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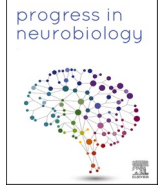
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The unconditioned fear response in vertebrates deficient in dystrophin

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ABSTRACT

Dystrophin loss due to mutations in the Duchenne muscular dystrophy (DMD) gene is associated with a wide spectrum of neurocognitive comorbidities, including an aberrant unconditioned fear response to stressful/threat stimuli. Dystrophin-deficient animal models of DMD demonstrate enhanced stress reactivity that manifests as sustained periods of immobility. When the threat is repetitive or severe in nature, dystrophinopathy phenotypes can be exacerbated and even cause sudden death. Thus, it is apparent that enhanced sensitivity to stressful/threat stimuli in dystrophin-deficient vertebrates is a legitimate cause of concern for patients with DMD that could impact neurocognition and pathophysiology. This review discusses our current understanding of the mechanisms and consequences of the hypersensitive fear response in preclinical models of DMD and the potential challenges facing clinical translatability.

1. Introduction

The largest human gene is located on the X-chromosome and encodes dystrophin protein (DMD gene). Dystrophin is expressed in several tissues, including brain and muscle cells, where it contributes to diverse cellular functions such as synaptic transmission (Anderson et al., 2003) and mechanotransduction (Kumar et al., 2004). Mutations in the gene encoding dystrophin can result in loss of expression of dystrophin – a disease referred to as Duchenne muscular dystrophy (DMD), which is characterized clinically by a progressive and severe muscle pathology and premature death. Patients with DMD may also suffer from a plethora of neurocognitive disorders due to the loss of brain-specific dystrophin, including attention hyperactivity disorder (ADHD), anxiety, autism spectrum disorders (ASD) and lower intelligence quotient (IQ) (Hendriksen et al., 2020). Maresh et al. recently described the “startle” response in patients with DMD, another neurocognitive comorbidity characterized by hypersensitivity to stressful/threat stimuli (Maresh et al., 2023). In the *mdx* mouse model of DMD, which does not express full-length dystrophin in brain (Lidov et al., 1993), a similar aberrant unconditioned fear response to stressful/threat stimuli is associated with disease pathophysiology (Lindsay et al., 2021b), can exacerbate some dystrophinopathy phenotypes (Lindsay et al., 2021a) and cause sudden

death due to sustained hypotension (Razzoli et al., 2020). Given the apparently similar clinical manifestations of this unconditioned fear response in *mdx* mice to patients with DMD, it appears prudent to examine and review the preclinical literature to understand the mechanistic basis of this behavior, the efficacy of targeted therapeutics and the challenges of clinical translatability.

2. Duchenne muscular dystrophy

DMD, a fatal neuromuscular disease, is an X-linked recessive disorder affecting 1 in 5000 boys worldwide (Crisafulli et al., 2020). DMD is caused by mutations in the DMD gene (Hoffman et al., 1987) that results in loss of expression of its main protein product of 427 kDa, the full-length dystrophin (Dp427). Loss of muscle Dp427 causes atrophy, degeneration, and weakness in striated muscle of patients (Allen et al., 2016; Blake et al., 2002; Bruin et al., 1997; Frankel and Rosser, 1976; Lerario et al., 2012; Nigro et al., 1990; Wokke et al., 2014). However, DMD is also associated with a spectrum of neurocognitive comorbidities attributed to the loss of brain-specific dystrophin-gene products normally expressed in the nervous system from independent internal promoters (see details on the dystrophin-gene products in the following section) (Banihani et al., 2015). The earliest symptoms of DMD,

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manifesting around 2 – 5 years of age, include altered gait with toe walking, frequent falls, and difficulties with climbing stairs (Birnkranz et al., 2018; Mercuri et al., 2019). At around 10 – 12 years of age, patients with DMD become wheelchair dependent because muscle strength deteriorates. Assisted ventilation is usually introduced at around 20 years of age to sustain diaphragmatic function and respiration, initially at night because of sleep-related breathing disorders and hypoventilation (Phillips et al., 2001). As the respiratory muscles continue to weaken, patients with DMD require assistance to breathe during the day (Bushby et al., 2010; Phillips et al., 2001). Eventually, even with optimal care, most patients with DMD die in the late second to third decade of life due to cardiorespiratory failure, yet improvements in multidisciplinary care have increased life expectancy by about 10 years (Birnkranz et al., 2018; Eagle et al., 2007).

2.1. Muscle and non-muscle roles of dystrophins

The DMD gene contains several internal promoters that encode multiple dystrophin products differentiated by their length and location of expression: Dp427, which is expressed in striated muscle, smooth muscle and brain neurons (Byers et al., 1991; Górecki et al., 1992; Hoffman et al., 1988; Muntoni et al., 2003), and shorter isoforms (Dp260, Dp140, Dp116, Dp71 and Dp40), which are expressed in non-muscle tissues (Bies et al., 1992; Muntoni et al., 2003), among which Dp140 and Dp71 likely have distinct roles in human brain developmental stages and in a variety of cellular processes (Ahn and Kunkel, 1993; Pilgram et al., 2010) (Fig. 1). Dp427 is an integral component of the dystrophin glycoprotein complex (DGC) that forms a costameric network with signaling and scaffolding proteins in striated muscle (Ervasti, 2003; Heydemann and McNally, 2007; Lapidus et al., 2004) (Fig. 2) and the neuronal/synaptic plasmalemma (Fig. 3) with mechanical and non-mechanical (signaling) roles (Doorenweerd et al., 2017; Ervasti and Campbell, 1991; Nelson and Ervasti, 2021; Perronnet and Vaillend, 2010).

In skeletal muscle, dystrophin acts as a molecular shock absorber, enables the lateral transmission of mechanical force generated by the sarcomere, and stabilizes the connections between the intracellular actin

cytoskeleton and the extracellular matrix (Bhasin et al., 2005; Dowling et al., 2021; Kumar et al., 2004; Le et al., 2018; Ramaswamy et al., 2011; Ramirez et al., 2022; Wilson et al., 2022). Loss of skeletal muscle-specific dystrophin causes destabilization of the sarcolemma (Petروف et al., 1993), increased sensitivity of fast(er) twitch muscle to eccentric contraction-induced damage (Head et al., 1992; Lindsay et al., 2019; Lynch, 2004; Moens et al., 1993; Petروف et al., 1993), replacement of contractile tissue by fat and fibrotic deposits (Cros et al., 1989; Desguerre et al., 2009; Kharraz et al., 2014; Naarding et al., 2020; Veegeer et al., 2021), centrally nucleated fibers and loss of muscular strength (Bulfield et al., 1984; Duan et al., 2021; Scott et al., 1982; Trost et al., 2021; Ziter et al., 1977).

The composition of the brain DGC is variable because of its interaction with distinct dystrophin products in multiple neural cell types (neurons, astrocytes, oligodendrocytes, endothelium) (Waite et al., 2012). The DGC enables proper neural cell function by linking the actin cytoskeleton to a scaffold of postsynaptic molecules and membrane receptors (Fig. 3) (Pilgram et al., 2010). This is critical for neurotransmitter receptor complex maturation and clustering at the neuromuscular junction (NMJ) and central inhibitory synapses (Pilgram et al., 2010). Brain-specific Dp427 is expressed in principal neurons of the cerebellum (Purkinje cells), cortex, hippocampus (pyramidal cells in CA1-CA4 areas) and amygdala at the postsynaptic site of inhibitory GABAergic synapses (Doorenweerd et al., 2017; Lidov et al., 1993). Thus, neuronal Dp427-associated DGC indirectly interacts with γ -aminobutyric acid type A (GABA_A) receptors, and it has an essential role in clustering and stabilizing these receptors to the postsynaptic membrane of synapses (Fig. 3) (Anderson et al., 2002; Knuesel et al., 1999). The loss of Dp427 expression in the brain causes structural abnormalities such as a reduction in grey matter and altered microstructure of white matter (Doorenweerd et al., 2014). In contrast, the absence of glial Dp71 alters the clustering of Kir4.1 potassium channels and aquaporin-4 (AQP4) channels in retinal and brain glial cells (Fort et al., 2008), demonstrating Dp71's central role in anchoring and localization of other essential cellular membrane channels (Fort et al., 2008). The Dp140 isoform, largely expressed in the brain, retina and kidney (Lidov and Kunkel, 1997; Lidov et al., 1995; Uecla et al., 2000), is suggested to be involved

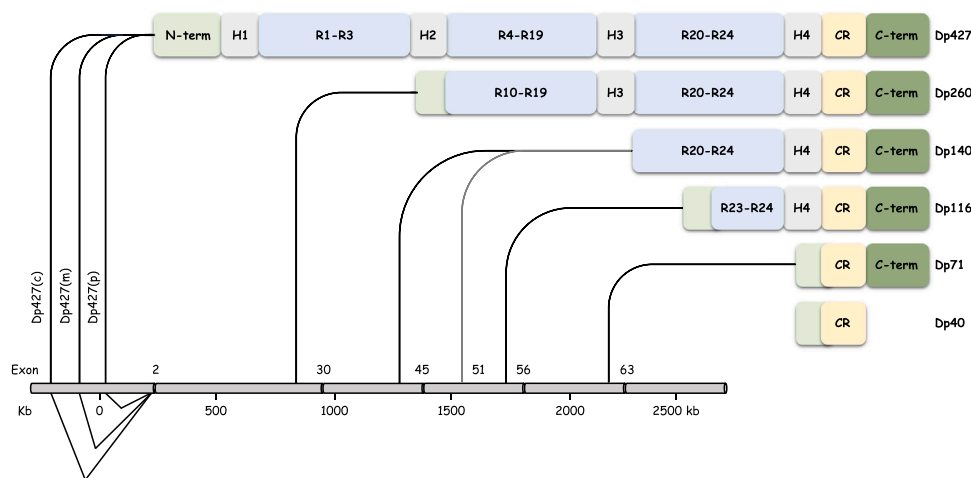


Fig. 1. Schematic of the genomic organization of the dystrophin gene and the dystrophin proteins. The length of the DMD gene is 2.5 Mb and encodes six dystrophin products. Lines represent the transcription start from different promoters encoding the dystrophins. There are three full-length dystrophin transcripts (14 kb) produced from the most upstream promoters that each have a specific first exon (Chelly et al., 1990; Górecki et al., 1992; Koenig et al., 1987; Monaco et al., 1986): Dp427m (muscle-specific), Dp427c (cerebral neurons) and Dp427p (Purkinje cells). Dp260 is expressed primarily in the retina and driven by the promoter in intron 29 (Pillers et al., 1993). Dp140's transcription starts at intron 44, but its translation starts at exon 51 (grey line), leaving a long untranslated region between intron 44 and exon 50. Dp140 is expressed in the kidney and central nervous system. Dp116 is expressed from a promoter in intron 55 and is primarily localized in peripheral-nerve Schwann cells (Lidov et al., 1995). In all cell types, a promoter in intron 62 is used to express Dp71, which is highly expressed in brain (Hugnot et al., 1992; Kesari et al., 2008). Therefore, the region of the mutation to the DMD gene can affect the expression of one to several dystrophins, which can differentially impact the phenotypic profile of patients with DMD. N-term; N-terminus, H; hinge region, R; spectrin-like repeats, CR; cysteine-rich domain, C-term; C-terminus.

This figure is adapted from Muntoni, Torelli & Ferlini (Muntoni et al., 2003).

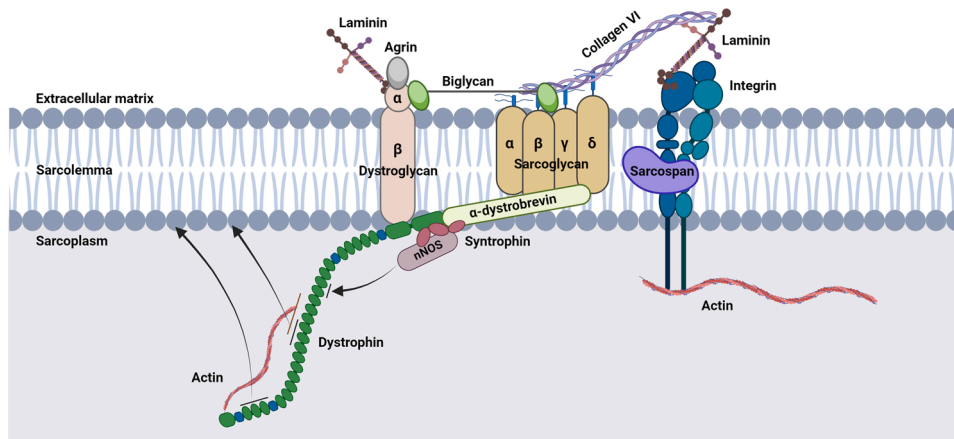


Fig. 2. Schematic depiction of the dystrophin glycoprotein complex in a muscle cell. Dystrophin is a protein with a rod-like structure that serves as a robust mechanical connection between the intracellular cytoskeleton network of muscle cells and the extracellular matrix. The illustration includes only the primary structural and signaling components of the striated muscle complex. nNOS; neuronal nitric oxide synthase.

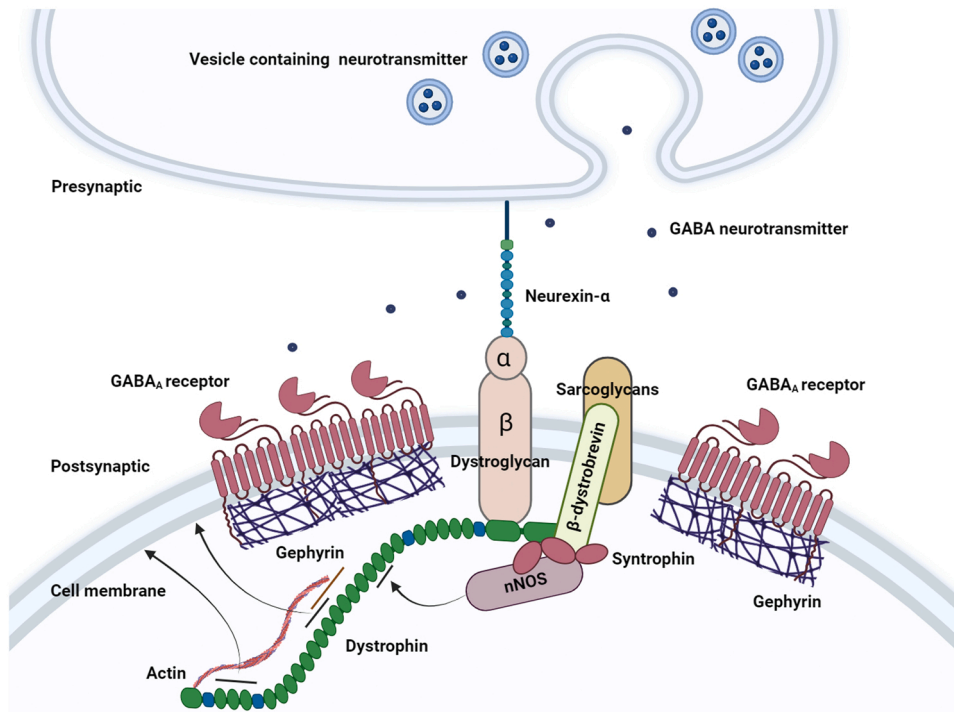


Fig. 3. Schematic depiction of the dystrophin glycoprotein complex (DGC) and GABA (gamma-aminobutyric acid) receptor clustering in neurons. The illustration includes only the primary structural and signaling components of the neuronal complexes. In inhibitory synapses of principal neurons, gephyrin is the main anchoring protein of GABA receptors, showing indirect interactions with the DGC that are likely facilitated by other scaffolding proteins, such as S-SCAM and IQsec3 (Zarrouki et al., 2022a). nNOS; neuronal nitric oxide synthase.

in glutamatergic transmission, presynaptic inhibition and regulation of neuron-astrocyte interactions at least in the basolateral amygdala (BLA) (Hashimoto et al., 2022). The absence of Dp140 and Dp71 is associated with a higher risk of cognitive impairment (e.g., ADHD) and other neurodevelopmental disorders in patients with DMD (Daoud et al., 2009; Lorusso et al., 2013; Pane et al., 2012, 2013). Dp260 expression is primarily in the outer plexiform layer of retina and needed for normal retinal electrophysiology (D'Souza et al., 1995). Dp116 is expressed mainly in the peripheral nerves and is known as a Schwann cell-specific isoform (Byers et al., 1993). Yet, Dp116 is also expressed in cardiac muscle (Yamamoto et al., 2018), and patients with DMD show lower expression of Dp116 in their skin fibroblast (Labarque et al., 2007), which results in Gs alpha subunit upregulation (type of G-protein that

stimulates the cAMP-dependent pathway) and increased Gs signaling (Labarque et al., 2007). Dp40, the smallest dystrophin product, is expressed from the same promoter of Dp71 (intron 62) up to exon 70 (Tinsley et al., 1993). Dp40 has been detected in excitatory synapses and is likely involved in synaptic functions (Fig. 1) (Fujimoto et al., 2014).

3. Neurocognitive disorders in DMD

3.1. Intellectual and neuropsychiatric problems

The location of the mutation in the DMD gene is correlated with the severity of intellectual problems affecting patients with DMD (Ricotti et al., 2016). Thirty percent of patients with DMD have intellectual

disability with full-scale IQ scores lower than 70 (Milic Rasic et al., 2014). The severity of the intellectual problems appears to depend on the position of the mutation to the DMD gene and number of dystrophin-gene products consequently lost. Proximal mutations affecting only Dp427 are associated with normal IQ and mild cognitive problems, while distal mutations leading to cumulative loss of several dystrophins are associated with significant reduction of the IQ (Taylor et al., 2010). DMD is also associated with a range of behavioral disturbances and neuropsychiatric disorders relative to the general population (Battini et al., 2021; Billard et al., 1992), which may include ASD (3.1–20.7%) (Banihani et al., 2015; Parisi et al., 2018; Ricotti et al., 2016) vs. 0.6% for the general population (Salari et al., 2022)). The etiology of ASD in patients with DMD is still unknown, but it might involve altered cell migration during central nervous system (CNS) development (Reiner et al., 2016). Moreover, ADHD, which occurs in 18% of patients with DMD (Pascual-Morena et al., 2022), might associate with an impaired central excitatory/inhibitory balance due to the absence of Dp427 and/or Dp71 (De Stefano et al., 2022; Grillner and Mercuri, 2002; Suzuki et al., 2017). Depression (prevalence: 7–11%) and obsessive-compulsive disorders (OCD; prevalence: 7–12%) are also reported in patients with DMD but their etiology is unknown (Pascual-Morena et al., 2022). Emotional disturbances in patients with DMD include both internalizing and externalizing problems. Interestingly, anxiety (prevalence: 24–25%) occurs relatively evenly among genotype subgroups in patients with DMD (Pascual-Morena et al., 2022), indicating abnormal emotional behaviors are mainly dependent on Dp427, as this dystrophin is invariably lost in all patients (Ricotti et al., 2016).

3.2. Progression of intellectual and emotional problems

According to most studies, cognitive impairments in patients with DMD are not progressive (Hellebrekers et al., 2020; Tyagi et al., 2019). However, computerized tomography neuroimaging of older patients with DMD showed a progressive cerebral degeneration (Yoshioka et al., 1980). The experimental data from postmortem evaluation of patients with DMD is also controversial because there is no clear relationship between cognitive abilities and brain structural abnormalities (Doornweerd, 2020).

3.3. Hypersensitivity to stressful stimuli

In addition to known cognitive and behavioral abnormalities, there are reports of the presence of sensory processing disorders (SPD) in patients with DMD (Backhouse et al., 2012; Poysky, 2007). SPD is described when a child has difficulties detecting, processing, and responding to sensory stimuli, which can manifest as hypo or hyper-reactivity. Maresh et al. recently identified a pathological unconditioned startle response in patients with DMD, which is described as a heightened physiological reaction to unpredictable visual and auditory stressful stimuli relative to age-matched unaffected controls (Maresh et al., 2023). The startle response is thought to reflect hyperarousal and enhanced anticipatory anxiety. All DMD participants showed increased levels of anxiety, threat-induced bradycardia, and a greater unconditioned skin conductance change, suggesting the higher startle response manifests independent of the type of dystrophin affected by mutations. While this suggests an outcome resulting from Dp427 deficiency, it was reported that in DMD participants lacking both Dp427 and Dp140, anxiety symptom scores were greater relative to participants that did not express Dp427 only (Maresh et al., 2023). This suggests mutations toward the 3' end of the DMD gene are associated with more severe emotional behavior problems. The neural basis of the startle response in patients with DMD is still unclear, but this likely provides an objective measure of anxiety that has implications for clinical management, translational research, and monitoring of central benefits of treatments in future clinical trials. Strikingly, this recent study supports the conclusions previously reached by the behavioral analyses of DMD mouse

models regarding the role of Dp427 in the genesis of pathological stress-induced unconditioned fear. Moreover, a range of preclinical investigations in relevant genetic mammalian models of DMD have elucidated stress-related effects that can exacerbate dystrophinopathy phenotypes (Lindsay et al., 2021a) and cause sudden death (Razzoli et al., 2020).

4. Strong association of brain Dp427 deficiency with severe and pathological fearfulness

4.1. Enhanced stress reactivity conserved across animal species

The association between dystrophin deficiency and an increased fear response to stress/threat stimuli (the terms fear, threat, and stress stimuli, often used interchangeably in different studies, will be collectively referred to as a stressful stimulus) was first described in the dystrophin-deficient *mdx23* mouse (referred to as the *mdx* mouse in this review) (Sekiguchi et al., 2009). In this model, a premature stop codon in exon 23 of the *dmd* gene, which results in the selective absence of Dp427 (Sicinski et al., 1989), causes enhanced freezing, also described as a tonic immobility, which likely reflects a pathological defensive response to a restraint-induced stress, as it is not observed in wildtype (WT) littermates in the same conditions. Sekiguchi et al. ameliorated this enhanced fear-related behavior by partially restoring brain Dp427 using an exon-skipping strategy (Sekiguchi et al., 2009). Because the morpholino phosphorodiamidate (morpholino) antisense oligonucleotide used to induce expression of a functional, yet slightly truncated, dystrophin was administered directly in the cerebral ventricles, this indicated a central origin of this phenotype. The same unconditioned fear response is evident in the dystrophin-deficient rat (Caudal et al., 2020). Importantly, several studies demonstrate that the relationships linking the *dmd* gene to abnormal responsiveness to stressful stimuli is conserved across animal species. Indeed, a high-throughput transcriptome analysis unveiled an association between the *dmd* gene and the unconditioned fear typically observed and also expressed as tonic immobility in the Japanese quail (Frésard et al., 2012). Moreover, *dmd* gene expression is reduced in tadpoles following morphological changes in response to a predation threat and nursery pigs with fear-related behavioral disturbances and heightened stress responses hold a polymorphism in exon 41 of the *dmd* gene associated with a porcine stress syndrome sometimes resulting in death (Mori et al., 2015; Nonneman et al., 2012). These observations and outcomes support the notion that the absence of dystrophin in vertebrates is associated with an enhanced fear/threat/stress behavior.

4.2. Stress-related phenotypes in Dp427-deficient mice

A range of laboratory stressors have been used to characterize the behavioral profile of *mdx* mice, which highlighted an enhanced emotional reactivity. Research groups have used the terms fear response, freezing response, stress response, emotional reactivity, and defensive behavior to explain the behavioral phenotype of *mdx* mice to various stressful stimuli. For the purposes of this review, and to avoid confusion, we will generally condense these descriptors of this behavior to the fear response, except when immobility cannot be undoubtedly interpreted as fear in specific experimental conditions.

4.2.1. Restraint stress

This intervention robustly induces a fear response in *mdx* mice relative to WT mice, that is often reported as a drastic reduction in their mobility (Goyenville et al., 2015; Lindsay et al., 2021b,a; Perronnet et al., 2012; Razzoli et al., 2020; Saoudi et al., 2021; Sekiguchi et al., 2009; Yamamoto et al., 2010). Restraint stress (10–30 s manual scruff-restraint; grasping the mouse by the nape between the index finger and thumb, the tail being placed between the fourth and fifth digits, and the mouse held in a supine orientation) was first applied by

Sekiguchi et al. to demonstrate that *mdx* mice display an enhanced defensive behavior (fear) relative to WT mice (Sekiguchi et al., 2009). Fear was calculated by the percent time spent in tonic immobility (freezing) after restraint (70%) (Sekiguchi et al., 2009). Vaillend and Chaussonot further demonstrated that freezing can extend for up to 12 h when *mdx* mice are maintained in a novel environment during a circadian cycle following the restraint test (Vaillend and Chaussonot, 2017). In contrast, Yamamoto et al. showed that the amount of freezing could be attenuated if other mice (regardless of sex) had been previously reared in the cage where test mice were released following restraint (Yamamoto et al., 2010), suggesting the fear response could be modulated by conspecific scent, perhaps due to its influence on mouse arousal state. Other distractive factors such as sudden noise or touching are also reported to temporarily interrupt the fear response of *mdx* mice (Vaillend and Chaussonot, 2017).

It is unknown which component of the restraint stress induces a fear response in *mdx* mice. It was tested whether titling the mouse in a supine position during restraint stress was an effector, as shown in Japanese quail, but this hypothesis was rejected because there were no differences in the level of physical activity between a mouse completing a scruff-restraint in a supine or prone position (Vaillend and Chaussonot, 2017). Placing *mdx* mice in a tube-restraint (a semi-customized tube to fit the dimensions of individual mice that prevents movement in any direction) for 5 min in a prone position resulted in a freezing response, yet it was lower relative to the response induced by a 10 s scruff-restraint (Table 1) (Vaillend and Chaussonot, 2017). This suggested (1) that pain sensitivity (the physical act of the scruff-restraint) was not a major effector of the fear response, which was further confirmed by showing that *mdx* mice display a similar repertoire of responses as WT mice following delivery of electric foot shocks of variable (mild) intensities (Chaussonot et al., 2015), and (2) that the stress intensity directly impacts the magnitude of freezing.

It is unknown if the startle response in patients with DMD emerges as an accumulative consequence from dystrophin deficiency or if it is evident from birth. In boys with DMD, the neuropsychological profile shows no change over time (Battini et al., 2021). In *mdx* and *mdx52* mice, where *mdx52* mice do not express the Dp427 and Dp140 isoforms in the brain (Araki et al., 1997), the fear response is weak at 20 days of age and indifferent to WT, but from 30 to 36 days postnatal to ~three months of age, the fear response gradually increases (Hashimoto et al., 2022; Sekiguchi et al., 2009; Yamamoto et al., 2010). In adulthood, the fear response does not differ between 3- and 9-month-old *mdx* mice and 3- and 7-month-old *mdx52* mice (Hashimoto et al., 2022; Saoudi et al., 2021; Vaillend and Chaussonot, 2017). The gradual increase in fear response disparity between WT and *mdx* mice can likely be attributed to changes in dystrophin expression in the CNS of WT mice as they develop (Kim et al., 1992). It could also be related to an abnormal postnatal development of the *mdx* mouse brain because the fear response gradually increases overtime (Sekiguchi et al., 2009), which could be linked to the timeline of brain developmental stages (Kerkerberg et al., 2021).

In normal physiology, an organism adapts to repeated stressors (Grissom and Bhatnagar, 2009). When a WT mouse is exposed to repeated scruff-restraints over six days, their level of physical activity, as a measure of fear, decreases (Vaillend and Chaussonot, 2017). While this could be an indicator that the WT mouse does not adapt to repeated scruff-restraints, it could also be a measure of decreased motivation to explore. In contrast, the fear response in *mdx* mice remains enhanced and resistant to extinction (Vaillend and Chaussonot, 2017), except when a reduction in the fear response occurs during extensive repetition of scruff-restraint (18 trials in a single day) (Vaillend and Chaussonot, 2017). When a scruff-restraint is applied daily for 12 weeks, the fear response in *mdx* mice does not change (Lindsay et al., 2021a). When a stressor is applied weekly (scruff-restraint and forced treadmill exercise), both female and male *mdx* mice displayed increased levels of physical inactivity (Lindsay and Russell, 2023). However, when *mdx* mice are repeatedly restrained inside a tube for 5 min, both sexes adapt,

becoming hypertensive instead of hypotensive (this phenotype can cause increased mortality in *mdx* mice, which is discussed in a later section) during the stress (Lindsay and Russell, 2023). With successive exposures, the mean arterial blood pressure and shock index (calculated by maximum heart rate/low systolic blood pressure) gradually became WT-like. It has been suggested that the nature of the stressor, rather than the intensity (Table 1) (Vaillend and Chaussonot, 2017), might impact the adaptive potential of *mdx* mice, with a tube-restraint minimizing pain and researcher interference relative to a scruff-restraint. These results indicate that the behavioral response of *mdx* mice does not habituate to repetitive stressors, but rather, when they are submitted to repetitive stimuli within a single day, it is likely that it triggers a different response to the stress (flight instead of freeze).

4.2.2. Forced swim test

A heightened immobility response was reported in *mdx* mice submitted to the forced swimming test (FST) (Comim et al., 2019; Vaillend and Chaussonot, 2017), a test used to assess depression in WT mice (Porsolt et al., 1977). FST induces episodes of freezing in mice as they experience an unescapable aversive stimulus. Two studies in *mdx* mice reported contrasting outcomes in behavioral responses to FST, likely due to some protocol variations. Vaillend et al. applied FST in two sessions to characterize learning and behavioral despair in *mdx* mice (Vaillend and Chaussonot, 2017). The test involved two exposures with a 24 h interval to an inescapable glass cylinder filled with 18 cm of water at 25 °C. The behavior was recorded for 5 min in each session for analyzing three parameters: climbing, staying afloat and immobility (freezing). Climbing was defined when mice had upward-directed movements with the forepaws striking along the walls of the container and repetitive movement of hind paws in the water. Staying afloat was defined when mice had minimal movement to keep the head above water and immobility was defined when mice showed complete immobility for at least one second. During exposure one, the latency and duration of freezing in WT and *mdx* mice were comparable but during the second exposure, each parameter decreased in both groups, which was interpreted as animals' learning about the inescapable context (learning helplessness). It was also noted that *mdx* mice spent more time climbing to escape relative to WT mice, suggesting a greater level of stress reactivity (Vaillend and Chaussonot, 2017). Comim et al. performed this test with two exposures to a cylindrical water tank filled with 30 cm of water at 22–23 °C. Each mouse completed a single 7-min trial (training 2 min; test 5 min). The behavior was manually recorded for total immobility, which was defined as a time duration when the movement was not for swimming but only to keep the body in balance and keep the head above water (Comim et al., 2019). They reported a marked increase in the immobility of *mdx* mice relative to WT mice following FST (Comim et al., 2019). In any case, the two studies revealed, through analysis of distinct behavioral parameters, a heightened stress reactivity in *mdx* mice.

4.2.3. Forced treadmill exercise

Forced treadmill exercise, level or with a slight decline (e.g., -15°), has been shown to induce a stress response with increased anxiety in WT mice (Svensson et al., 2016) and it causes higher levels of physical inactivity in *mdx* mice relative to WT mice (Belanto et al., 2014; Kobayashi et al., 2008; Leasure and Jones, 2008; Lindsay and Russell, 2023; Lindsay et al., 2021b). Forced treadmill exercise induces physical inactivity in WT mice due to the forced nature of the assay, not the running component (Svensson et al., 2016). Mice are stressed on a treadmill by forcing them to run at 15 m/min for 15 min, sometimes without any prior familiarization (Belanto et al., 2014; Kobayashi et al., 2008; Lindsay and Russell, 2023; Lindsay et al., 2021b). In response to forced treadmill exercise, *mdx* mice display higher levels of physical activity in the open field relative to scruff-restraint (Kobayashi et al., 2008; Leasure and Jones, 2008; Lindsay and Russell, 2023; Lindsay et al., 2021b), suggesting forced treadmill exercise is a less intense stress

Table 1

The physical activity response to stressful stimuli in WT and dystrophin-deficient mouse models of DMD with or without peripheral and/or central dystrophin or utrophin restoration.

Genotype	Reference	Stressor	Freezing (%) ^a	Activity (%) ^b	Dp/Up restoration	Methods for restoration	Freezing (%) after restoration ^a	Activity (%) after restoration ^b	
Male WT	(Sekiguchi et al., 2009)	SR 10–30 s	0						
	(Yamamoto et al., 2010)		1						
	(Goyenville et al., 2015)		21						
	(Vaillend and Chaussonot, 2017)		9.5						
	(Relizani et al., 2017)		15						
	(Razzoli et al., 2020)			44					
	(Saoudi et al., 2021)			9					
	(Lindsay and Russell, 2023)			56					
	(Saoudi et al., 2023)			10.5					
	(Johnson et al., 2024)			37 ^d					
	(Vaillend and Chaussonot, 2017)		TR 5 min	5					
	(Belanto et al., 2014)		Treadmill 15 min		53				
(Lindsay and Russell, 2023)			94						
Female WT	(Yamamoto et al., 2010)	SR 10–30 s	2						
	(Razzoli et al., 2020)			44					
	(Lindsay and Russell, 2023)			53					
	(Lindsay and Russell, 2023)		Treadmill 15 min		107				
Genotype	Reference	Stressor	Freezing (%) ^a	Activity (%) ^b	Dp/Up restoration	Methods for restoration	Freezing (%) after restoration ^a	Activity (%) after restoration ^b	
Male <i>mdx</i>	(Sekiguchi et al., 2009)	SR 10–30 s	71*						
			61*,##		Dp427; Brain: 27%	ICV (central) morpholino ^c	33**		
	(Perronet et al., 2012)		50*,#		Overexpression brain Up395	Six-week IP, AB; 600 mg/kg/day	53*		
	(Yamamoto et al., 2010)		71*		Dp427; Brain: < up to 6%; striated muscle: up to 50%	IV (peripheral); 200 mg/kg/week 15-mer tcDNA	17**		
	(Goyenville et al., 2015)		71*		Dp427; Brain: < 6%; striated muscle: < 27%	IV (peripheral); 200 mg/kg/week 13-mer tcDNA	36.5**		
	(Relizani et al., 2017)		72*						
	(Vaillend and Chaussonot, 2017)		78*						
	(Razzoli et al., 2020)			7*		Overexpression Dp427/Up495	Dys ^{ΔMTB} - <i>mdx</i>		34**
	(Lindsay et al., 2021a)			26*		Overexpression muscle Up495	Fiona- <i>mdx</i> mice		34.5**
	(Lindsay et al., 2021b)			10*					
(Saoudi et al., 2021)		86*							
(Hashimoto et al., 2022)		76*							
(Zarrouki et al., 2022)		70*,#			Dp427; Brain: 25%	ICV (central) Palm-TcDNA	30		
(Lindsay and Russell, 2023)			10*****						

(continued on next page)

Table 1 (continued)

Genotype	Reference	Stressor	Freezing (%) ^a	Activity (%) ^b		
	(Johnson et al., 2024)			1 ^{*,d}	Skeletal muscle: overexpression muscle Up495	Fiona- <i>mdx</i> mice 23 ^{*,*,d}
	(Vaillend and Chaussonot, 2017)	TR 5 min	63 [*]		Overexpression Dp427/Up495	Dys ^{ΔMTB} - <i>mdx</i> 38 ^{*,*,d}
	(Belanto et al., 2014)	Treadmill 15 min		1 [*]	Skeletal muscle: overexpression Up495	Fiona- <i>mdx</i> mice 20 ^{*,*,*}
					Skeletal muscle: overexpression Dp Δ71–78	Dys-TG- <i>mdx</i> mice 43.5
					Skeletal muscle: overexpression Dp minigene	mini-Dys-TG- <i>mdx</i> mice 25 ^{*,*,*}
	(Lindsay et al., 2021b)			43 [*]		
	(Lindsay and Russell, 2023)			45 [*]		
Female <i>mdx</i>	(Yamamoto et al., 2010)	SR 10–30 s	44.5 ^{*,*,*}	20 ^{**}		
	(Lindsay et al., 2021b)			20 ^{*,*,*,*,*}		
	(Lindsay and Russell, 2023)			80 ^{**}		
	(Lindsay et al., 2021b)	Treadmill 15 min		84 ^{*,*,*,*,*}		
	(Lindsay and Russell, 2023)					
Female <i>mdx</i> -het	(Yamamoto et al., 2010)	SR 10–30 s	1.5			
	(Lindsay and Russell, 2023)			57		
	(Lindsay and Russell, 2023)	Treadmill 15 min		81 ^{*,*,*,*}		
Male <i>mdx52</i>	(Saoudi et al., 2021)	SR 10–30 s	90 [*]			
	(Hashimoto et al., 2022)		82 [*]			
	(Aupy et al., 2020)		85 ^{*,#}		Skeletal muscle: 25%	IV (peripheral); AAV9-U7Ex51 92 [*]
	(Saoudi et al., 2023)		86.5 ^{*,###}		Brain: 3–10%	ICV (central); TcDNA 62 ^{**}
	(Saoudi et al., 2023)		92 ^{#,*}		Muscle: 15% in TA and TRI, 50% in heart; Brain: 3–10%	ICV (central); TcDNA + IV (peripheral); AAV9-U7Ex51 71 ^{**}

Note: Values that were not explicitly stated in the referenced publications were estimated using Plotdigitizer. Re-expression of dystrophin or utrophin expressed as % of WT levels. scAAV9: self-complementary adeno-associated virus serotype 9; scAAV9-U7Ex51M: AAV9 encoding the U7snRNA targeting dystrophin exon 51; AON: antisense oligonucleotide; AB: arginine butyrate; het: heterozygous; DMD: Duchenne muscular dystrophy; Dp: Dystrophin protein; Dp427: full-length dystrophin; DpΔ71–78: Dystrophin protein lacking C terminus (Δexon 71–78); Dp427/Up495: Full length dystrophin with repeats 20–24 replaced by repeats 18–22 of utrophin; ICV: intracerebroventricular; IP: intra-peritoneal; IV: intravenous; TA: tibialis anterior; TRI: triceps; SR: scruff-restraint; TR: tube-restraint; tcDNA: tricyclo-DNA; Up: utrophin protein; Up395: brain full-length utrophin; Up495: muscle full-length utrophin; WT: wild-type.

Mdx: Dp427-deficient mouse model. *Mdx52*: Mouse model lacking Dp140, Dp260 and Dp427. ^a Percentage of time spent immobile following a stressor. ^b Physical activity post-stress relative to physical activity pre-stress (% of pre-intervention). ^c 150 μL of morpholino oligonucleotide solution (0.5 mM in saline). ^d Data extracted from figure. 6E (Johnson et al., 2024). * Significantly different from male WT. ** Significantly different from male *mdx*. *** Significantly different from female WT. # Injected with phosphate buffered saline (PBS) or sodium chloride rather than as a standard control. ## *Mdx* mice injected with control sense morpholino AON rather than as a standard control. ### *Mdx52* mice injected with control sense tcDNA rather than as a standard control.

for *mdx* mice. It was suggested that a reduction in physical activity levels in *mdx* mice compared to WT mice after forced treadmill exercise was due to a deficit in sarcolemma-localized nNOS, which reduces vasomodulation in the blood vessels of muscles (Kobayashi et al., 2008). However, overexpressing a mini-dystrophin or utrophin in the skeletal muscle of *mdx* mice, an intervention that does not restore nNOS localization to the sarcolemma, reduced stress-induced physical inactivity to approximately 50% of WT mice (Belanto et al., 2014). In the same study, transgenic overexpression of a nearly full-length dystrophin successfully reduced the immobility of *mdx* mice following forced treadmill exercise (Belanto et al., 2014). These outcomes are challenging to interpret

because there is no conclusive evidence to indicate whether muscle quality (improved with mini-dystrophin and utrophin overexpression in *mdx* mice), muscle/motor performance or stress, drive the immobility to forced treadmill exercise in the *mdx* mouse.

4.2.4. Electric foot shocks

Foot shock-induced stress has been used to reflect depression, anxiety, and post-traumatic stress disorder in rodents (Bali and Jaggi, 2015). Foot shock may also induce a heightened level of fear in WT and *mdx* mice, with the latter experiencing a larger response (De Boer and Koolhaas, 2003; Sekiguchi et al., 2009; Siegmund and Wotjak, 2007).

Sekiguchi et al. also reported a stronger fear memory in *mdx* mice when animals were placed back in the same foot shock protocol 24 h later (contextual fear memory) (Sekiguchi et al., 2009). In this test, mice are placed in the conditioning box, which consists of a grid floor through which an electric current can be passed. However, the intensity of the stressful stimulus, which can vary by duration and amperage, did not distinguish a difference in the fear response between a WT and *mdx* mouse in two other studies (Comim et al., 2019; Vaillend and Chaussonot, 2017). Comim et al. suggested that the inability of foot shock stress to distinguish between WT and *mdx* mice could be due to a similar flinch response and nociceptive threshold to the stressor in their experimental conditions (Comim et al., 2019).

4.2.5. Auditory stress

In contrast with contextual fear conditioning, the phenotype reported in *mdx* mice submitted to auditory-cued fear conditioning paradigms was unexpected, as it was characterized by reduced conditioned freezing as compared to WT littermate controls (Chaussonot et al., 2015; Vaillend and Chaussonot, 2017). However, this amygdala-dependent task was not aimed to measure spontaneous fear responses nor contextual fear memory, and the reduced freezing in response to conditioning stimuli rather reflected a deficit or delay in learning aversive cue-outcome Pavlovian associations. In this task, a 30 s auditory tone was used as the conditioning stimulus (CS) delivered just before an electric foot shock (unconditioned stimulus, US). Both WT and *mdx* mice displayed high and rapid ceiling amounts of freezing following the electric foot shocks. The delivery of CS-US pairs was repeated in successive trials at variable intervals of 60 – 180 s to allow mice to resume running. In both WT and *mdx* mice, the amount of freezing specifically expressed during the 30 s auditory tone delivery was initially low. It rapidly increased in WT mice across trials, as they progressively learnt that the tone predicts delivery of the electric foot shock. This progressive improvement of performance was significantly delayed in *mdx* mice, indicating delayed associative fear learning. Although *mdx* mice may finally reach comparable learning performance as WT mice on the last trial, they also expressed lower freezing amounts when the tone was delivered 24 h later in a different experimental box, suggesting an additional deficit in auditory-cued fear memory.

4.2.6. Social subordination

Social stress has been applied in WT rodents to recapitulate human psychopathological impairments, including major depression (Kampen et al., 2002), and is developed based on natural behavior to establish a territory by male mice and its defense against male intruders (Bartolomucci et al., 2001). A social subordination stress can be categorized as a severe stressful stimulus because Razzoli et al. showed that *mdx* mice exposed to social defeat (mice were exposed to a 10-min social defeat from a CD1 mouse, while also being in continuous sensory contact) displayed excessive amounts of stress-induced motor inhibition, sustained hypotension, severe cardiac damage, and a lethality rate of 70%. In contrast, the scruff-restraint caused transient hypotension with no report of lethality (Razzoli et al., 2020).

4.3. Anxiety-related phenotypes in Dp427-deficient mice

Significant yet debated distinction lies in the concepts of fear and anxiety. Fear and anxiety share substantial similarities in how they impact subjective experiences, behaviors, physiological responses, and neurological functions. Nevertheless, specific differences set the two apart. Fear is commonly characterized as a sudden and intense fight-or-flight response triggered by an immediate and identifiable threat. On the other hand, anxiety is often described as a more prolonged state of tension, worry, and apprehension concerning uncertain and potentially negative future events. While fear is associated with specific and immediate threats, anxiety is conceptualized as an emotional response to vague and potential threats that may generate conflicting motivations

and emotions. It is crucial to underscore that fear and anxiety still display significant overlap in subjective, behavioral, physiological, and neurological characteristics (Duval et al., 2015). This detailed insight underscores the complex nature of fear and anxiety, exposing the various dimensions of these emotional reactions. Existing evidence also indicates shared central mechanisms and partially overlapping neural bases for fear and anxiety in both animals and humans. However, more research is necessary to comprehend the specific circuits that regulate anxiety behaviors.

The distinction between mouse models of DMD appears to explain variations in the manifestation of anxiety and fear responses. The *mdx52* mouse model, characterized by the absence of the Dp427 and Dp140 isoforms in the brain (Araki et al., 1997), exhibits a more severe anxiety response compared to *mdx* mice, which solely lack the Dp427 isoform. However, both mouse models demonstrate comparable fear responses. Another illustrative instance is the *mdx^{3cv}* model, lacking the Dp71 and Dp140 isoforms, which displays heightened anxiety-related behaviors. While these observations underscore the potential overlap in central origin, they also suggest that fear responses may involve distinct mechanisms dependent on Dp427. Interestingly, Saudi et al. used exon-skipping therapy and successfully restored Dp427 dystrophin protein expression in the hippocampus, cerebellum, and cortex of *mdx52* mice in the range of 3–10% of WT levels through the administration of tricyclo-DNA antisense oligonucleotides. Specifically targeting dystrophin exon 51, this approach exclusively restored Dp427 and did not reinstate Dp140 expression (Saudi et al., 2023). However, it notably led to a significant reduction in anxiety and fear responses in *mdx* mice. This observation underscores the crucial involvement of Dp427 in emotional responses. Conversely, a comparable pattern is evident in patients with DMD, where the absence of both Dp427 and Dp140 isoforms correlates with higher anxiety symptom scores compared to patients lacking only Dp427 (Maresh et al., 2023). This alignment with the observed mouse model distinctions further underlines the potential role of specific dystrophin isoforms in anxiety responses, emphasizing the need for comprehensive investigation into the distinction interplay between dystrophin isoforms and emotional behaviors.

A recent study, using the DBA/2 J (D2) *mdx* mice (show earlier onset and worsened pathology relative to *mdx* mice (Coley et al., 2015)), also provided indirect evidence that distinct mechanisms could underlie the anxiety and unconditioned fear phenotypes (Copeland et al., 2022). They investigated kynurenine (KYN) metabolism, which has emerged as a metabolic pathway to explain the hypersensitive stress phenotype in *mdx* mice because KYN is increased during chronic stress, in various mood disorders (Agudelo et al., 2014; Huang et al., 2020; Kim and Jeon, 2018; Wang et al., 2018) and it has a crucial role in governing communication between skeletal muscles, brain, vasculature, and other peripheral organs (Agudelo et al., 2014; Pedersen, 2019; Savitz, 2020). D2-*mdx* mice had altered KYN metabolism, presenting lower levels of circulating kynurenic acid (KYNA) and a reduced KYNA:KYN ratio relative to WT mice. The KYNA:KYN ratio is indicative of KYN aminotransferase activity and the balance between the neuroprotective [KYNA] and neurotoxic [KYN-NAD] arms of the KYN pathway. The KYNA arm is considered neuroprotective, as it converts KYN into the non-blood-brain barrier transportable metabolite KYNA. The KYN-NAD arm, regulated by KYN mono-oxygenase, is considered neurotoxic, as it transforms KYN into metabolites capable of inducing free radical production and neuronal apoptosis (Wu et al., 2018). Lower KYNA:KYN ratios have also been associated with increased risk of depression and anxiety symptoms (Liu et al., 2018; Wu et al., 2018). However, findings by Johnson et al. suggest that the KYN pathway and associated NAD⁺ metabolic pathways are not associated with the fear response in *mdx* mice (Table 1) (Johnson et al., 2024). These studies suggest that there is a distinction between anxiety and fear in dystrophin-deficient mice, the latter not associated with KYN metabolism.

4.3.1. Bright light

Anxiety-like behavior is also enhanced in *mdx* mice (Vaillend and Chausse-not, 2017) and can vary in severity depending on the type of anxiety test used. This spectrum of anxiety-like behavior could be attributed to the nature of anxiogenic stimuli in terms of strength and intensity. The dark/light box stressor is a test commonly used in animal research to evaluate anxiety-like behavior in rodents. The test involves a box with two compartments, one dark (<10 lux) and one brightly lit (~600 lux). The dark/light box is a low-motor demand test and is designed according to the natural aversion of mice to the brightly illuminated environment despite their motivation to investigate novel spaces (Crawley and Goodwin, 1980). Time spent in the light compartment, number of entries and latency to visit this compartment are used as a measure of anxiety-like behavior, as animals with higher anxiety levels tend to avoid anxiogenic areas (brightly lit). There are contrasting data presented on the response of dystrophin-deficient mice to the dark/light box. Vaillend et al. observed no differences in any dark/light box parameter between WT and *mdx* mice (Vaillend et al., 1995) but Vaillend and Chausse-not later showed that *mdx* mice spend shorter time in the lit area relative to WT mice (Vaillend and Chausse-not, 2017), which could be attributed to a different experimental setup (100-W desk lamp compared to a 600 lux light source, respectively). Rimmelin et al. revealed that female *mdx* mice displayed a similar response to the dark/light box as male *mdx* mice, with significantly less time spent in the lit compartment relative to WT mice (Rimmelin et al., 2016). However, the same group, applying the same protocol, also observed a comparable dark/light response between male *mdx* mice and WT mice (Engelbeen et al., 2021). *Mdx52* mice, which have a comparable fear response to *mdx* mice (scruff-restraint) (Saoudi et al., 2021), spend less time, with higher latency and less number of entries to the lit compartment relative to WT and *mdx* mice (Saoudi et al., 2021). This suggests that the absence of shorter brain dystrophin isoforms might be responsible for the heightened levels of anxiety in *mdx52* (Saoudi et al., 2021). These data further suggest that the dark/light box is not an optimal assay to assess differences between *mdx* and WT mice and that if researchers were to use this assay in the future, a standardized protocol should be adhered to.

4.3.2. Open space and void

The elevated plus maze is a common test used to assess anxiety by producing strong approach-avoidance conflict in rodents (Walf and Frye, 2007). This test is designed based on the tendency of rodents to avoid open spaces and fear void, leading them to prefer seeking shelter in closed areas (Walf and Frye, 2007). Despite variations in light intensity between studies, the number of entries and time spent in the open arms in the elevated plus maze test was reported to be comparable in *mdx* mice relative to WT mice (Comim et al., 2019; Saoudi et al., 2021; Sekiguchi et al., 2009; Vaillend and Chausse-not, 2017). In the *mdx52* mouse, the total number of visits to all arms of the apparatus, as well as the number of entries and the percentage of time spent in open arms, was lower relative to WT mice (Saoudi et al., 2021). During open field exploration, anxiety may also relate to open-space avoidance and can be evaluated by measuring the time spent by mice in the central area of the field. Here again, *mdx52* mice display an anxiety-related phenotype, while milder anxiety-related responses are expressed by *mdx* mice only when the experimental condition is more anxiogenic (no sawdust, brighter illumination). These data suggest that *mdx* mice lacking Dp427 display borderline anxiety behaviors, while a cumulative deficiency in Dp140 and Dp427 exacerbates anxiety-like behavior in the *mdx52* mouse model of DMD.

5. Neural and physiological bases of the fear response

Being among the most robust phenotypes expressed by Dp427-deficient *mdx* mice, the fear response is currently viewed as a major outcome measure, useful in preclinical studies to evaluate the potential

of various treatments to overcome behavioral disturbances associated with dystrophin deficiency. However, the altered mechanisms and brain circuits underlying the fear response are still unclear. Recent studies have raised several working hypotheses suggesting a multifactorial origin involving a complex interplay between central and peripheral physiological processes that we describe in this section (Fig. 4).

5.1. Brain dystrophin deficiency and its rescue

Dp427 is expressed in principal neurons of several brain structures that play roles in the processing of aversive and fearful stimuli, at least including the amygdala as well as cortical, cerebellar, and hippocampal regions. This suggests that Dp427 loss is, at least in part, responsible for the impairment of the brain's circuit of fear and fear response in *mdx* mice. The comparable fear response of the *mdx* and *mdx52* mouse (Aupy et al., 2020; Hashimoto et al., 2022; Saoudi et al., 2021), along with evidence from the *mdx*^{3Cv} mouse (may have low-level residual expression of all dystrophin isoforms due to a "leaky" point mutation in intron 65 (Daloz et al., 2001; Li et al., 2008, 2010b)) that does not have more noticeable deficits than the *mdx* mouse (Vaillend and Ungerer, 1999), are consistent with studies investigating emotional behavioral problems in patients with DMD that are mainly affected by Dp427 deficiency (Ricotti et al., 2016; Vaillend and Ungerer, 1999).

In the study by Sekiguchi et al., the fear response of *mdx* mice to scruff-restraint was partially rescued following intracerebroventricular administration of a morpholino oligonucleotide that induced skipping of the *dmd*-gene exon 23 that contains an aberrant stop codon (Sekiguchi et al., 2009). This allowed recovery of brain Dp427 expression as a truncated but functional form to approximately ~27% of WT levels in the postsynaptic density fraction of brain tissue (Sekiguchi et al., 2009). In the study by Goyenville et al., the systemic delivery of tricyclo-DNA antisense oligonucleotides successfully restored Dp427 expression in many tissues of the *mdx* mouse, including skeletal muscle, heart, and brain (approximately less than 6% in brain and up to 50% in diaphragm) (Table 1) (Goyenville et al., 2015; Relizani et al., 2017). In these studies, the fear response was ameliorated completely in *mdx* mice that received tricyclo-DNA treatment at 200 mg/kg/week for 12 weeks (Goyenville et al., 2015; Relizani et al., 2017). In another study, exploring the therapeutic potential of systemically delivering an adeno-associated virus (AAV) vector containing a single antisense sequence targeting exon 51 in *mdx52* mice, Aupy et al. restored the level of peripheral dystrophin (skeletal and smooth muscles) to 10–25% of WT levels but failed to detect dystrophin in the brain (Table 1) (Aupy et al., 2020). These peripheral dystrophin levels improved skeletal muscle and motor function in *mdx52* mice but no improvement was observed in the fear response of *mdx52* mice to scruff-restraint stress relative to untreated mice (Aupy et al., 2020). In another study, Saoudi et al. investigated exon-skipping therapy effects using tricyclo-DNA antisense oligonucleotides to restore dystrophin expression in the brain of *mdx52* mice (Saoudi et al., 2023). Restoring brain dystrophin to 3–20% of WT levels in the hippocampus and cerebellum led to a significant reduction in anxiety, unconditioned fear, and improved fear conditioning acquisition. However, restoring Dp427 in skeletal and cardiac muscles through systemic treatment did not further improve the unconditioned fear response (Saoudi et al., 2023). These results suggest that the fear response in mouse models of DMD likely has a central origin. Still, it is important to highlight that further experimental evidence is needed to determine the putative contribution of muscle Dp427.

5.2. GABA_A receptor dysfunction in the brain fear circuit

It has been suggested that the fear response of *mdx* mice to stressful stimuli is not associated with greater pain sensitivity, spontaneous catalepsy, obvious depressive-like behavior and neophobia, but rather with an impaired coping strategy (Vaillend and Chausse-not, 2017). The amygdala is a part of the brain that is crucial for regulation of defensive

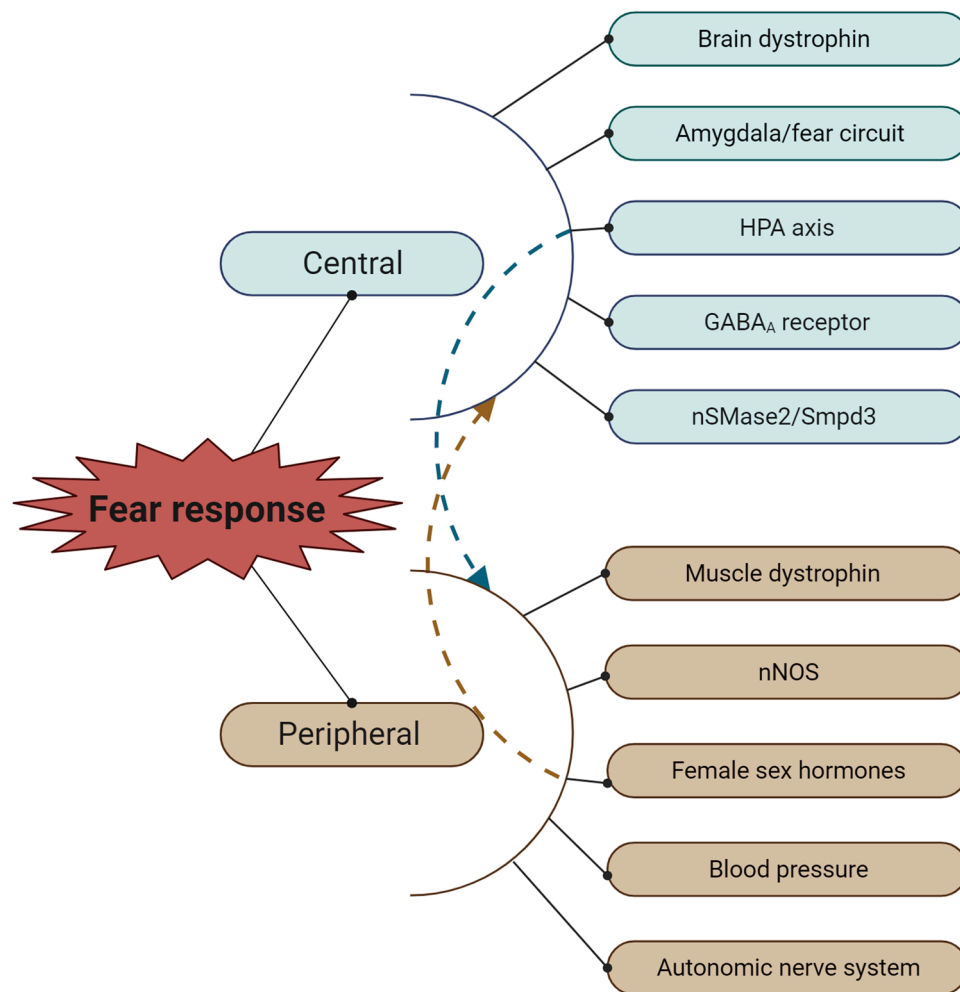


Fig. 4. Schematic representation of the mechanisms involved in the fear response in dystrophin-deficient mice. The fear response in mouse models of DMD may result from a coordinated interplay between its central and peripheral origins. Metabolites produced due to the activation of a pathway in the central origin could potentially exert an impact on the peripheral origin, and vice versa (illustrated by a dashed line). HPA; hypothalamic-pituitary-adrenal, nNOS; neuronal nitric oxide synthase.

behavior and the transition between different modes of defense in rodents (Gozzi et al., 2010). A theory to explain the fear response of *mdx* mice is that in the absence of brain-specific dystrophin, the aversive brain system modulated by the hippocampus and amygdala that controls defensive and fear-related motor behaviors is altered.

Norepinephrine is released in response to stressful stimuli, and in the basolateral amygdala (BLA) of *mdx* mice, the norepinephrine-induced GABAergic inhibitory synaptic current is decreased, which might explain impaired controlling of the excitability of BLA in response to stressful stimuli (Sekiguchi et al., 2009). Local GABAergic inhibition has a critical role in controlling amygdala circuit activity and dystrophin deficiency induces depression in amygdala GABAergic synaptic activity, which has been associated with a reduced clustering of distinct subunits of GABA_A receptors (Sekiguchi et al., 2009). GABA_A receptor clustering might contribute to activity-dependent synaptic plasticity, as shown in hippocampal circuits (Vaillend et al., 2010). In fact, GABA_A receptor α_1 and α_2 subunits in the cerebral cortex of *mdx* mice, as well as the α_1 subunit in Purkinje cells and α_2 subunit in the CA1 and CA3 regions of the hippocampus and BLA, are reduced (Knuesel et al., 1999, 2001; Kueh et al., 2008; Sekiguchi et al., 2009). This reduction of GABA_A receptors at postsynaptic sites is not associated with a decrease in the total amount of receptors, but is rather attributed to an impaired stabilization and clustering due to dystrophin deficiency (Kueh et al., 2008). As shown in the hippocampus, the deficiency in GABA_A receptor clustering

of the *mdx* mouse can be successfully restored to WT levels utilizing an exon-skipping strategy that rescues dystrophin (Vaillend et al., 2010). These results strengthen the hypothesis of dystrophin's role in reduced GABAergic inhibition and altered synaptic plasticity through disrupted GABA_A receptors clustering and lateral diffusion to extrasynaptic sites.

Enhanced behavioral reactivity to natural aversive stimuli, harm avoidance behavior and ambiguous cue discrimination learning is reported in mice heterozygous for the GABA_A receptor γ_2 -subunit that causes reduced synaptic GABA_A receptor clustering (Crestani et al., 1999). Altered composition of receptors in synaptic and extrasynaptic sites has also been shown in *mdx* mice (Zarrouki et al., 2022a), and an electrophysiological study revealed a high sensitivity of cerebellar neurons to 4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridine-3-ol hydrochloride (THIP), a selective activator of extra-synaptic subunit-containing GABA_A receptors (Kueh et al., 2011). Extra-synaptic subunit-containing δ GABA_A receptors have a high affinity to THIP and are responsible for mediating tonic inhibition. Alteration in the expression of δ GABA_A receptors and tonic inhibition is linked to some neuropsychiatric phenotypes that have also been reported in patients with DMD and associated with stress-related phenotypes in *mdx* mice (Vaillend and Chaussonet, 2017). The results obtained from testing the behavioral effect of *in vivo* administration of THIP by Vaillend et al. revealed that *mdx* mice display more sensitivity to 10 and 30 mg/kg doses of THIP, with sedative/ataxic and hypotonic effects, respectively (Vaillend and Chaussonet, 2017).

These observations suggest a contribution of altered tonic inhibition mediated by extrasynaptic receptors on the fear response in *mdx* mice.

5.3. *nSMase2/Smpd3*

Neutral sphingomyelinase 2/sphingomyelin phosphodiesterase 3 protein (*nSMase2/Smpd3*) is expressed predominantly in the brain and is involved in the regulation of lipid raft integrity, thus affecting membrane trafficking, receptor clustering, and signal transduction, as well as cellular stress response, cell growth and inflammation signaling pathways (Wu et al., 2010). Recently, *nSMase2* has been shown to reduce muscle degeneration and inflammation in young *mdx* mice, and was also proposed as a potential modulator of the fear response in *mdx* mice (Matsuzaka et al., 2020). In the study by Matsuzaka et al., the heightened fear response in *mdx:Smpd3*^{-/-} mice at 11–12 weeks of age was partially rescued. This study suggested that the congenital loss of *nSMase2*'s possibly improved the behavioral phenotype by normalizing brain-derived neurotrophic factor (BDNF), which is known to modulate GABAergic synaptogenesis and synaptic plasticity (Matsuzaka et al., 2020). This raised a new potential strategy for treatment of central deficits associated with DMD by modulating lipid-raft integrity and/or BDNF-dependent signaling.

5.4. The hypothalamic-pituitary-adrenal (HPA) axis

The body maintains a balance in the physiologic niche through processes collectively known as homeostasis. Homeostasis is constantly challenged through the intrinsic or extrinsic forces defined as stressors (Chrousos and Gold, 1992; de Kloet et al., 1993a). To overcome changes in homeostasis, the body utilizes the stress system; a central and peripheral biomechanism encapsulating metabolites and hormones derived from the efferent sympathetic/adrenomedullary system (SAM) and the hypothalamic-pituitary-adrenal (HPA) axis at both the cellular and molecular level (Tsigos et al., 2020). The SAM pathway plays a vital role in the fight-or-flight response by synthesizing catecholamines to increase alertness, metabolic function and excite the cardiovascular system through β -adrenergic receptors (Godoy et al., 2018; Ulrich-Lai and Herman, 2009). The HPA axis develops an appropriate response to any stressor or threat and is comprised of the paraventricular nucleus of the hypothalamus, pituitary gland and the adrenal cortices, which act synergistically to generate biosynthetic end products, such as glucocorticoids (cortisol/corticosterone) (Bornstein and Chrousos, 1999; De Kloet, 2013). Glucocorticoids impact several hormone-responsive genes to change metabolism via the glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) (Pratt, 1990; Weikum et al., 2017), which are crucial for cardiovascular and sodium homeostasis (De Kloet et al., 1993b) as well as stress activated neural metabolism and circuitry (de Kloet et al., 1993a; Feldman and Weidenfeld, 1999).

The HPA axis has repeatedly been shown to be associated with the fear response of *mdx* mice. In WT and *mdx* mice, serum corticosterone increases to a similar level following a 10 s scruff-restraint indicating that both genotypes experience comparable levels of HPA axis activation (Yamamoto et al., 2010). However, the lower level of circulating adrenocorticotrophic hormone (ACTH) observed in *mdx* mice under non-stressed conditions suggests a possible disruption in the processes involving the HPA axis in response to stressful stimuli (Vaillend and Chausseot, 2017). Thus, the heightened fear response in *mdx* mice might not be independent from HPA axis activation. In contrast, abnormal HPA axis activity in *mdx* mice relative to WT mice was shown by Razzoli et al. where a single episode of social subordination stress followed by sensory housing with an aggressive CD1 male mouse induced a consistent increase in plasma corticosterone six hours after the exposure that remained above baseline for up to 24 hours (Razzoli et al., 2020). HPA activity has also been attributed to the exacerbation of dystrophinopathy phenotypes in *mdx* mice, where daily, repeated scruff-restraint leads to an enlarged adrenal cortex, suggesting chronic

activation of the HPA axis (Lindsay et al., 2021a). This view was further supported by evidence that urinary corticosterone is higher in *mdx* mice relative to WT mice three hours following a 30 s scruff-restraint (Lindsay et al., 2021a). Pharmacological inhibition of the HPA axis in *mdx* mice, however, failed to affect scruff-induced physical inactivity and likewise, exogenous injection of corticosterone did not affect this phenotype either (Lindsay et al., 2021a).

Interestingly, studies of large cohorts of WT and *mdx* mice revealed sex-dependent and inter-individual variability, as female *mdx* mice, relative to male *mdx* mice, showed less sensitivity to stress. These differences in the fear response were linked to HPA and renin-angiotensin-aldosterone system (RAAS) activity, where terminal hormones of these axes (corticosterone and aldosterone, respectively) were between 6- and 69-fold greater in the most physically active mice relative to the least physically active mice (Lindsay et al., 2021b). Contrary to previous reports (Razzoli et al., 2020), these data indicate the stress-induced physical inactivity is not driven by HPA axis, but that greater HPA and RAAS activity is required to overcome the inability of *mdx* mice to generate an adequate fight-or-flight response to stress.

5.5. Functional interplay between central and peripheral processes

The question was raised as to whether brain-specific dystrophin is sufficient to rescue the fear response of *mdx* mice to more severe stressful stimuli (e.g., social subordination) and whether peripheral dysfunction also contributes to this phenotype.

In a study by Razzoli et al., both *Fiona-mdx* and *Dys* ^{Δ MTB}-*mdx* mice, which transgenically express utrophin (dystrophin homologue) and full-length dystrophin/utrophin chimera in skeletal muscle respectively, presented with WT-like physical activity levels after scruff-restraint, while the cardiovascular response to subordination stress in *Fiona-mdx* mice showed an intermediate phenotype in harmony with its moderate mortality rate (Table 1) (Razzoli et al., 2020). In contrast, systemic injections of arginine butyrate (AB) at adulthood, which upregulates muscle and brain utrophin expression in *mdx* mice, failed to improve the fear response (Perronet et al., 2012). Thus, it is possible that peripheral dysfunction, which includes striated muscle functionality, also contributes to the development of the fear response in *mdx* mice and that peripheral interventions may represent an alternative or complementary route to provide protection against moderate (scruff-restraint) and severe (social subordination) stressful stimuli (Fig. 4).

5.6. Autonomic nervous system (ANS)

The ANS is comprised of the sympathetic and parasympathetic branches, and it plays a pivotal role in the regulation of heart rate and cardiac output. Alterations in the distribution and function of parasympathetic and sympathetic nerves can disrupt the delicate balance between these two branches of the ANS. This imbalance can lead to excessive sympathetic activation and reduced parasympathetic tone, which can increase the risk of arrhythmias and sudden cardiac death (Vaseghi and Shivkumar, 2008).

Freezing is a defensive behaviour that animals demonstrate to stressful stimuli, which is considered as a state of avoiding detection by predators and increasing perceptual acuity (Lojowska et al., 2015). Freezing includes physical inactivity and parasympathetic-induced bradycardia. In contrast, fight-or-flight is driven by sympathetic stimulation that induces tachycardia. The flexible shifting between freezing and active defensive responses is required for a proper fear response. During a threat or stress, both the sympathetic and parasympathetic branches of the ANS are activated concurrently, but it is only when the parasympathetic response predominates that defensive freezing occurs (Roelofs, 2017).

Patients with DMD often display abnormalities in circadian rhythm and heart rate variability, suggesting disturbances in both sympathetic and parasympathetic activities (Inoue et al., 2009). Autonomic

dysfunction in patients with DMD is characterized by a reduction in the parasympathetic aspect and an increased dominance of the sympathetic component. This impairment intensifies as the disease advances with age (da Silva et al., 2018; Yotsukura et al., 1998). As muscle fibrosis escalates in proportion to age, a continuous inflammatory process might prompt the brain to address the threat through the ANS. This involves sending out a signal through the vagus-inhibiting pro-inflammatory cytokine synthesis (Tracey, 2002).

In the Maresh study, it was observed that unconditioned responses to aversive threat stimuli in patients with DMD resulted in a significant drop in heart rate, a phenomenon not observed in the non-DMD control group (Maresh et al., 2023). Similarly, the Razzoli study observed threat-induced tachycardia followed by chronic bradycardia in *mdx* mice. Hypotension developed over the 24 hours following a single subordination stress, leading to a subsequent decrease in heart rate, ultimately resulting in the demise of 5 out of 9 *mdx* mice (Razzoli et al., 2020).

This information suggests that dysfunction of the ANS in *mdx* mice exposed to stressful stimuli might result in a different imbalance in sympathetic and parasympathetic nerves. This underscores the importance of understanding the roles of both sympathetic and parasympathetic nerves in fear responses in the absence of dystrophin, an area that has received limited attention.

5.7. Blood pressure

Patients with DMD have lower resting blood pressure compared to unaffected controls. This condition is associated with worse diaphragmatic muscle strength and cardiac status, including elevated cardiac biomarkers and the absence of nocturnal dipping, which can lead to premature death (Cheeran et al., 2017; Marui et al., 2018; Masood et al., 2015). Lower resting blood pressure is likely associated with the loss of dystrophin expression in smooth muscle cells, where it can regulate functionality via nitric oxide (NO) signaling and α -adrenergic processes (Ito et al., 2006). The importance of dystrophin in the vascular system is further highlighted by reports regarding abnormalities in blood vessel structure and function in both patients and animal models of DMD (Kaplan and Morgan, 2022). Additionally, irregularities in the mechanical properties of the carotid arteries have been observed in *mdx* mice, along with changes in the spontaneous motor activity of their portal veins (Dye et al., 2007; Mancinelli et al., 1999). These peripheral blood flow abnormalities are present centrally, where a decrease in cerebral blood perfusion has been observed in the brains of individuals with DMD (Doorenweerd et al., 2014). This decrease is primarily attributed to the absence of dystrophin, a link that could also extend to the reported reduction in cerebral diffusion observed in both young and adult *mdx* mice (Goodnough et al., 2014). Therefore, the concurrent presence of low blood pressure and inadequate cerebral blood perfusion could potentially contribute to brain-related comorbidities among patients with DMD. In *mdx* mice, social subordination stress leads to 70% lethality within 48 hours, which is associated with a hypotensive response (Razzoli et al., 2020). Similarly, the higher levels of physical inactivity in *mdx* mice relative to WT mice in response to scruff-restraint was associated with a transient loss in mean arterial blood pressure relative to a hypertensive response, respectively (Razzoli et al., 2020). This hypotensive phenotype of *mdx* mice is likely associated with abnormal HPA axis activation and depression in amygdala activity (Gianaros et al., 2008; Razzoli et al., 2020), with female *mdx* mice exhibiting higher HPA and RAAS activation following stress relative to male *mdx* mice (Lindsay and Russell, 2023; Lindsay et al., 2021b). The hypotensive phenotype in response to a five min tube-restraint in male *mdx* mice closely resembles the hypotensive response to scruff-restraint and social subordination stress (Lindsay and Russell, 2023). Female *mdx* mice were hypertensive in response to a five min tube-restraint, with higher mean arterial blood pressure relative to male *mdx* mice, suggesting the possible role of female sex hormones in regulating blood

pressure in response to stressful stimuli (Lindsay and Russell, 2023). Heterozygous female *mdx* mice, which express 30–60% brain and skeletal muscle dystrophin (Weller et al., 1991; Yamamoto et al., 2010), manifest a WT-like hypertensive response to a five min tube-restraint (Table 1) (Lindsay and Russell, 2023). These data suggest that this level of dystrophin expression, in brain and skeletal muscle, is adequate to develop a normal physiological response to a stress stimulus.

5.8. nNOS

It has been suggested that low levels of physical activity in *mdx* mice relative to WT mice after forced treadmill exercise were due to a deficit in sarcolemma-localized nNOS, which reduces vasomodulation in the blood vessels of muscles (Kobayashi et al., 2008). However, transgenic overexpression of a mini-dystrophin (mini-Dys-TG-*mdx*) or utrophin (Fiona-*mdx*) in the skeletal muscle of *mdx* mice, which does not restore nNOS localization to the sarcolemma (Lai et al., 2009; Li et al., 2010a), restored physical activity levels of *mdx* mice to 25% of their pre-stress levels relative to ~50% in WT mice and ~2% in *mdx* mice (Belanto et al., 2014) (Table 1). In the same study, transgenic overexpression of a nearly full-length dystrophin in skeletal muscle only, one that restored nNOS to the sarcolemma, fully rescued the immobility response of *mdx* mice to forced treadmill exercise (approximately 45% of physical activity levels). These data suggest that nNOS, and by association, the capacity to manipulate blood flow in skeletal muscle, is partially associated with physical inactivity in *mdx* mice following forced treadmill exercise.

5.9. Female sex hormones

The fear response has been primarily investigated in dystrophin deficient male vertebrates because DMD is an X-linked disease and females carrying one copy of a mutant DMD gene have long been considered non-affected. However, although female carriers express more than 60% of normal levels of dystrophin (Pegoraro et al., 1995), they are at risk of dilated cardiomyopathy (Ogata et al., 2000), altered gait, fatigue, muscle weakness (Bushby et al., 1993) and some also manifest similar neurocognitive disorders as boys with DMD, including increased anxiety and a deficiency in cognitive flexibility (Demirci et al., 2020). Variation in clinical severity among female carriers might be a result from either partially efficient skewed X-inactivation (Soltanzadeh et al., 2010) or the mutation profile leading to the loss of several brain dystrophin isoforms (Demirci et al., 2020). The heterozygous female *mdx* mouse manifests a comparable response to scruff-restraint as WT mice (i.e., no fear response) (Lindsay and Russell, 2023; Yamamoto et al., 2010). It is inferred, based on dystrophin expression in the amygdala of heterozygous female *mdx* mice ($49.3 \pm 12.2\%$ relative to WT), as well as dystrophin restoration studies in *mdx* mice ($6\% \pm 30$) (Goyenville et al., 2015; Relizani et al., 2017; Zarrouki et al., 2022b), that low to medium level dystrophin expression in the brain is sufficient to ameliorate the fear response (Yamamoto et al., 2010). Homozygous female *mdx* mice, on the other hand, also suffer from a heightened fear response relative to WT female mice (Lindsay and Russell, 2023; Lindsay et al., 2021b; Yamamoto et al., 2010). However, homozygous female *mdx* mice are more resistant to stressful stimuli relative to male *mdx* mice (Lindsay et al., 2021b), suggesting the female sex hormones could play an important role in this behavioral phenotype. For example, relative to male *mdx* mice, female *mdx* mice have higher activation of the HPA and SAM axes following a scruff-restraint, which might be explained by larger adrenal glands (Lindsay et al., 2021b). Capitalizing on anti-anxiety and anti-depressive properties of estrogen and progesterone (P4) (Burgess and Handa, 1992; Gaskin and Kitay, 1971; Lund et al., 2006; Viau and Meaney, 1991; De Lignières and Vincens, 1982), Lindsay et al. ovariectomized (OVX) female *mdx* mice and showed that they were less physically active after stress (scruff-restraint and forced treadmill exercise) relative to sham operated mice (Lindsay et al.,

2021b). The OVX resulted in lower adrenal mass and zona glomerulosa area, which lowered aldosterone and corticosterone synthesis during a scruff-restraint (Lindsay and Russell, 2023). Estradiol (E2) replacement completely rescued the physical activity response of OVX female *mdx* mice to forced treadmill exercise but not scruff-restraint, suggesting P4 also plays an important role in the fear response and/or that the immobility displayed following treadmill exercise and scruff restraint rely on distinct mechanisms (Lindsay et al., 2021b). Furthermore, replacement of E2 in OVX female *mdx* mice restored adrenal gland mass, zona glomerulosa area and aldosterone synthesis during a stressor, but it did not restore corticosterone synthesis. E2 also lowered the area of the zona fasciculata in OVX female *mdx* mice that was associated with elevated cortisol synthesis. Cortisol is believed to be a faster responder to stress relative to corticosterone, while corticosterone acts more like an adaptation-related biomarker during stress (Gong et al., 2015). E2 can regulate the activity of the HPA axis (Carey et al., 1995; Shupnik, 2002), RAAS pathway (Maric-Bilkan et al., 2014) and cardiovascular system (Mendelsohn and Karas, 1999; White et al., 2005), and female *mdx* mice hyperactivate the HPA and RAAS pathways following a stress relative to male *mdx* mice (Lindsay et al., 2021b). These observations provide further support for the hypothesis that E2 partially regulates the heightened fear response of *mdx* mice.

5.9.1. Potential therapies to ameliorate fear response involving female sex hormones

Estrogen-based treatments, such as diethylstilboestrol, have historically demonstrated anabolic effects and reduced muscle permeability in patients with DMD (Cohen and Morgan, 1976; Rudman et al., 1972). A recent study has also shown the safety and effectiveness of using a selective estrogen receptor modulator (SERM; ER agonists; tamoxifen) to maintain motor and lung function in patients with DMD (Tsabari et al., 2021). These clinical trial findings align with previous research indicating the potential of estrogen-based therapies to enhance pathophysiological outcomes in *mdx* mice. Dorchie et al. showed that treatment of male *mdx* mice with tamoxifen can improve muscle strength, increase specific force and fatigue resistance in male *mdx* mice (Dorchies et al., 2013). Vang et al. showed that estrogen can improve the recovery capacity of muscle following physiological injury in *mdx* mice (Vang et al., 2021). In contrast, estrogen supplementation could not improve muscle strength in old female *mdx* mice (Hourde et al., 2013). While these studies have shown promise on pathophysiological outcomes, estrogen has also rescued the physical activity response of female *mdx* mice to mild stressful stimuli (Lindsay et al., 2021b). Clinical trial findings align with studies on *mdx* mice, illustrating that tamoxifen can enhance muscle strength, specific force, and fatigue resistance, possibly through the upregulation of estrogen receptor expression. This suggests a potential role for estrogen-based therapies in improving both pathophysiological outcomes and neurocognitive responses in DMD. However, a clinical trial (NCT03354039) aimed at evaluating the safety and efficacy of tamoxifen as an adjunct to corticosteroid therapy failed to achieve its primary endpoint, exhibiting no clinical or statistical effects when compared to a placebo (Henzi et al., 2023). Consequently, these findings do not lend support to the application of tamoxifen as an adjunct to corticosteroid therapy in routine clinical practice. This study not only fell short in demonstrating the intended therapeutic effect of delaying disease progression but also lacked the implementation of neurological assessments aligned with physiological measurements. Therefore, there exists a possibility for repurposing tamoxifen to improve neurological disorders in patients with DMD, particularly in the context of mitigating the fear response.

6. Impact on DMD pathophysiology

The fear response in *mdx* mice can negatively impact pathophysiological phenotypes of DMD (Lindsay et al., 2021a, 2021b), including sustained hypotension, cardiac damage and death (Razzoli et al., 2020).

Mdx mice that are exposed to acute and repetitive stress have greater levels of skeletal muscle fibrosis than non-stressed *mdx* mice (Lindsay et al., 2021a). Hydroxyproline concentration, as an indicator of fibrotic content, is elevated in the diaphragm as well as slow and fast twitch hindlimb skeletal muscles of *mdx* mice, except the extensor digitorum longus. Additionally, repetition of daily scruff-restraint slows the relaxation rate and increases the passive stiffness of isolated skeletal muscles and enlarges the adrenal gland mass and adrenal cortex area in *mdx* mice (Lindsay et al., 2021a). There is further evidence to suggest that the heterogeneity observed in the fear response in male *mdx* mice can impact dystrophinopathy phenotypes, because *mdx* mice with higher levels of physical activity after stress present with greater skeletal muscle oxidative capacity, capillary density, utrophin expression, fewer centrally nucleated fibers and a greater uniformity in fiber size relative to *mdx* mice with the low levels of physical activity after stress (Lindsay et al., 2021b). These data suggest that the fear response in *mdx* mice is associated with, and can exacerbate, DMD pathogenesis and cause death.

7. Challenges for patients with DMD

Understanding the mechanistic basis of the startle response in patients with DMD is experimentally and ethically challenging. First, the spectrum of behavioral stressors applied to animal models of DMD may inadvertently exacerbate dystrophinopathy phenotypes and have life-threatening consequences for patients, thus preventing the possibility of attaining ethical approval. Maresh et al. further suggested that challenges underlying the assessment of the startle response in patients with DMD included repeated testing across multiple days and a cognitive and neurodevelopmental problem selection bias (Maresh et al., 2023). It is also possible that the startle response could be greater in patients with DMD who are at an advanced disease state or have greater neurodevelopmental comorbidities because some individuals show a tendency to develop a deterioration in anxiety and OCD even though evidence suggests the neuropsychiatric profile of patients in general does not change over time (Battini et al., 2021; Cotton et al., 2005; Hellebrekers et al., 2020). Considerable heterogeneity in behavioral and physiological phenotypes in the DMD population, lack of specific neurocognitive diagnostic assessment tools, variable physician-prescribed care plans, educational status of the patients, as well as medical history and current treatment can also influence the results of studies investigating cognitive and neurodevelopmental problems in DMD (Sayed et al., 2022; Snow et al., 2013). For example, and with relevance to the startle response in patients with DMD, participants were not stratified according to corticosteroid administration (Maresh et al., 2023). Corticosteroids are the primary treatment for boys with DMD, improving muscle strength and motor function through their anti-inflammatory properties (Angelini and Peterle, 2012). However, it is known that corticosteroid therapy in the general population can impact the stress response via HPA axis activity (Bennett, 1954; Ortega et al., 1976; Webb and Clark, 1981). Similarly, the iatrogenic suppression of the HPA axis induced by corticosteroid therapy in patients with DMD results in impaired pituitary adrenocorticotrophic hormone secretion and reduced stimulation of the adrenal cortex and consequently, atrophy of the adrenal gland and adrenal insufficiency (Krasner, 1999). Therefore, adrenal insufficiency might limit the ability of patients with DMD to develop an appropriate response to stressful stimuli. This view can be supported by the reports of unexplained deaths amongst patients with DMD in response to severe physiological stress (Kinnett and Noritz, 2017), which could be attributed to instances of adrenal insufficiency independent of cardiorespiratory failure (Van Ruiten et al., 2016a). Thus, corticosteroid-induced adrenal insufficiency will likely affect our understanding of the startle response because we will not be able to discriminate between a stress response or a stress response influenced by corticosteroid therapy. A further caveat to our limited understanding of the startle response in patients with DMD is the lack of blood pressure data during stressful

stimuli (Maresh et al., 2023), thus limiting our ability to understand the translatability of hypotension findings in *mdx* mice (Lindsay and Russell, 2023; Razzoli et al., 2020).

7.1. Pharmacological consideration for anxiety-related phenotypes

The altered GABA_A receptors, characterized at least by reduced distribution in the prefrontal cortex in patients with DMD (Suzuki et al., 2017), pose challenges in perioperative stress management, necessitating a tailored approach due to potential variations in responses to SSRIs and benzodiazepines, as well as heightened stress sensitivity. Anesthetic considerations should involve careful selection to account for altered GABAergic function, requiring careful plans to mitigate risks. Psychopharmacological interventions, such as SSRIs and benzodiazepines, may be beneficial in managing stress during the perioperative period (Hendriksen et al., 2016; Horikoshi et al., 2021). Beyond traditional measures like steroids and hyperthermia precautions, a comprehensive stress management strategy, including anxiolytics and psychological support, is crucial (Al-Mfarej et al., 2023; Kim et al., 2021). Close monitoring for adverse reactions, recognizing the variability in responses to medications affecting GABA receptors, is imperative. The transient use of anxiolytics could be beneficial during anxiogenic clinical interventions, and while benzodiazepine (Hendriksen et al., 2016; Horikoshi et al., 2021) and serotonin (Spinazzola et al., 2020) administration has shown some benefits, further understanding of the neurobiology of these deficits and the status of GABA and serotonin neuromodulation is essential before proposing specific treatments based on these pharmacological interventions. Additionally, chronic benzodiazepine administration may further complicate stress management, as it has been shown to result in decreased postsynaptic sensitivity to GABA (Gallager et al., 1984), requiring careful consideration in the perioperative context. Nevertheless, there remains a lack of research examining the impact of these medications concerning acute anxiety or fear responses in patients with DMD.

8. Future directions

The mechanism(s) regulating the heightened fear response in vertebrates deficient in dystrophin, and its association with physical inactivity and loss of blood pressure, are complex, multi-faceted, and not fully elucidated. Further preclinical investigations in mouse models will be beneficial, but studies on larger animal models of DMD (e.g., rat, dog, and pig) might also provide a more physiologically relevant model to patients with DMD that has greater clinical translatability. These larger animal studies should focus on the relationship between impaired vascular function during a stressful stimulus, dystrophin product expression and the peripheral and central origins of this behavioral phenotype (e.g., brain and skeletal muscle). This will provide a greater mechanistic understanding of the fear response and these advancements in knowledge will reduce the risk associated with stress sensitivity in patients with DMD.

GABA_A receptor activity and GABAergic synaptic transmission in the amygdala is impaired during a stressful stimulus in *mdx* mice and it is restored with brain-specific dystrophin expression (Sekiguchi et al., 2009; Vaillend and Chaussonot, 2017; Vaillend et al., 2010). Therefore, it appears this neurotransmitter pathway plays an integral role in the fear response in *mdx* mice. It would be beneficial to investigate the effects of GABA_A receptor modulators (agonists and antagonists) to determine if this impacts the fear response of *mdx* mice, that could ultimately be exploited as a potential therapy to attenuate or prevent stress-induced complications associated with DMD. These studies will improve our understanding of the relationship between alteration in amygdala function and stressor-evoked blood pressure regulation in the heightened fear response in *mdx* mice. Furthermore, it will be important to explore the potential impact of these GABA_A receptors modulators in combination with currently prescribed drug treatments for patients with

DMD, such as prednisone and deflazacort, to understand any complications or synergistic benefits that may arise with a dual therapy approach.

Neuroimaging techniques could provide valuable insight into the neural mechanisms underlying the heightened fear response in *mdx* mice. For example, functional magnetic resonance imaging could assess changes in blood flow in the brain during a stressor, which could help identify regions of the *mdx* mouse brain that are activated or inhibited during a stressor. Electroencephalography, a recording of brain activity in response to stressful stimuli, could similarly provide information on patterns of neural activity associated with fear in *mdx* mice. Likewise, positron emission tomography could assist to assess metabolic brain activity during a stressor in *mdx* mice using neural tracers or radioisotopes.

Treatment with female sex hormones could be investigated as a strategy to rescue the startle response in boys with DMD. Estrogen availability appears to regulate the fear response of female *mdx* mice and female *mdx* mice are less sensitive to stress than male *mdx* mice (Lindsay and Russell, 2023; Lindsay et al., 2021b; Yamamoto et al., 2010). Additionally, female sex hormone-based interventions in boys with DMD (Cohen and Morgan, 1976; Rudman et al., 1972; Tsabari et al., 2021) and male *mdx* mice (Dorchies et al., 2013) have shown a safe and efficacious profile in physiological phenotypes, thus providing evidence that they could also be successful at attenuating a neuropsychological phenotype.

The primary treatment for DMD is the administration of glucocorticoids (McDonald et al., 2018). Because glucocorticoids can cause adrenal insufficiency in patients with DMD (Van Ruiten et al., 2016b; Wittlieb-Weber et al., 2020), and adrenal gland function is associated with the fear response in *mdx* mice (Lindsay et al., 2021a; Lindsay and Russell, 2023), preclinical and clinical studies on the startle or fear response in vertebrates deficient in dystrophin should consider the synergistic impact or contraindications/limitations of glucocorticoid therapy on this phenotype. Additionally, other current treatments for DMD, such as angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor-neprilysin inhibitor (ARNI), which aim to restore cardiac function and prevent premature heart failure (Duboc et al., 2005; Li and Chen, 2019), also inadvertently lower resting blood pressure in patients (Bruno and Taddei, 2017; Li and Chen, 2019). Because stress-induced hypotension can kill *mdx* mice during severe stress (Razzoli et al., 2020), it is possible these therapies may further predispose patients with DMD to stress-associated complications that could have life-threatening ramifications. Thus, future studies on the startle or fear response could benefit from including groups with ACE/ARNI treatment, or at minimum, controlling for ACE/ARNI treatment when investigating the fear response phenotype.

Clinical trials in patients with DMD should have an arm that specifically incorporates an assessment of stress sensitivity (physiological reaction to unpredictable visual and auditory stressful stimuli (Maresh et al., 2023)) to ensure that any potential treatment (e.g., exon-skipping (Charleston et al., 2018), corticosteroids [NCT05185622], micro-dystrophin [NCT05096221] (Mendell et al., 2020) or GALGT2 [NCT03333590]) improves pathophysiological outcomes but also mitigates the possibility of a mortality phenotype driven by fear.

9. Summary

Dystrophin-deficiency hypersensitizes vertebrates to stressful stimuli (Maresh et al., 2023; Saoudi et al., 2021; Sekiguchi et al., 2009; Vaillend and Chaussonot, 2017; Vaillend and Ungerer, 1999). Patients with DMD also suffer from enhanced anxiety and threat-induced bradycardia independent of dystrophin isoform deficiency (Maresh et al., 2023). This neuropsychiatric phenotype was first described in the dystrophin-deficient *mdx* mouse and was later reported in patients with DMD. In the *mdx* mouse, a heightened fear response is characterized by HPA axis activation and freezing or physical inactivity following a

stressor. The mechanism(s) behind the heightened fear response to stressful stimuli in *mdx* mice is not fully understood but the absence of brain-specific dystrophin leads to impaired GABAergic inhibitory synaptic activity due to disrupted GABA_A receptor clustering and possibly to lateral diffusion to extrasynaptic sites. Additionally, dysfunctional blood pressure regulation, which can cause death in 70% of *mdx* mice following social subordination stress, suggests a peripheral mechanism. The female sex hormones also appear to be involved in the heightened fear response phenotype because female *mdx* mice are less sensitive to stress than male *mdx* and OVX female *mdx* mice. Finally, *mdx* mice are not capable of habituating to repetitive stressors and exposing them to acute but repetitive stress can exacerbate dystrophic physiological phenotypes. This emphasizes the importance of future studies to develop a better understanding of the consequence of stress sensitivity in patients with DMD and the relationship between dystrophin deficiency and the heightened fear response.

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CRedit authorship contribution statement

Angus Lindsay: Conceptualization, Supervision, Writing – review & editing. **Cyrille Vaillend:** Conceptualization, Writing – review & editing. **Saba Gharibi:** Conceptualization, Data curation, Writing – original draft.

Declaration of Competing Interest

Except for funding agencies detailed above, the authors have no interests to declare.

Data Availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.pneurobio.2024.102590](https://doi.org/10.1016/j.pneurobio.2024.102590).

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