced with UCS (~80% reinforcement schedule).

Electrodermal activity (EDA) was recorded via surface skin electrodes on the non-dominant hand and ECG via standard three-lead electrodes. A Biopac MP160 unit recorded physiological parameters using AcqKnowledge 5.0.3 software (Biopac Systems, Inc., Aero Camino, CA).

Full-scale intelligence quotient (FSIQ) was assessed using the Wechsler Abbreviated Scale of Intelligence-II (WASI-II)⁴⁴ that uses verbal and non-verbal reasoning tests without working memory and executive function tasks, which can be more impaired in DMD and thus could confound general IQ estimation.⁴⁵ Participants’ parent/carers completed neuropsychiatric symptom questionnaires: anxiety (Screen for Child Anxiety Related Emotional Disorders, SCARED)⁴⁶; internalizing and externalizing problems (Child Behavior Checklist, CBCL)⁴⁷; social communication problems (Social Communication Disorder Checklist, SCDC)⁴⁸; and inattention and hyperactivity (Conners’ Parent Rating Scale—Revised, CPRS-R).⁴⁹ Several control group scores were higher than published population mean scores (FSIQ mean difference 14.0, $P < 0.001$); hyperactivity ($P = 0.047$) (Supplementary Table 1); therefore, we also compared DMD scores against age-/gender-matched normative data for each scale.^{44,47,49–51}

DMD participants were stratified into subgroups determined by whether the mutation site predicted expression of shorter dystrophin isoforms: upstream of intron 44 predicts absent Dp427/intact Dp140 (‘Dp140+’); intron 44 to exon 50 predicts absent Dp427/indeterminate Dp140 expression (depending whether Dp140 promoter/long 5’UTR are affected; ‘Dp140_unk’); exon 51 to 62 predicts absent Dp427/Dp140 (‘Dp140–’); and exon 63 to 79 predicts absent Dp427/Dp140/Dp71 (‘Dp140–/71–’).

A subgroup of DMD participants ($n = 11$) repeated the fear-conditioning task after 3 months, confirming the test-retest reliability of the skin conductance response (results previously reported).⁴³

Data processing

A detailed description of data processing parameters is available in our previous work.⁴³ EDA and ECG data were extracted from 0–12 s post-CS onset, and EDA alone from 0–6 s (First Interval Response, FIR) and 6–12 s (Second Interval Response, SIR) windows in acquisition to evaluate conditioned response acquisition and unconditioned response habituation, respectively. EDA represents the absolute skin conductance level (SCL) in microSiemens (μS), from which the skin conduction response (SCR) amplitude is derived: $SCR = (SCL_{max}) - (SCL_{base})$. SCL_{base} is baseline SCL at start of the trial, SCL_{max} is maximal SCL after CS onset. SCR metrics were defined for CS+ trials (SCR_{CS+}), CS– trials (SCR_{CS-}) and differential SCR, $SCR_{Diff} = SCR_{CS+} - SCR_{CS-}$ in contiguous trials. All SCRs were visually inspected for artefacts by a blinded researcher, cross-referencing digital event markers. Where artefacts affected data, responses were re-measured manually wherever possible, and if the SCR was completely obscured by artefacts, these data were excluded

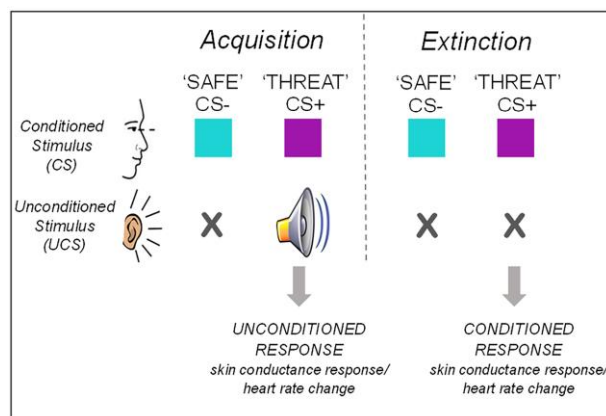


Figure 2 Schematic representation of the fear-conditioning task. Two different neutral stimuli, the CS, are presented to the subject in randomized trials during the response acquisition phase (‘Acquisition’). One is a ‘threat’ cue (CS+), which is paired with an aversive noise stimulus, the UCS. A ‘safe’ cue (CS–) is presented alone with no UCS. At the initial presentation of the UCS, behavioural and physiological responses, termed ‘unconditioned responses’, are elicited. With repeated presentation of trials in the Acquisition phase, a learned association develops between the CS+ cue and the unconditioned response, which then becomes ‘conditioned’. After 1–2 h, an Extinction phase is conducted in which both CS+ and CS– cues are presented, but with no UCS. The CS+ cue initially elicits the conditioned response, but after repeated trials not reinforced by the UCS, this association is extinguished and the CS+ cue no longer elicits the conditioned response, termed extinction. Prior to the Acquisition phase, a pre-task Calibration phase and Familiarization phase also occur, to allow physiological calibration manoeuvres, habituation to neutral stimuli and baseline measurements.

(see Maresh et al.⁴³ for further details). All exclusions were reviewed independently by another researcher. Non-responders were defined as participants with SCR <0.01 μ S in pre-task calibration manoeuvres and in $\geq 50\%$ CS+/UCS trials, based on published recommendations.⁵² The non-responder rate was 1.9% ($n=1$), lower than the quoted SCR non-responder rate of 10%.⁵³

ECG data were transformed into R-R intervals (in seconds), the time interval between each heartbeat, where 'R' is the maximal positive peak of the ECG complex. Heart rate (HR) $\text{HR} = 60/(\text{R-R interval})$ beats per minute (bpm). Heart rate in CS+ trials = $\text{HR}_{\text{CS+}}$; heart rate in CS- trials = $\text{HR}_{\text{CS-}}$. Change in HR (ΔHR) = (mean HR in sampling window) – (mean baseline HR), where baseline is mean HR in the familiarization phase.

Statistical considerations

The sample size ($n=56$, DMD $n=31$, controls $n=25$) was determined primarily from previous literature and pragmatic considerations rather than formal power calculations, as there were no previous studies in this clinical population and measuring these outcomes. Previous similar studies have examined fear responses in anxious versus non-anxious children, with $n=35$ (subgroups $n=17/18$)⁵⁴; $n=54$ (subgroups $n=16/38$)⁵⁵; $n=60$ (subgroups $n=8/11/19/16$)⁵⁶. As an additional guide from healthy paediatric data,⁵⁷ a sample size of $n=56$ ($n=28$ per group) predicts 80% power to detect clinically important differences, although does not account for a healthy versus clinical population; therefore this pilot aimed to gather data in this population to guide future studies. Based on these considerations, we predicted the study had adequate power to detect significant differences in skin conductance responses for DMD versus control group comparisons, although was not powered to detect differences in the exploratory analyses of Dp140+ versus Dp140- subgroup comparisons.

Analysis was conducted by a researcher blinded to participants' group and performed using IBM SPSSv.26, with a two-tailed significance level, alpha, of $P=0.05$ and, where appropriate, Bonferroni adjustment for multiple comparisons.

Neuropsychiatric mean scores were compared with two-tailed independent samples t -tests. Chi-square tests compared task completion status. Analysis of block data used linear mixed model analysis, accounting for missing/unbalanced data, for (i) SCR_{Diff} and ΔHR startle response metrics (fixed effects = Group (DMD, control)/Block; random effect = StudyID; Group \times Block pairwise comparisons); (ii) $\text{SCR}_{\text{CS+}}$ and $\text{SCR}_{\text{CS-}}$ in Extinction blocks (fixed effects = Group/Block/Stimulus (CS+/CS-); random effect = StudyID). Conditioned response extinction was defined as no significant discrimination between $\text{SCR}_{\text{CS+}}$ and $\text{SCR}_{\text{CS-}}$. The Residual Maximum Likelihood Estimation method was used with 'Unstructured' covariance structure.

IQ differed significantly between the DMD and Control groups ($P<0.001$), Dp140+ and Dp140- groups ($P=0.03$), and at the combined group level, IQ was significantly negatively correlated with anxiety, internalizing, externalizing, social communication and inattention symptoms, and moderately correlated with SCR_{UC} in the Control group ($\rho=0.39$, $P=0.06$). Therefore, to avoid confounding, we adjusted the analysis of primary outcomes for IQ (group differences in startle responses and association of startle responses with neuropsychiatric measures).

Between-groups univariate ANOVA evaluated primary physiological outcomes (unconditioned responses to first reinforced CS+ trial, SCR_{UC} , $\Delta\text{HR}_{\text{UC}}$; conditioned SCR to first Extinction CS+ trial, SCR_{EXT}), and within-groups repeated measures ANOVA evaluated

$\Delta\text{HR}_{\text{UC}}$, taking IQ as a covariate. Between-group effect sizes, η^2 were derived from univariate ANOVA output; within-group effect sizes were calculated from within-group general linear model analysis, where $\eta^2 = (\text{treatment sum of squares})/(\text{total sum of squares})$ with outcomes $\eta^2 \geq 0.01$ (small effect), $\eta^2 \geq 0.06$ (medium effect); and $\eta^2 \geq 0.14$ (large effect). Partial correlations (controlling for IQ) evaluated neuropsychiatric scores and startle response metrics associations.

A sensitivity analysis was conducted for primary outcome metrics to account for missing data, excluding 'Non-completer' participants who terminated the task early (completing <4/5 Extinction blocks) (Supplementary Table 2), showing no change in statistical significance for all the primary SCR metrics (unconditioned and conditioned $\text{SCR}_{\text{CS+}}$) and an increase in significance for $\Delta\text{HR}_{\text{UC}}$, thus justifying use of the full dataset. A further sensitivity analysis for HR metrics excluded one DMD participant taking a beta-blocker (which can lower HR), which showed no difference in outcomes (Supplementary Table 2).

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary material. In view of the rarity of DMD and that genotype data is included, only aggregated data is available on reasonable request.

Results

Participants

Fifty-six participants (31 DMD and 25 Controls) took part in the study from the 63 initially recruited (Supplementary Fig. 1). Groups were age-matched [DMD 9.6 years (SD 1.4); Controls 9.7 years (SD 1.8)] and did not differ in mean pubertal stage or socioeconomic status (Table 1). In the DMD group, 29/31 were ambulant (mean NorthStar score 21.2/34), 30/31 were taking corticosteroid treatment and 7/31 were taking cardiac medication as part of routine DMD treatment. Four DMD participants had diagnosed ASD and one ADHD.

Of the 56 physiological recording data sets, four were excluded from SCR analysis due to: physiological 'Non-responder' (DMD $n=1$); protocol deviation (control $n=1$); and technical equipment problems (DMD $n=2$). No control participants requested early task termination compared to eight 'Non-completer' DMD participants ($P=0.007$), of whom two were Dp140+ participants (2/12), five were Dp140- (5/11) and one was Dp140-/71- (1/1) (Table 1). There were no differences in other characteristics of the 'Completer' and 'Non-completer' groups (Supplementary Fig. 2 and Supplementary Table 3). Data exclusion due to artefacts affected 3.8% Control and 7.5% DMD Acquisition trials and 16.5% Control and 21.3% DMD Extinction trials (Supplementary Fig. 2).

The impact of dystrophin deficiency on neuropsychological profile

Dp427 deficiency: DMD versus Controls

We evaluated emotional problems (anxiety and internalizing problems), neurodevelopmental features (IQ), social communication problems, inattention, hyperactivity and externalizing problems (Table 2). The DMD group IQ was 20–25 points lower than that of the Controls on all IQ measures, including full-scale IQ (FSIQ: DMD 90.5 versus Controls 115.4; $P<0.001$), in line with work from

Table 1 Participants' baseline demographic information and available data for each group

	Control	DMD	DMD isoform subgroups			
			Dp140+	Dp140-	Dp140-/71-	Dp140_unk
Demographics						
Participants, n	25	31	12	11	1	7
Mean age, years (SD)	9.7 (1.8)	9.6 (1.4)	9.6 (1.6)	9.6 (1.5)	–	9.8 (1.1)
Non-ambulant, n	–	2	0	2	0	0
Mean NorthStar score (0–34) ^a	–	21.2	27.4	20.4	–	14.0
Cardiac medication, n (ACEi/BB)	–	ACEi n = 7; BB n = 1	ACEi n = 2; BB n = 1	ACEi n = 3; BB n = 0	ACEi n = 0; BB n = 0	ACEi n = 2; BB n = 0
Corticosteroid treatment, n	–	30 (D = 10; I = 19; A = 1)	11 (D = 8; I = 2; A = 1)	11 (D = 6; I = 5, A = 0)	1 (I = 1)	7 (D = 4; I = 3; A = 0)
Autism spectrum disorder diagnosis	0	4	0	4	0	0
ADHD diagnosis	0	2	0	0	1	1
Mean socio-economic status rank decile (1–10) ^b	6.4	7.1	7.8	7.4	–	5.8
Mean Pubertal Stage (1–5) ^c	1.6	1.5	1.3	1.6	–	1.6
Available data						
Parent-report neuropsychiatric scores, n	25	31	12	11	1	7
Participant self-report anxiety score, n	25	30	12	11	0	7
Intelligence quotient assessment, n	25	30	12	11	0	7
Skin conductance response data, n	24	28	10	11	1	6
HR data, n	25	31	12	11	1	7
Non-completers ^d , n	0	8	2	5	1	0

A = Alternate; ACEi = angiotensin converting enzyme inhibitor; BB = beta blocker; D = Daily; I = Intermittent.

^aNorthStar score is a 17-item lower limb functional assessment scale used in DMD, scored out of 34.

^bSocio-economic status mean decile score was derived from the Index of Multiple Deprivation for England⁴¹, available for participants living in England (n = 52; DMD = 28, Control = 24).

^cPubertal stage was assessed using the parent/self-report 'Growing and Changing' questionnaire⁴². Scores range from 1 (pre-pubertal) to 5 (adult stage).

^dNon-completers were participants who terminated the task early, completing <4/5 extinction blocks.

our group and others.^{11–13} Control group FSIQ was higher than the population normative score (mean difference 14.0, $P < 0.001$), but DMD group FSIQ was also lower than age-matched normative data (mean difference –10.9, $P < 0.001$; [Supplementary Table 1](#) and [Supplementary Fig. 3](#)).

Most neuropsychiatric co-morbidity scores were higher in the DMD group than Controls and population norms, including anxiety, internalizing, externalizing, social communication and inattention symptoms, and excepting only hyperactivity. Hyperactivity scores in both Control and DMD groups were higher than the typical population ([Supplementary Table 1](#) and [Supplementary Fig. 3](#)).

In the combined cohort, there were weak-moderate significant correlations ($\rho = 0.3–0.4$) between IQ and all neuropsychiatric symptom scores apart from hyperactivity ([Supplementary Table 4](#)), but not in the separate DMD and Control groups, apart from a significant negative correlation of inattention with IQ in the DMD group.

Dp427 and Dp140 deficiency: Dp140+ versus Dp140–

Neuropsychological assessment scores for isoform subgroups, stratified by involvement of the Dp140 isoform (Dp140+, n = 12, and Dp140–, n = 11) and uncertain Dp140 expression (Dp140_unk, n = 7), are shown in [Supplementary Table 1](#) and [Supplementary Fig. 3](#). Deficiency of Dp140 was associated with lower FSIQ (Dp140+ 96.4 versus Dp140– 82.8; $P = 0.03$). Compared with the population mean, Dp140+ FSIQ was not significantly different (mean difference –5.0, 95% CI –14.0, 4.0; $P = 0.25$), whilst Dp140_unk FSIQ was lower

by 9.0 points (–13.7, –4.3; $P = 0.003$) and Dp140– FSIQ was lower by 18.6 points (–27.2, –10.0; $P < 0.001$). That the Dp140_unk FSIQ lies approximately in between Dp140+ and Dp140– FSIQ highlights that this is a mixed group regarding the expression of Dp140.

Inattention and hyperactivity scores were significantly higher in the Dp140– compared with the Dp140+ subgroup, and trends of higher anxiety and internalizing scores in Dp140– compared to Dp140+. The Dp140– subgroup also scored significantly higher compared with population means for both emotional problems (anxiety, internalizing problems) and neurodevelopmental problems (social communication problems, inattention and hyperactivity), whereas the Dp140+ subgroup had higher scores only in internalizing problems ([Supplementary Fig. 3](#) and [Supplementary Table 1](#)). Internalizing problems encompass anxiety, depression and somatic symptoms, the latter of which can lead to higher scores in children with chronic physical conditions.⁵⁸

Impact of a loss of dystrophin on psychophysiological outcomes

Baseline physiological responses

DMD versus Control

Baseline data showed similar mean SCRs in DMD and Control groups [mean difference 0.008 μ S (–0.18, 0.20), $P = 0.93$] and higher mean HR in the DMD group [mean difference 17.9 bpm (10.9, 24.8), $P < 0.001$] ([Fig. 3](#) and [Supplementary Fig. 4](#)), consistent with the well-recognized phenomenon of resting sinus tachycardia in DMD.⁵⁹

Table 2 Neuropsychological assessment mean score comparisons between groups and subgroups

Neuropsychological assessment ^a	DMD versus Control				DMD Dp140– versus Dp140+			
	Raw score, mean difference (95% CI)	t	Sig. ^b , P		Raw score, mean difference (95% CI)	t	Sig. ^b , P	
FSIQ	–24.9 (–32.5, –17.4)	–6.7	<0.001		–13.6 (–25.3, –1.9)	–2.4	0.03	
Anxiety	5.4 (0.09, 10.8)	2.0	0.046		3.1 (–5.4, 11.5)	0.8	0.46	
Internalizing problems	4.6 (1.2, 7.9)	2.7	0.009		4.3 (–1.5, 10.0)	1.6	0.14	
Externalizing problems	6.5 (0.9, 12.2)	2.3	0.02		4.3 (–7.7, 16.3)	0.7	0.47	
Social communication problems	4.8 (1.9, 7.8)	3.2	0.003		4.4 (–0.9, 9.6)	1.7	0.10	
Inattention	2.0 (0.06, 4.0)	2.1	0.04		3.9 (0.6, 7.2)	2.5	0.02	
Hyperactivity	–0.12 (–2.3, 2.1)	–0.1	0.91		3.4 (0.2, 6.7)	2.2	0.04	

^aFSIQ-4 assessed using WASI-II. The other neuropsychological/neuropsychiatric scores were obtained using SCARED-P, CBCL, SCDC and CPRS-R.

^bBetween-group comparisons performed with independent samples t-tests, showing mean difference and 95% CI. Two-tailed significance calculated using alpha level of $P = 0.05$. P -values <0.05 are highlighted in bold.

Dp140+ versus Dp140–

There was no difference in either mean baseline SCR and HR between the Dp140+ and Dp140– subgroups [baseline SCR mean difference 0.08 μ S (–0.28, 0.44), $P = 0.64$; baseline HR mean difference –7.7 bpm (–19.0, 3.7), $P = 0.18$].

Unconditioned startle responses

DMD versus Control

The mean unconditioned SCR to the initial threat stimulus (SCR_{UC}) was higher in the DMD group compared with the Controls throughout the Acquisition phase (Fig. 3), most notably on the first CS+ trial [mean difference 3.0 μ S (1.0, 5.1); $P = 0.004$; $\eta^2 = 0.16$] and first Acquisition block [mean difference 2.2 μ S (0.9, 3.5), $P = 0.001$] (Table 3 and Fig. 4).

The unconditioned change in HR after the initial threat stimulus, ΔHR_{UC} , showed a fall in mean HR of 6.1 bpm in the DMD group (–10.7, –1.6; $P = 0.006$; $\eta^2 = 0.04$), but no change in the Control group [–0.8 bpm (–7.1, 5.8); $P = 0.99$; $\eta^2 = 0.001$] (Fig. 4). There was also a significant difference in ΔHR_{UC} between groups [mean difference –8.7 bpm (–0.51, –16.9); $P = 0.04$; $\eta^2 = 0.08$] (Table 3). This indicates a bradycardic response to threat in the DMD group only, which is more notable since heart rate variability is typically reduced in the DMD population.⁶⁰

Due to the baseline group differences in IQ, all primary outcomes were adjusted for IQ (described in the 'Materials and methods' section). This adjustment did not affect the SCR_{UC} data findings, as significant group differences remaining after adjustment. ΔHR_{UC} group comparisons were only significant after adjustment. Unadjusted data are presented in Supplementary Table 5.

These data indicate that SCR_{UC} is effective in discriminating between the DMD and Control groups, and ΔHR_{UC} may be a useful secondary outcome.

Dp140+ versus Dp140–

There was no difference in unconditioned startle responses between Dp140+ and Dp140– subgroups [SCR_{UC} mean difference 0.61 μ S (–3.0, 4.2), $P = 0.73$; ΔHR_{UC} mean difference 3.5 bpm (–6.3, 13.2), $P = 0.47$].

Acquisition of conditioned responses in both groups

DMD and Control groups showed successful discrimination between 'threat' CS+ and 'safe' CS– cues in all Acquisition blocks, indicating that the paradigm was effective (Supplementary Table 6). Acquisition of the conditioned response was similar in both groups,

with no difference in differential SCR between CS+ and CS– trials, SCR_{Diff} , in the FIR window (Fig. 5A and Supplementary Table 6). This suggests similar fear learning in both groups. Habituation of the unconditioned response, indicating reduced salience of the threat stimulus with repeated presentations, was also seen in both DMD and Control groups with significant reduction in SCR_{CS+} from Acquisition block 1 to block 3 (Fig. 5B and Supplementary Table 6). Habituation is a well described phenomenon in fear-conditioning paradigms.^{61–63}

Retention and extinction of conditioned skin conductance responses

DMD versus Control

The conditioned response was not successfully retained in either the DMD or Control groups at the start of the Extinction phase, with no discrimination between SCR_{CS+} and SCR_{CS-} in the first Extinction block in either group [DMD mean difference 0.18 μ S (–0.1, 0.4), $P = 0.21$; Control mean difference 0.09 μ S (–0.2, 0.4), $P = 0.52$] and no difference in SCR_{EXT} in the first Extinction trial between DMD and Control groups [mean difference 0.37 μ S (–0.23, 0.96), $P = 0.22$] (Table 3), although there was a trend of completed extinction in the DMD group by the third block (Fig. 5C).

Dp140+ versus Dp140–

In contrast to the whole group data, the DMD Dp140– subgroup showed significant retention of the conditioned response with higher SCR_{EXT} compared to Controls [mean difference 1.1 μ S (0.2, 2.1), $P = 0.02$, $\eta^2 = 0.18$] and a clear pattern of conditioned response extinction in Fig. 5C, neither of which were seen in the Dp140+ subgroup (Table 3). The difference in SCR_{EXT} between the Dp140+ and Dp140– subgroups was not statistically significant, however this may have been due to the small group sizes [mean difference 0.84 μ S (–0.1, 1.8), $P = 0.08$, $\eta^2 = 0.18$]. Behavioural avoidance responses during Extinction were only observed in DMD participants: averting gaze during CS+ presentation and early termination in eight DMD boys (six Dp140 deficient). These findings suggest stronger conditioning in participants lacking the Dp140 isoform.

Associations between unconditioned startle responses and the neuropsychiatric phenotype

Unconditioned physiological startle responses, SCR_{UC} and ΔHR_{UC} , for the combined cohort correlated positively with Anxiety and Internalizing problems scores with SCR_{UC} (Anxiety versus SCR_{UC} : $\rho = 0.38$, $P = 0.01$; Internalizing problems versus SCR_{UC} : $\rho = 0.33$, $P =$

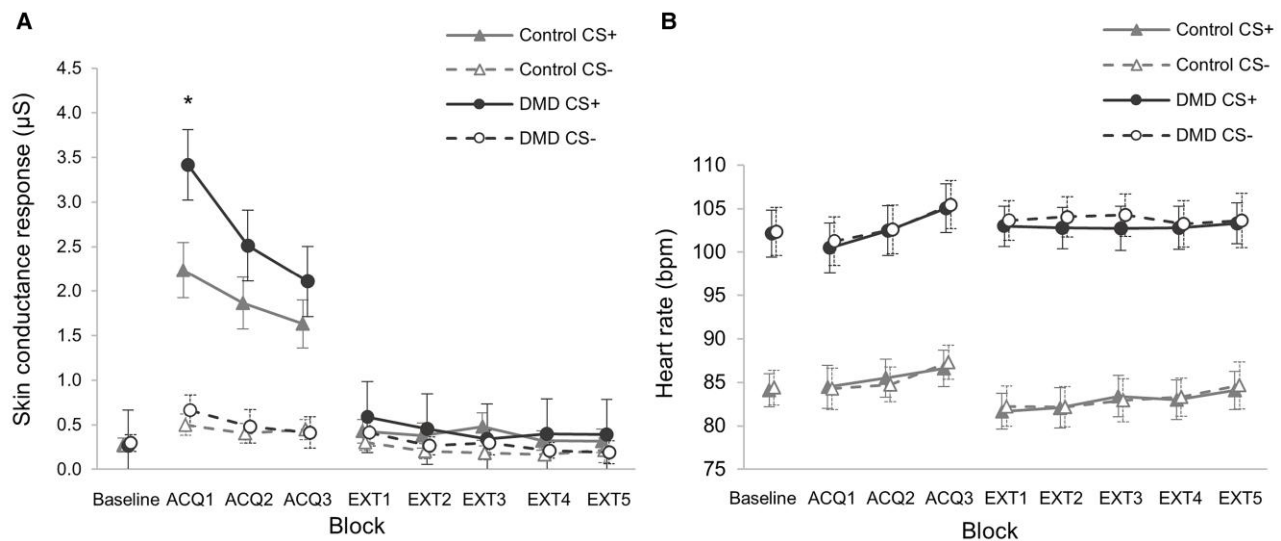


Figure 3 Mean physiological responses to ‘threat’ and ‘safe’ conditioned stimuli (CS+ and CS–) by block for all fear conditioning task phases in DMD and Control groups. (A) SCRS (measured in microSiemens, μ S). Mean SCR in first Acquisition block (ACQ1) was significantly higher in the DMD compared to the Control group (* $P=0.03$). (B) HR (bpm). SCR derived from EDA recorded from the palmar surfaces of digits 2 and 3, and defined as the baseline-to-peak EDA in the 12 s window following CS presentation. HR derived from the inter-beat interval from three-lead ECG recording. Error bars show the 95% CI.

0.03; [Supplementary Table 4](#)). At the group/subgroup level the strongest correlations occurred for Anxiety versus SCR_{UC} in the Dp140– subgroup ($\rho=0.75$, $P=0.01$), with non-significant positive correlations in the other groups (DMD: $\rho=0.37$, $P=0.08$; Dp140+: $\rho=0.32$, $P=0.40$; Control: $\rho=0.22$, $P=0.32$). This may be due to a ‘floor effect’ from lower scores in the latter groups.

In contrast, neurodevelopmental problems scores for Inattention and Hyperactivity did not correlate with SCR_{UC} , suggesting that this association is specific to anxiety alone and not related to more general psychopathology. Social communication problems scores showed positive but non-significant correlations in the combined cohort ($\rho=0.27$, $P=0.07$), most marked in the Dp140– subgroup ($\rho=0.54$, $P=0.11$), which may be a confounding effect of the well-established relationship between anxiety and social communication disorders.⁶⁴

ΔHR_{UC} did not significantly correlate with any neuropsychiatric outcomes, although we noted moderate, non-significant correlations of ΔHR_{UC} with both Anxiety ($\rho=0.60$, $P=0.07$) and Internalizing problems ($\rho=0.46$, $P=0.18$) in the Dp140– group.

The positive correlations with Anxiety and Internalizing problems scores support the validity of the unconditioned skin conductance response, SCR_{UC} , as a biological correlate for trait anxiety. These relationships were strongest for Dp140 deficient DMD participants, most likely reflecting the higher anxiety scores in this subgroup.

Discussion

In recent years there has been an increasing understanding of the role that the multiple dystrophin isoforms have in brain function, both in human and various animal models.^{11,12,65–67} However, some aspects of the complex DMD neurobehavioural phenotype are only now beginning to be elucidated. There is growing evidence implicating dystrophin in fear and stress responses in experimental and naturally occurring animal models, including exaggerated startle responses in the *mdx* mouse,¹⁹ but until now there has been no systematic study of equivalent responses in humans

with DMD. This study is the first to obtain objective evidence for a pathological unconditioned startle response in boys with DMD using a psychophysiological fear-conditioning task.

We found DMD participants had increased anxiety symptoms, with higher Anxiety and Internalizing problems scores compared to the Control group. Furthermore, the DMD subgroup lacking the Dp140 dystrophin isoform (Dp140–) had higher anxiety scores than the normal population, whilst the subgroup retaining the Dp140 isoform (Dp140+) did not. Previous studies have shown higher anxiety prevalence in DMD than the typical population, and whilst some observed that anxiety was greater in those with more distal 3’ mutations, in these studies the genotype stratifications did not allow determination of whether the Dp140 isoform was expressed or not.^{11–13} Recent work by Saoudi et al.²¹ showed that mice deficient in both Dp427 and Dp140 isoforms displayed increased anxiety compared to mice lacking only Dp427, further supporting our findings that associate Dp140 deficiency with a heightened anxiety phenotype.

Anxiety is the anticipation of a perceived future threat, associated with more long-lasting increased arousal and apprehension,⁶⁸ whilst fear is a rapid-onset emotional response to an immediate threat, mediated by the amygdala, and well-conserved across vertebrate species.^{38,69} Human anxiety disorders are thought to be caused by excessive activation of innate fear circuits.³⁷ In fear-conditioning tasks, anxious individuals typically show increased unconditioned startle responses compared to non-anxious individuals,^{54,55} and greater retention of conditioned responses in extinction.²⁸

The psychophysiological responses we found in the DMD group have similarities with those seen in anxiety disorders; the DMD group had greater startle responses than Controls, most notably the unconditioned skin conductance response (SCR_{UC}) but also the change in HR (ΔHR_{UC}). SCR_{UC} , but not ΔHR_{UC} , also correlated with anxiety symptom scores (Anxiety and Internalizing problems) in the combined cohort, suggesting SCR_{UC} is a valid physiological correlate of trait anxiety.

Our study was designed to investigate the impact of loss of Dp427 dystrophin on the startle response in DMD, as Dp427

Table 3 Primary outcomes: skin conductance and HR response metrics in DMD group and DMD isoform subgroups compared with the Control group

Group	DMD versus Control				DMD Dp140+ versus Control				DMD Dp140– versus Control				DMD Dp140– versus Dp140+			
	Outcome measure	Mean difference ^d (95% CI)	η^2 ^e	Sig., P	Mean diff. (95% CI)	η^2	Sig., P	Mean difference (95% CI)	η^2	Sig., P	Mean difference (95% CI)	η^2	Mean difference (95% CI)	η^2	Sig., P	Sig., P

SCR_{UC} (μS)^a 3.0 (1.0, 5.1) 0.16 0.004 3.4 (1.3, 5.6) 0.25 0.003 3.9 (1.1, 6.6) 0.20 0.008 0.61 (–3.0, 4.2) 0.01 0.73

ΔHR_{UC} (bpm)^b –8.7 (–16.9, –0.51) 0.08 0.04 –10.0 (–19.8, –0.19) 0.11 0.046 –4.2 (–17.6, 9.1) 0.01 0.53 3.5 (–6.3, 13.2) 0.03 0.47

SCR_{EXT} (μS)^c 0.37 (–0.23, 0.96) 0.03 0.22 0.34 (–0.21, 0.89) 0.05 0.22 –1.1 (–2.1, –0.21) 0.18 0.02 0.84 (–0.11, 1.8) 0.18 0.08

Bonferroni adjustment made for multiple comparisons. Covariate values for FSIQ = 101.9 for DMD versus Control; FSIQ = 109.5 for Dp140+ versus Control; FSIQ = 104.8 for Dp140– versus Control; FSIQ = 89.3 for Dp140– versus Dp140+.

FSIQ data available for 30/31 DMD, 25/25 Control, 12/12 Dp140+ and 11/11 Dp140– participants.

^aSCR_{UC} = Unconditioned skin conductance response to the first ‘threat’ trial with conditioned stimulus, CS+, in microSiemens (μS).

^bΔHR_{UC} = Unconditioned change in HR response to the first CS+ ‘threat’ trial (bpm).

^cSCR_{EXT} = Conditioned skin conductance response to the first CS+ trial of the Extinction phase (μS).

^dAdjusted mean difference from analysis of covariance pairwise comparisons including IQ as a covariate.

^e η^2 = effect size of Group.

^fSignificance testing using univariate analysis of variance between groups/subgroups, adjusted for FSIQ, taking alpha of P = 0.05; P-values < 0.05 are highlighted in bold.

deficiency is a feature of all individuals with DMD. The increased startle responses we have demonstrated occurred for all genotypes of DMD, irrespective of whether the Dp140 isoform expression was also affected, and therefore can be attributed solely to deficiency of the full-length Dp427 isoform. This is consistent with animal studies: the *mdx* mouse, lacking only Dp427 dystrophin, displays an abnormal unconditioned startle response.²⁰ We therefore conclude that the SCR_{UC} startle response is a useful biomarker of the deficiency of full-length Dp427 dystrophin.

In view of the known association of Dp140 deficiency with greater prevalence of neurodevelopmental problems,^{11,12} also confirmed in our cohort with lower IQ and increased inattention and hyperactivity symptoms, we also conducted an exploratory analyses comparing Dp140+ and Dp140– subgroups to investigate the influence of Dp140 on unconditioned startle responses and conditioned responses. Intriguingly, whilst there was no differential effect of the Dp140 isoform on the unconditioned startle response magnitude, the Dp140– subgroup showed increased retention of the conditioned SCR compared with Controls, which is a typical feature of anxiety disorders.^{28,70} Half of those lacking Dp140– terminated the Extinction phase early, which could be attributed to heightened arousal. Persistence of the conditioned response in Dp140– participants may also relate to increased salience of auditory stimuli, given the increased tendency to neurodevelopmental symptoms in this group that can be associated with sensory over-reactivity.⁷¹ These exploratory analyses were not powered to identify differences in startle responses between the isoform subgroups, therefore we cannot draw definite conclusions from the findings. However, our data indicate that while the deficiency of Dp427 is associated with increased anxiety prevalence and heightened activity in fear circuits, distal 3′ mutations that also affect Dp140 isoform expression may further exacerbate these symptoms. This is in line with recent studies in the *mdx*52 mice lacking Dp140 as well as Dp427 demonstrating increased anxiety behaviours and pathophysiological differences between these mice and the *mdx* lacking only Dp427.^{21,72} Our observation warrants attention in future studies.

GABAergic synapse dysfunction has been demonstrated in the *mdx* mouse, lacking full length Dp427 dystrophin. In wild-type mice, GABA_A-receptors (GABA_A-R) on post-synaptic membranes co-localise with dystrophin at inhibitory synapses in the hippocampus, cerebellum, and amygdala.⁷³ In the *mdx* mouse there is reduced clustering of GABA_A-Rs and disrupted synaptic function in the basolateral amygdala and hippocampus.^{20,73,74} CNS dystrophin-restoration (either by direct injection or viral vector administration) of AONs^{19,20,26,75,76} corrects both the synaptic abnormalities and the exaggerated startle response. In a different mouse model with a heterozygous mutation in the γ_2 -GABA_A-R subunit, reduced GABA_A-receptor clustering also occurs and is associated with heightened responses in fear-conditioning tasks, increased harm avoidance behaviour and an explicit memory bias to threat cues.⁷⁷ These findings suggest that reduced post-synaptic GABA_A-R density is a key factor in the enhanced fear phenotype.

In typical humans, dystrophin is expressed in the so-called ‘limbic’ structures, including the amygdala, the hippocampal formation and the parahippocampal gyrus.^{3,38,78} Imaging studies have suggested limbic dysfunction occurs in DMD: hippocampal and medial temporal lobe hypometabolism has been observed by FDG-PET⁷⁹; there is hippocampal hyperconnectivity in the default mode network fMRI⁸⁰; GABA_A-receptor distribution is abnormal in the prefrontal cortex.^{22,79,80} Dysfunction at GABAergic inhibitory

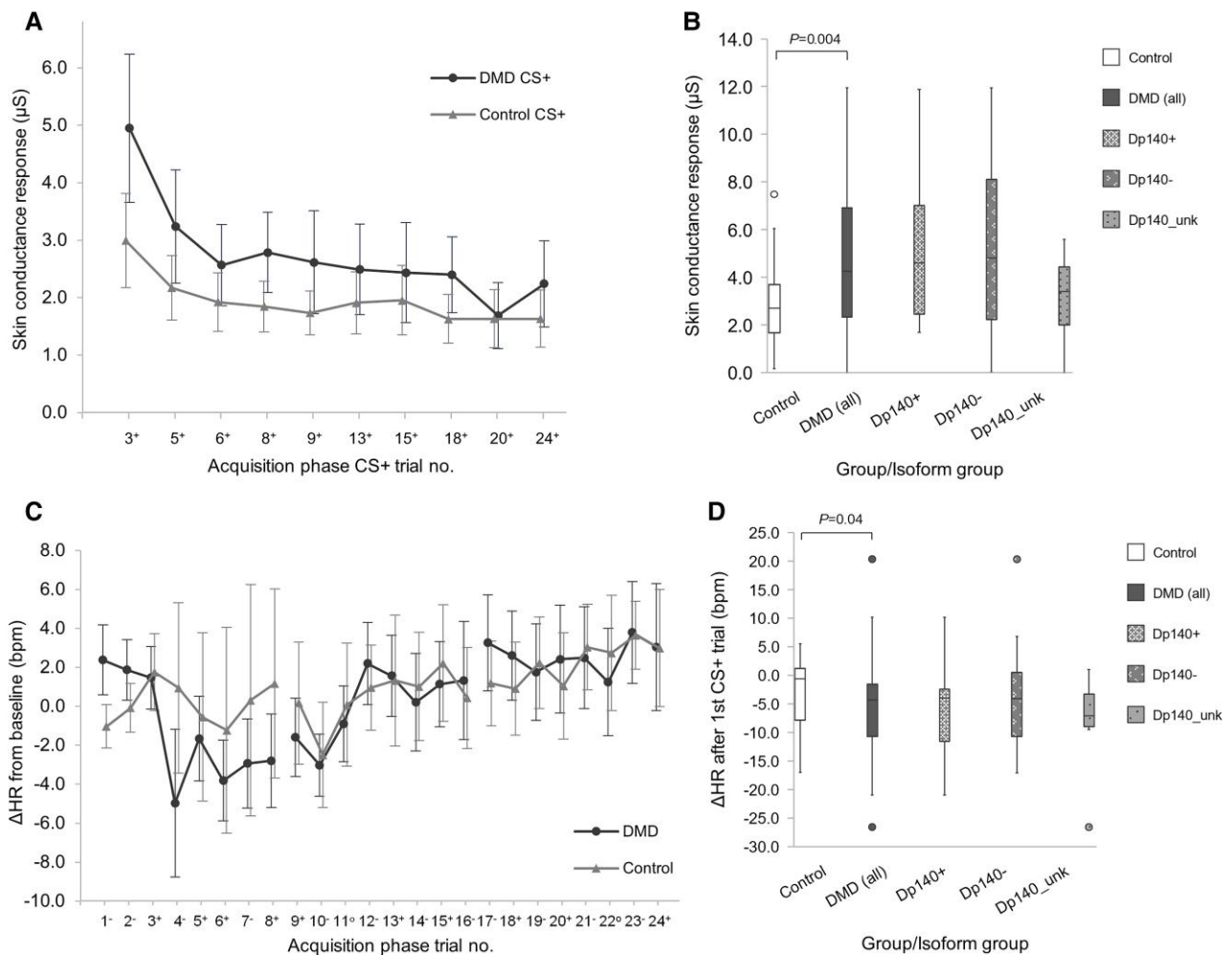


Figure 4 Unconditioned responses to the aversive threat stimulus. (A) SCR_{CS+} for DMD and Control groups in the Acquisition phase. Two ‘threat’ conditioned stimulus (CS+) trials were omitted (Acquisition trials 11 and 22) as these were unreinforced CS+ trials (no aversive noise presented). (B) Box plot of unconditioned SCR_{CS+} for the first CS+ trial (SCR_{UC}) for Control, DMD and DMD isoform subgroups categorized by Dp140 isoform status. The Dp140+ group comprised DMD participants who retain the Dp140 isoform ($n=10$); the Dp140– group comprised DMD participants who lack the Dp140– isoform (including Dp140– and Dp140–/71–; $n=12$); and the Dp140_unk group contained DMD participants whose Dp140 status is uncertain ($n=6$). (C) ΔHR from baseline (bpm) for DMD and Control groups for all Acquisition phase CS+ and CS– trials in the order as presented in the task, measured in bpm. $\Delta HR > 0$ indicates tachycardia relative to baseline; $\Delta HR < 0$ indicates bradycardia relative to baseline. The DMD group had a significant drop in HR from the first CS+ trial to the subsequent trial, the unconditioned ΔHR , ΔHR_{UC} (mean $\Delta HR_{UC} -6.1$ bpm; $P=0.006$) but not the Control group (mean $\Delta HR_{UC} -0.8$ bpm; $P=0.99$). (D) Box plot of ΔHR_{UC} showing a deceleration in HR in the DMD group but not the Control group, with a significant difference between groups in univariate analysis of variance: mean difference -8.7 bpm (95% CI $-16.9, 0-0.51$; $P=0.04$). Error bars in line graphs indicate 95% CI (DMD, solid line; Control, dashed line). Solid bars in box plots indicate median values; error bars show the interquartile range calculated with inclusive median; outliers indicated with markers.

synapses caused by loss of Dp27 dystrophin may therefore underlie the pathogenesis of at least some of the complex neuropsychiatric phenotype in DMD. Impaired GABAergic transmission has been implicated in the pathogenesis of autism spectrum disorder and depression.^{81,82} Accordingly, it is possible that some mental health issues associated with DMD are potentially reversible in humans following CNS-targeted dystrophin restoration, as in the *mdx* mouse.

The outlook for individuals with DMD now is very different to that in previous decades. Following improved standards of care, life-expectancy has increased by approximately 10 years,^{83,84} and genetic therapies addressing dystrophin deficiency in muscle are likely to further improve functional status and survival. However, the neuropsychiatric aspects of DMD impact on the daily functioning of individuals living with DMD and increase carer-burden.^{36,85–87} Current care recommendations advise consideration of

neuropsychological referral at diagnosis of DMD, and if neurodevelopmental problems arise,⁸⁵ although there is no specific guidance for assessment, monitoring or treatment of psychiatric disorders and specific treatment for psychiatric disorders in DMD is uncommon.⁸⁶ Therefore, it is increasingly important to identify and manage neuropsychiatric symptoms with therapeutic interventions, and potentially with future disease-modifying CNS-acting therapies.

CNS-targeted intrathecal AON therapy is effective in spinal muscular atrophy,⁸⁸ however in DMD systemic AON therapy is the primary requirement to ameliorate the muscle pathology. The current peripherally administered AONs in DMD do not cross the blood-brain barrier, but systemically delivered AONs with improved CNS penetration are under development,^{26,89} which could potentially enable dystrophin restoration in muscle and CNS from the same systemically administered therapy.

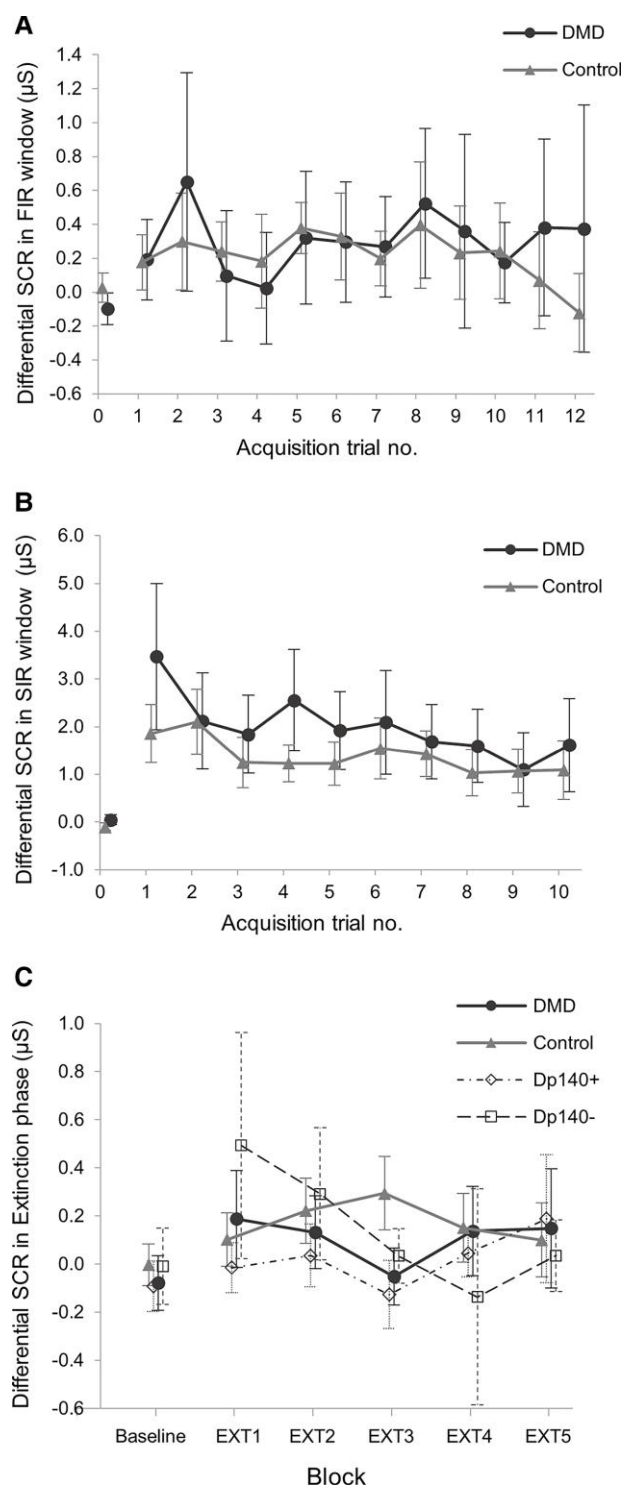


Figure 5 Conditioned response acquisition, habituation to unconditioned stimulus and retention of conditioned responses. SCRs shown as the differential SCR (SCR_{Diff}) to represent the degree of discrimination between the ‘threat’ conditioned stimulus (CS+) and ‘safe’ conditioned stimulus (CS-) cues. $SCR_{Diff} = SCR_{CS+} - SCR_{CS-}$ in contiguous trial pairs; $SCR_{Diff} = 0$ indicates no difference in response to the CS+ and CS- cues. (A) Mean SCR_{Diff} in the FIR window (0–6 s after CS onset) for the DMD and Control groups. This shows SCR_{Diff} after CS presentation but before the aversive noise, indicating the degree of learned response acquisition. For Acquisition trial 1, the FIR measurement occurred before participants had been presented with the first aversive stimulus,

(Continued)

We acknowledge several limitations in this study. As this was the first study of its kind in this clinical population, sample sizes were primarily based on previous literature and pragmatic considerations as accurate *a priori* power calculations were not possible, although power estimates from healthy paediatric data supported our sample size. Isoform subgroup analyses were exploratory outcomes and subgroup sizes were underpowered, therefore the subgroup findings should be interpreted with caution, however as DMD is a rare disorder and some genotypes are uncommon it can be practically difficult to recruit to all genotype subgroups. We compared DMD participants against age- and sex-matched healthy control subjects in this proof-of-principle investigation, however we did not control for steroid use or physical disability, which could potentially be confounding factors. Data loss due to artefacts and early termination limited Extinction phase data interpretation, although had little impact on primary startle responses. Early termination of the task in ‘Non-completer’ participants may have been partly due to some DMD participants finding the task challenging, particularly those deficient in Dp140 dystrophin, therefore modifications to further adapt the task for the DMD population should be considered for future studies. Conditioned response retention may have been enhanced if the extinction task was delayed, as optimal retention occurs after 24 h⁹⁰; however, for practical reasons all task phases were performed on the same day to avoid repeat reduce visits, which may be more difficult especially for DMD participants. Selection bias may have occurred in the Control group, given the higher FSIQ scores compared to the normal population, and in the DMD group a potential bias towards participants without significant cognitive or neurodevelopmental problems. The emotional and behavioural screening instruments used are not diagnostic measures and may have limitations in DMD,⁹¹ therefore caution should be taken in interpreting neuropsychiatric data.

In conclusion, we have demonstrated for the first time that boys with DMD show increased physiological startle responses to threat, using the principle of ‘back-translation’ from the exaggerated startle response phenotype in *mdx* mice. This has implications both for the further understanding of the neurobiology of DMD and clinical management. We propose that a lack of full-length Dp427 dystrophin leads to a dysfunctional fear system which predisposes individuals with DMD to develop anxiety disorders. In keeping with recent data in Dp140 deficient *mdx* mice,²¹ our data also provides some evidence that the lack of the Dp140 dystrophin isoform may further increase this risk. To date, anxiety in DMD has received little attention in both research and clinical practice therefore robust prevalence data are lacking, however given the increased risk of anxiety due to underlying neurobiological changes we suggest that young boys with DMD should be actively monitored for anxiety

Figure 5 Continued

therefore this is lower than for the subsequent trials in both groups. (B) Mean SCR_{Diff} in the SIR window (6–12 s after CS onset, the start of which corresponds to the onset of the aversive ‘threat’ stimulus) for the DMD and Control groups. These are unconditioned responses to the aversive stimulus, which are shown to reduce or ‘habituate’ with repeated presentations of the aversive stimulus. For SIR data, one trial pair in each of Acquisition blocks 2 and 3 was omitted, as these included unreinforced CS+ trials (no aversive noise presented). (C) Mean SCR_{Diff} in the Extinction phase blocks for the Control, DMD and DMD isoform subgroups (Dp140+ and Dp140-). Extinction of the conditioned response (SCR_{CS+}) is defined at the point at which there is no discrimination between the CS+ and CS- cues, i.e. when $SCR_{Diff} = 0$. Extinction data were extracted from the whole response window (0–12 s after CS+ presentation). Mean SCR for familiarization phase is included as a baseline comparison in all plots. Error bars show 95% CI for each data point.

and emotional problems from an early age to enable support, interventions and treatment where appropriate. Further investigation of anxiety and other neuropsychiatric disorders is needed to inform clinical practice and, where necessary, refine clinical guidelines to optimize the care of young people with DMD. Our findings also have implications for translational research, as to date no CNS biomarker of dystrophin has been identified that could be used in a clinical trial. We propose that the unconditioned skin conductance startle response is a useful objective physiological biomarker that could be used in future clinical trials aimed at evaluating CNS dystrophin restoration, analogous to the *mdx* startle response as an outcome in preclinical studies of CNS-targeted antisense oligonucleotide therapies. The findings from this study may inform current clinical management of anxiety disorders in DMD, and potentially be a first step towards evaluating future CNS-disease modifying therapies.

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Competing interests

F.M. has received grants, speaker and consultancy honoraria from Sarepta Therapeutics, Avexis, PTC Therapeutics, Roche, Biogen, Dyne Therapeutics, Novartis and Pfizer. D.S. has current grant funding from the Medical Research Council (UK), Sarepta Therapeutics, the European Research Council, and the Patrick Paul Foundation. W.M. is supported by the National Institute of Health Research, Autistica, the Dunhill Medical Trust, the Medical Research Council and the European Research Council. N.H. has received consulting fees from Janssen and GSK. The remaining authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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