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

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CHAPTER 2

Central nervous system involvement in Duchenne muscular dystrophy

A review of behaviour and brain pathology in patients and mouse models

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Abstract

The most common neuromuscular disorder, Duchenne muscular dystrophy (DMD) is caused by mutations in the *DMD* gene, resulting in a lack of dystrophin. Next to severe and progressive muscle wasting, patients experience cognitive deficits including a lower IQ, behavioural problems and neurological comorbidities such as autism spectrum disorder, obsessive compulsive disorder and attention deficit hyperactivity disorder. Neuroimaging has identified widespread pathology including structural, physiological and connective alterations. DMD mouse models exhibit similar behavioural problems, including anxiety, social deficits and learning disabilities and have been used to delve deeper into the DMD brain pathology. While there are currently no therapies to treat DMD brain pathology, advances have been made in genetic approaches aimed at restoring dystrophin expression. Most advanced is the exon skipping approach which partially restores dystrophin expression, thereby ameliorating some of the behavioural deficits. However, many challenges remain to be addressed before patients can benefit from these approaches.

Keywords

Dystrophin, comorbidities, DMD mouse models, , exon skipping therapy

Introduction

Duchenne muscular dystrophy (DMD) is a severe neuromuscular disorder affecting 1:5000 newborn boys. It is caused by mutations in the X-chromosomal *DMD* gene, which prevent the synthesis of dystrophin. Lack of dystrophin in muscle renders muscle fibres more prone to exercise induced damage, leading to progressive loss of muscle tissue and function with age. Consequently, patients become wheelchair dependent in their teens and die prematurely due to cardiorespiratory failure between the age of 30-40 years in the Western World (reviewed in (Guiraud et al. 2015, Duan et al. 2021)). It is underrecognized, however, that approximately 30% of DMD patients also suffer from cognitive and behavioural problems that are caused by the absence of brain-specific dystrophin isoforms.

The *DMD* gene is the largest gene in the human genome and consists of 2.4 million base-pairs, containing 79 exons (Bello and Pegoraro 2019). Multiple promotors, spread throughout the gene, give rise to several dystrophin isoforms with distinct functions, sizes and sites of expression (Figure 1). In muscle, the full-length dystrophin isoform Dp427m (Dp427 standing for dystrophin protein with a size of 427kDa) is expressed. Two other unique promotors give rise to the full-length isoforms Dp427c and Dp427p, which are expressed in the brain (Perronnet and Vaillend 2010, Waite et al. 2012). Several promotors located more downstream of the gene, give rise to shorter dystrophin isoforms. Of these, Dp140, Dp71 and Dp40 are expressed in the brain, while Dp260 and Dp116 are expressed in the retina and peripheral nerves respectively.

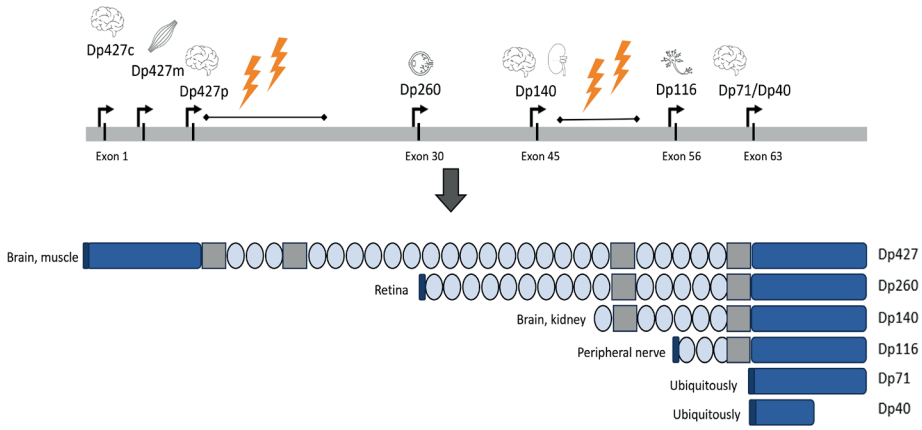

Figure 1. The DMD gene and the different dystrophin isoforms. *The DMD gene contains seven promotors (indicated by the arrows), which give rise to different dystrophin proteins expressed in diverse tissues (indicated by the pictograms above the arrows). Dp427c, Dp427p, Dp140, Dp71 and Dp40 are expressed in the brain. There are two mutation hotspots, indicated by the lightning icons (exons 2-22 and 45-55).*

Knowledge of where and when dystrophin, and more specifically individual isoforms, are expressed in the brain is limited. Using the Allen Brain Atlas data we showed that dystrophin is highly expressed in the human amygdala and hippocampus, while lower levels are found throughout the cortex. Here, levels in the temporal and frontal cortex exceed those of the occipital and parietal cortex (Doorenweerd et al. 2017). Expression is low in the pons and cerebellum. Due to the nature of the data, no distinction could be made between the dystrophin isoforms, as such, these observations represent the sum of all isoforms. Utilization of the BrainSpan database allowed us to study the expression profiles of individual dystrophin isoforms throughout development, ranging from 8 weeks post conception to 40 years of age (Doorenweerd et al. 2017). We showed that expression of Dp427c and Dp427m is low before birth, slightly increases around two years of age and then remains low throughout adulthood. In contrast to previous reports on the mouse (Górecki et al. 1992, Kueh et al. 2008, Snow et al. 2014), Dp427p is virtually absent in the human brain. Notably, expression of Dp140 is high in the foetal brain, while levels drop from the late foetal stages onwards. Levels in the cerebellum exceed those of the cortex. Dp71 and Dp40 are ubiquitously expressed throughout the brain at high levels during the foetal stages, which remain till adulthood. See Tetorou *et. al.* (2024) (Tetorou et al. 2024) for a review of dystrophin and its interactors in the brain.

The number of dystrophin isoforms lacking in DMD patients depends on the position of their mutation. Notably, mutations within the *DMD* gene are clustered in two mutation hotspots, located between exons 2-22 and exons 45-55. Mutations at the proximal end of the gene (exon 1 – 43) lead to the absence of Dp427m,c,p. Distal mutations (intron 44 – exon 79) are expected to not only affect the expression of the full-length isoforms but also that of some or all shorter isoforms. Based on the mutation frequency (Bladen et al. 2015), all DMD patients lack the full-length isoforms, while ~40-50% also lack Dp140. Up to 10% of patients lack all dystrophin isoforms (Desguerre et al. 2009, Taylor et al. 2010, Pane et al. 2012, Rasic et al. 2014, Ricotti et al. 2016).

Whereas muscle pathology has been studied in great depth since the discovery of the *DMD* gene in the 1980s (Hoffman et al. 1987), research on the DMD brain has only gained attention in the last decade. This review focuses on the brain pathology in DMD, summarizes the mouse models that have been used to unravel disease mechanisms and overviews the therapeutic opportunities for patients.

Section 1: Behavioural deficits and brain pathology in DMD patients

The first description of the disease, dating back to the 1860s, already acknowledged the occurrence of brain deficits in a subset of DMD patients (Tyler 2003). Approximately 30% of patients have a higher risk for cognitive impairment, learning disabilities and delayed developmental milestones (Ricotti et al. 2016, Colombo et al. 2017, Darmahkasih et al. 2020). The average IQ of the DMD population (mean=85) is one standard deviation below the general population norm (Hinton et al. 2000, Cotton et al. 2001, Cyrulnik et al. 2008, Connolly et al. 2013, Weerkamp et al. 2022). Learning and behavioural problems occur in patients with cognitive impairments, but also in those with a normal to high range IQ (Battini et al. 2018). Difficulties have been described with respect to information processing, (verbal) working memory, math and reading. In addition, numerous patients are reported with comorbidities such as autism spectrum-, obsessive compulsory-, and attention deficit hyperactivity disorder (ADHD) (Billard et al. 1992, Hendriksen and Vles 2008, Pane et al. 2013, Ricotti et al. 2016, Vicari et al. 2018, Darmahkasih et al. 2020). Patients also have a higher chance of epileptic seizures (Hoogland et al. 2019). The severity of cognitive and behavioural defects largely differs between DMD patients. Increasing evidence reveals that the number of affected dystrophin isoforms seems to correlate with severity, in which patients lacking all isoforms are most severely affected (Taylor et al. 2010, Chamova et al. 2013, Ricotti et al. 2016). However, these observations are evident in a group, but not on a patient level. The neuropsychological and neurobehavioral impairments are reviewed in Snow *et. al.* (2013) (Snow et al. 2013).

Anatomical and functional consequences of dystrophinopathy in the human brain

How dystrophinopathy affects brain morphology and function is not well understood. The very sparse and inconsistent post-mortem data that is available from DMD patient brains was acquired decennia ago (Rosman and Kakulas 1966, Dubowitz and Crome 1969, Jagadha and Becker 1988). In summary, no gross structural abnormalities were found. Some patients had increased cortical thickness, neuronal and Purkinje cell loss, gliosis in the grey matter, and reduced dendritic length and branching. More recent data is non-existent due to the world-wide unavailability of DMD brain tissues.

Neuroimaging techniques enabled structural, physiological and connectivity analyses. DMD patients show a decrease in total brain volume and global reductions in grey matter volume, most prominently in the left primary sensorimotor cortex (Yoshioka et al. 1980, Al-Qudah et al. 1990, Lv et al. 2011, Doorenweerd et al. 2014). White matter volume seems unaffected, but multiple microstructural deficits have been found. Diffusion Tensor Imaging studies, analysing the structural connectivity

of the white matter fibres via water diffusion, found increased overall and radial diffusivity in DMD patients paired with a lower fractional anisotropy. These changes suggest reduced fibre density, increased membrane permeability and/or decreased structural organization of the tissue (Doorenweerd et al. 2014, Preethish-Kumar et al. 2020, Biagi et al. 2021).

Additionally, functional abnormalities have been described. Resting-state functional MRI shows alterations in default-mode network functional connectivity, resulting in hyperconnectivity spread throughout the whole network, similar to what has been reported in ADHD (Doorenweerd et al. 2021). Proton spectra studies have shown conflicting data, reporting both increased and decreased choline compounds in different brain regions (cerebellum, hippocampus, frontal- and temporoparietal regions) (Rae et al. 1998, Kreis et al. 2011, Doorenweerd et al. 2017). Choline influences the synthesis of the neurotransmitter acetylcholine (Prado et al. 2017), which impacts brain network activity (reviewed in (Colangelo et al. 2019)). Alterations in choline could play a role in the overall reduction in network efficiency (i.e. reduced speed of information transfer) reported in DMD (Preethish-Kumar et al. 2022). DMD patients exhibit decreased local synchronization in neurons in the motor related brain areas during spontaneous firing (Lv et al. 2011). Furthermore, reductions in glucose metabolism in the sensorimotor cortex (Bresolin et al. 1994, Lee et al. 2002), and decreased excitability of the motor cortex have been reported (Di Lazzaro et al. 1998). Taken together, these results point towards impaired functionality of the motor cortex in DMD patients. Lastly, DMD patients also show reduced cerebral blood flow, independently of age or reductions in grey matter volume (Doorenweerd et al. 2017).

The reduced grey matter volume, altered structural connectivity and lower perfusion are more pronounced in patients also lacking Dp140 (Doorenweerd et al. 2014, Doorenweerd et al. 2017, Preethish-Kumar et al. 2022). However, these results are inconclusive, as some studies did not find a relation or suffered from a low sample size. In addition, no clear correlation between the neuroimaging outcomes and the severity of cognitive and behavioural defects has been found. It should however be noted that the vast majority of DMD patients chronically uses corticosteroids, which are known to negatively affect behaviour (Angelini 2007, Counterman et al. 2022). Recent studies even revealed correlations between corticosteroid dose and regiment and the extent of alterations of brain volume and white matter microstructure (van der Meulen et al. 2022, Geuens et al. 2023).

Section 2: DMD mouse models

As cognitive problems limit the social participation of DMD patients, elucidating brain pathology is vital to provide optimal care and ensure rehabilitation with the development of treatments. Here, animal models are instrumental. The majority of our knowledge on the DMD brain has been obtained from mouse models which are therefore the focus of this review.

Mouse models lacking Dp427

The C57BL/10ScSn-*Dmd*^{mdx}/J (*mdx/*bl10) mouse is the first DMD model in which cognitive abnormalities have been reported (Muntoni et al. 1991) and it has been widely used ever since. The *mdx*/bl10 mouse has a point mutation in exon 23 of the *Dmd* gene and consequently lacks Dp427 (Sicinski et al. 1989). Although *mdx*/bl10 mice exhibit muscle pathology, which could potentially influence behaviour in terms of reduced exploration or activity (Vaillend et al. 1995, Vaillend et al. 2004), it is rather mild compared to DMD patients. *Mdx* mice have also been generated on the C57BL/6J genetic background. *Mdx*/bl6 mice have the same mutation as the *mdx*/bl10 model and a comparable muscular involvement.

B6Ros.Cg-*Dmd*$^{mdx\text{-}5Cv}$/J *(mdx*^{5cv}) mice have an ENU-induced point mutation in exon 10 and consequently lack Dp427 (Im et al. 1996). In contrast to the *mdx*/bl10 and *mdx*/bl6 models, their muscle pathology and functioning are slightly more impaired, likely due to the absence of revertant muscle fibres (Danko et al. 1992, Beastrom et al. 2011). The genetic background of animals proves to be an important component for assessing behaviour, also in the context of DMD (Wolfer and Lipp 2000, Jacobson and Cryan 2007, Seemiller et al. 2021). Differences between distinct C57BL strains have been reported in expression profiles, sensitivity to seizure induction and in the brain glutamatergic system (McLin and Steward 2006, Deacon et al. 2007, Flynn et al. 2021, Mortazavi et al. 2022). This probably adds to the inconsistencies reported between *mdx/*bl10, *mdx*/bl6 and *mdx*^{5cv} mice, discussed in this review.

Mouse models lacking Dp427 and Dp140

The consequences of a lack of Dp427 and Dp140 have been primarily studied in the B6Ros.Cg-*Dmd*$^{mdx\text{-}4Cv}$/J (*mdx*^{4cv}) mouse, which carries a nonsense mutation in exon 53 (Im et al. 1996), and the *mdx52* mouse which has a deletion of exon 52. As of yet, no functional assessment has been done in *mdx*^{4cv} mice to assess muscle performance. Even though Dp140 is not expressed in muscle, *mdx52* mice display poorer executive motor function than *mdx*/bl10 mice (Chesshyre et al. 2022). The cerebellum (being a main site of Dp140 expression) and/or the cerebellar thalamic cortical connectivity are hypothesized to play an important role in this phenomenon, considering the crucial role of the cerebellum in the timing and control of goal-directed

movements. In DMD patients, similar correlations between the mutation site and motor function have been found in one (Chesshyre et al. 2022), but not in another study (Thangarajh et al. 2021). *Mdx52* mice also have altered visual processing, probably due to the lack of Dp260 (Barboni et al. 2021). *Mdx^{4cv}* mice are expected to suffer from similar deficits in visual processing but studies are lacking. These alterations highlight the importance of recognizing CNS involvement in motor executive function in DMD, which in term influences measurable behaviour during assessments and should therefore be taken into consideration when reviewing behaviour in DMD mice, especially those lacking Dp260 and Dp140.

Mouse models lacking all dystrophin isoforms or Dp71 only

The *DMD-null* mouse lacks all dystrophin isoforms due to the deletion of the entire genomic region of the *Dmd* gene (Kudoh et al. 2005). They display severe muscle hypertrophy, but functional deficits are similar to that of the *mdx52* model, which does not translate well to DMD in humans, as patients lacking all dystrophin isoforms show worse functional deficits than those lacking only Dp427 and Dp140 (Chesshyre et al. 2022).

The Dp71-null mouse was created by replacing part of the first exon of Dp71 (Dalloz et al. 2003). This model does not translate to the human condition as no mutations have been identified that exclusively affect Dp71. However, it provides insight into the specific functions of Dp71. Dp71-null mice have healthy muscles, due to unaffected expression of Dp427m (Helleringer et al. 2018), but suffer from altered retinal functioning (Barboni et al. 2020). This deficit has not been studied yet in *DMD-null* mice.

Behavioural deficits in DMD mouse models

A brief overview of all behavioural deficits per DMD mouse model can be found in Table 1.

Emotional reactivity

The most prominent phenotype of the *mdx*/bl10 mouse is the severe fear response, where a short stressor (i.e. manual restraint) instantly causes freezing behaviour lasting up to at least one hour (Sekiguchi et al. 2009, Yamamoto et al. 2010, Vaillend and Chaussenot 2017, Razzoli et al. 2020, Saoudi et al. 2021). Only intensive repetition (>16 times per day (Vaillend and Chaussenot 2017)) or activation of the territorial drive via strong odours of unfamiliar mice can reduce, but not extinguish, freezing behaviour (Yamamoto et al. 2010). The altered freezing response can be found as early as 36 days after birth (Sekiguchi et al. 2009). The underlying mechanism

Behaviour			Lack of Dp427			Lack of Dp427+Dp140		Lack of all isoforms	Lack of Dp71
Domain		Type of test	*mdx*/bl10	*mdx*/bl6	*mdx*^{5cv}	*mdx*^{4cv}	*mdx52*	*DMD-null*	Dp71-null
Pathology	Muscle function		↓	↓	↓↓		↓↓	↓↓	–
Motional reactivity	Fear	Restrained, shock	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑↑	
	Anxiety	DL, EPM, OF, light stimulus	↑	↑	↑↑	↑↑↑	↑↑↑	↑↑↑↑	↑
	Depressive behaviour	FST, TST	↑#		↑		↑		
	Spontaneous behaviour	Pheno-Typer cages						↓	
Working memory	Working memory	X-maze, T-maze		↓		↓			↓
Passive avoidance	Passive avoidance	Foot shock	↓						
Learning	Spatial learning	MWM, BM	–	–	–	–	–	–	↓
	Fear learning	LA, NPA, CF, ACF	↓		↓↓		↓↓		
	Food reward learning	CW, BPT, NP, radial maze	↑	–	–		–	–	
Recall	Short term recall	T-maze, NOR	?						
	Long term recall (spatial)	T-maze, MWM, BM	↓	–	–	–	–	–	↓
	Long term recall (recognition)	NOR	↓		↓#		↓#	–	
Cognitive flexibility	Learning flexibility	BM	–	–	–	–	–	↓	↓
	Food rewarded flexibility	CW, NP	?		↓		↓	–	
Extinction	Extinction learning	BPT	–						
Social inter-action	Sociability	3C	↓	–	↓	↑	↑		
	Social novelty seeking	3C	–	–	–	–	–	↑	
	Social stress response	Social defeat	↑						
	USV	Pup separa-tion	↓	–			↓↓		

Table 1. Behavioural deficits per DMD mouse model. *The arrow direction indicates if behaviour is increased (up) or decreased (down) compared to WT mice. Differences between mouse models are represented by the colour and amount of arrows, with one yellow arrow indicating the least severe deficit and 4 dark red arrows indicating the strongest deficit. Black arrows represent single data which does not allow for direct comparisons between models. A question mark indicates conflicting data in the literature. The combination of an arrow and a hash sign indicates a trend. Horizontal grey bars mean no differences were found. Empty cells indicate that the behaviour has not been investigated in this model. DL: dark light choice test. OF: open field. EPM: elevated plus maze. FST: forced swim test. TST: tail suspension test. MWM: Morris water maze. BM: Barnes maze. LA: light avoidance. NPA: Nose poke avoidance. CF: contextual fear. ACF: auditory cued fear. CW: Cognition wall. BPT: bar pressing task. NP nose pokes. NOR: novel object recognition task. 3C: 3 chamber social interaction task.*

is not fully understood. Vaillend *et. al.* (2017) showed that *mdx*/bl10 mice exhibit similar physiological stress responses to the restraint itself as wildtypes (reflected by comparable ACTH levels), indicating that the increased freezing stems from a more downstream reaction to the stress response (Vaillend and Chaussenot 2017). Similar reactions in freezing response have been found in *mdx/*bl6 (Hashimoto et al. 2022) and *mdx*^{5cv} mice (Verhaeg et al. 2025), and in *mdx52* and *mdx*^{4cv} mice, also lacking Dp140 (Zhang et al. 2020, Saoudi et al. 2021, Hashimoto et al. 2022, Verhaeg et al. 2025). Interestingly, this freezing response is further aggravated in *DMD-null* mice (Verhaeg et al. 2025).

The strong influence of Dp427 on the unconditioned fear response does not translate well to other types of emotional behaviour. Studies have struggled to conclusively detect anxiety-like behaviour in *mdx*/bl10 mice all together (Vaillend et al. 1995, Sekiguchi et al. 2009, Comim et al. 2019) or had inconsistent results within their study, detecting anxiety-like behaviour in one but not another test (Manning et al. 2014, Remmelink et al. 2016, Vaillend and Chaussenot 2017, Saoudi et al. 2021). This highlights the subtle nature of the anxiety phenotype in *mdx*/bl10 mice and suggests that the environment and test protocols play a crucial role in the proper detection of this deficit. Lack of Dp140 further deteriorates the anxious behaviour, as both *mdx*^{4cv} and *mdx52* mice show stronger anxiety responses compared to *mdx*/bl6 and *mdx*^{5cv} mice respectively (Saoudi et al. 2021, Verhaeg et al. 2025). This anxious behaviour is even further enhanced in *DMD-null* mice (Verhaeg et al. 2025) and to some extent in Dp71-null mice (Daoud et al. 2009), indicating worsening of anxiety with the lack of each brain related dystrophin isoform. Depressive-like behaviour has also been studied in *mdx*/bl10 mice, however, changes in despair or learning of inescapable nature of the task have been reported in one study (Comim et al. 2019), but could not be replicated in another (Vaillend and Chaussenot 2017), possibly due to high stress reactivity and/or impaired muscle functionality. *Mdx*^{5cv} and *mdx52* mice seem to have enhanced behavioural despair but no alterations in

learned helplessness. Depressive-like behaviour has not been studied in *DMD-null* mice, but in Dp71-null mice, no alterations have been observed (Helleringer et al. 2018).

DMD-null males show restless behaviour, altered day/night rhythms and movement and rest patterns, which was not found in *mdx*^{5cv} or *mdx52* mice (Verhaeg et al. 2025). Restlessness has also been observed in *DMD-null* females, accompanied by abnormalities in maternal behaviour(Kudoh et al. 2005).

Learning and memory

As DMD patients often exhibit learning difficulties, learning and memory has been extensively studied in DMD mouse models. Working memory is impaired in *mdx*/bl6, *mdx*^{4cv} and Dp71-null mice (Chaussenot et al. 2019, Verhaeg et al. 2024), but it remains unclear whether its severity differs between models. Initial learning of a task or location is unaffected in *mdx*/bl10 and *mdx*^{5cv} mice (Sesay et al. 1996, Vaillend et al. 2004, Remmelink et al. 2016, Bagdatlioglu et al. 2020), however, when needing to recall this information later on, depending on the delay time, deficits become apparent. Data on the short-term recall are inconclusive for *mdx*/bl10 mice (Vaillend et al. 1995, Vaillend et al. 2004, Bagdatlioglu et al. 2020). However, their long term recall (>24 hour delays) seems to be more robustly affected in both spatial memory (hippocampus dependent; (Sesay et al. 1996, Vaillend et al. 2004, Remmelink et al. 2016, Bagdatlioglu et al. 2020)) and recognition memory (hippocampus independent; (Vaillend et al. 1995, Vaillend et al. 2004, Comim et al. 2019, Bagdatlioglu et al. 2020). Spatial learning and memory are not affected in *mdx52*, *mdx*^{4cv} or *DMD-null* mice (Verhaeg et al. 2024, Verhaeg et al. 2025), whereas deficits have been found in Dp71-null mice (Daoud et al. 2009, Chaussenot et al. 2019).

Long term recall deficits in other types of memory have also been observed in *mdx*/bl10 mice (Muntoni et al. 1991, Coccurello et al. 2002, Vaillend and Chaussenot 2017, Comim et al. 2019). Opposite to WT mice, they fail to show strong reactions in fear learning and passive avoidance in response to an auditory stimulus. Interestingly, this lack of reactive is not observed in response to a visual stimulus, here similar responses to WT have been found (Daoud et al. 2009, Remmelink et al. 2016, Lewon et al. 2017, Vaillend and Chaussenot 2017, Saoudi et al. 2021). In *mdx*^{5cv} mice, the lack of response to auditory stimuli seems even more impaired, matching observations in *mdx52* mice (Saoudi et al. 2021). Notably, since 5-week-old *mdx*/bl10 mice already suffer from partial hearing loss (Raynor and Mulroy 1997, Chen et al. 2002), the lack of response to auditory, but not light, stimuli could result from a lower impact of the auditory cue instead of an actual learning deficit. Since aggravated deficits have been observed in *mdx*^{5cv} and *mdx52* mice, the genetic background might play a role in either fear learning and/or auditory processing.

In contrast to the lack of direct behavioural changes in response to negative reinforcement learning, positive reinforcement, such as a food reward after a short

period of food deprivation, might lead to improved performance in *mdx*/bl10 mice compared to WTs (Vaillend et al. 1998, Lewon et al. 2017, Dickson and Mittleman 2019), although some studies failed to replicate this increased performance (Vaillend et al. 1995, Vaillend and Ungerer 1999, Remmelink et al. 2016). Lewon *et al.* showed that this increased performance only occurs when mice are food deprived before the start of the task (Lewon et al. 2017). It can be hypothesized that *mdx*/bl10 mice are more motivated for food collection due to their increased metabolic rate caused by the continuous need to repair their muscle tissue (Radley-Crabb et al. 2011, Radley-Crabb et al. 2014, Stapleton et al. 2014) and consequently, perform better after food deprivation. The nature of the stimulus and the motivational drive play an important role in learning motivation and therefore the ability to investigate learning capabilities, making it hard to draw conclusions about learning capabilities in tasks involving positive or negative stimuli.

Cognitive flexibility, i.e. learning new information that is in conflict with earlier acquired information, does not seem to be impaired in any DMD model lacking Dp427 or Dp427 and Dp140 (Sesay et al. 1996, Chaussenot et al. 2015, Engelbeen et al. 2021). However, *mdx*/bl10 mice seem to retain old information better than WT mice and rely on this when newly learned behaviour does not yield the desired result (Remmelink et al. 2016). *DMD-null* and Dp71-null mice show a delay in reversal learning (Chaussenot et al. 2019, Verhaeg et al. 2025).

When introducing positive food reinforcement in cognitive flexibility tasks, results are contradictive, having shown both increased performance (Lewon et al. 2017), decreased performance (Vaillend et al. 1995, Remmelink et al. 2016) or no differences between *mdx*/bl10 and WTs during reversal tasks (Dickson and Mittleman 2019, Engelbeen et al. 2021).

Extinction learning, i.e. de-learning earlier information without presenting new options, does not seem to be affected in *mdx*/bl10 mice (Vaillend and Ungerer 1999, Dickson and Mittleman 2019), neither does taste aversion learning (Vaillend and Chaussenot 2017).

Social interaction and ultrasonic vocalization

With autism spectrum disorder being one of the DMD comorbidities, interaction experiments with different social contexts have been performed. Eight week, but not five month, old *mdx*/bl10 mice do not show the preference for social interaction that has been reported in WT mice (Miranda et al. 2015, Alexander et al. 2016). *Mdx*^{5cv} mice show a similar deficit at 5 weeks of age (Alexander et al. 2016). The behaviour of *mdx*/bl10 mice is strongly influenced by the sex and genotype of the interacting mouse, as they exhibit abnormal behaviour in direct interactions with male and female *mdx* mice, but not with WT males. This suggests a submissive response of *mdx*/bl10 mice, which is more easily influenced than in controls. Interestingly, *mdx52* and *mdx*^{4cv} mice show increased tendencies towards sociability

compared to WT and *mdx*/bl10 mice (Hashimoto et al. 2022).

Mdx/bl10 mice seem specifically vulnerable to social stress. When confronted with the odour of an intruder in their home cage they show signs of stress, indicated by a freezing response (Miranda et al. 2015). When experiencing social defeat followed by prolonged sensory housing, *mdx*/bl10 mice develop heart damage and other cardiovascular responses resulting in death within two days (Razzoli et al. 2020). This highlights a correlation between stress vulnerability and cardiomyopathy. Similar correlations have been made between shock and myocardial damage in patients with ST-elevation myocardial infarction (Reinstadler et al. 2016). Hypotension, as a result of shock, is associated with conditions like stroke, cardiac arrest and respiratory failure, which are among the most common causes of death in DMD (Winterholler et al. 2016, Cheeran et al. 2017). DMD could therefore be associated with a failure to mediate proper autonomic/cardiovascular responses, leading to extensive physical consequences in response to stress.

Lastly, alterations in ultrasonic calls, which are similar to those seen in autism spectrum disorder mouse models, have been found in *mdx/*bl10, but not *mdx*/bl6, pups and adult mice (Miranda et al. 2015, Hashimoto et al. 2022). In *mdx52* mice, these alterations are even more apparent (Hashimoto et al. 2022).

Neuroanatomical and physiological consequences of dystrophinopathy in the murine brain

Neuroimaging has also been utilized to study the DMD mouse brain. No or little abnormalities have been found in the whole brain or individual brain region volumetrics in young adult *mdx/*bl10 and *mdx52* mice (Bagdatlioglu et al. 2020, Saoudi et al. 2021). However, from 12 months of age onwards, total brain volume is increased in *mdx*/bl10 mice (Dunn and Zaim-Wadghiri 1999, Miranda et al. 2009, Bagdatlioglu et al. 2020), which contradicts observations in DMD patients (Doorenweerd et al. 2014). This is accompanied by increases in certain cortical structures, such as the basolateral amygdala and the ventricles (Miranda et al. 2009, Bagdatlioglu et al. 2020). Furthermore, a rounder head (as also seen in patients [120]), shorter nasal plate and wider parietal plate were seen in 12 month old *mdx/*bl10 mice.

Whereas medial diffusion is increased in DMD patients, it is decreased in *mdx/*bl10 mice (Goodnough et al. 2014). It should be noted that in patients only the white matter tracts were analysed, while in mice the whole brain was measured. Regional increases in medial diffusion have been reported in the cortex of *mdx/*bl10 mice along with decreases in fractional anisotropy in the hippocampus (Xu et al. 2015).

To our knowledge, resting state functional MRI has never been performed in DMD mice. However, multiple studies have been conducted on the choline/acetylcholine network in the brain. Increased choline compounds, mostly restricted to the cerebellum and hippocampus, are present in older *mdx*/bl10 mice (> 6 months) (Rae et

al. 2002). Reductions of acetylcholinesterase (responsible for breaking down acetylcholine) have also been reported (Tracey et al. 1996, Comim et al. 2011). Alterations in glucose metabolism, as described in DMD patients, are also present in *mdx/*bl10 mice, resulting in increased cellular flux of ^{13}C via oxidative glucose metabolism (Rae et al. 2002). Furthermore, *mdx/*bl10, *mdx52* and Dp71-null mice show reductions in docked vesicular glutamate (Daoud et al. 2009, Hashimoto et al. 2022), leading to enhanced glutamatergic transmission and altered synaptic responses.

Excitability of Purkinje cells and the range of synaptic transmission is reduced in *mdx/*bl10 mice (Kreko-Pierce and Pugh 2022). Many studies have focused on excitability and the role of GABA in the excitatory/inhibitory balance of the *mdx/*bl10 brain. In short, subunits of the GABA receptor show reduced clustering in multiple brain regions, resulting in changes in neuronal firing and altered synaptic plasticity (Knuesel et al. 1999, Vaillend and Billard 2002, Sekiguchi et al. 2009, Anderson et al. 2010, Dallérac et al. 2011, Vaillend and Chaussenot 2017, Zarrouki et al. 2022). *Mdx52* mice show even greater alterations in excitatory and inhibitory potentials (Hashimoto et al. 2022). Dp71-null mice show alterations in firing patterns, but not in synaptic plasticity (Daoud et al. 2009).

*Mdx/*bl10 mice present with reduced cerebral blood flow, similar as seen in DMD patients, however in mice this appears at an advanced age (>10 months) (Goodnough et al. 2014). Furthermore, blood-brain barrier permeability is increased (Goodnough et al. 2014, Verhaeg et al. 2024) and many proteins related to the blood-brain barrier show altered expression, including ZO-1, GFAP and AQP4 (Frigeri et al. 2001, Nico et al. 2003). Lack of Dp71 further alters the blood-brain barrier by reducing AQP4 clustering in Dp71-null mice (Blake et al. 1999, Daoud et al. 2009, Chaussenot et al. 2019).

Section 3: Therapy development for the brain:

For many DMD patients, their parents and caretakers, the CNS related impairments are considered to outweigh the muscular phenotype and therefore treatment options targeting the CNS are warranted. Psychopharmacological treatments have been given to DMD patients to address psychiatric symptoms such as ADHD, ASD and depression (Hendriksen et al. 2016, Lee et al. 2018, Lionarons et al. 2019, Darmahkasih et al. 2020, Noda et al. 2021). Therapeutic approaches to restore dystrophin expression have been investigated for decades in muscle, but they could also hold promise to treat the brain. This could be achieved by either stop-codon readthrough (reviewed in (Politano 2021)), gene therapy (reviewed in (Eslahi et al. 2023)), gene editing (reviewed in (Hanson et al. 2021)) or exon skip therapy (reviewed in (Aartsma-Rus et al. 2017, Niks and Aartsma-Rus 2017)). Only the exon skipping and gene therapy have been investigated in the DMD brain in mouse models to date. This review focuses on the exon skipping therapy approach.

The potential promise of the exon skipping therapy to treat the DMD brain

Exon skipping aims to restore the disrupted open reading frame of dystrophin pre-mRNA transcripts by hiding particular exons from the splicing machinery through the utilization of antisense oligonucleotides (AONs). Consequently, the targeted exon is spliced out together with its flanking introns, which restores the open reading frame and allows for the production of shorter, but partly functional, dystrophin proteins (Figure 2). Exon skipping is a mutation-specific approach. Depending on the location and size of the mutation, different exons need to be skipped to restore the open reading frame. Due to mutation hotspots, skipping of some exons applies to larger patient groups. The largest group of DMD patients could benefit from AONs targeting exon 51 (13%), exon 45 (8.1%) or exon 53 (7.7%) (reviewed in (Aartsma-Rus et al. 2009)). For treatment of the musculature, four uncharged phosphorodiamidate morpholino oligomer (PMO) AONs targeting exons 45 (Casimersen), 51 (Eteplirsen) and 53 (Viltolarsen and Golodirsen) have been approved by the Food and Drug Administration (FDA) in the USA and one AON (Viltolarsen) by the Japanese Ministry of Health, Labour and Welfare. Since these AONs cannot cross the blood-brain barrier, the brain is left untreated. Notably, charged AONs are very efficiently taken up by brain cells when delivered intrathecally. AON treatment for spinal muscular atrophy (SMA) has been approved by the FDA and European Medicine Agency (EMA) based on very convincing data (Haché et al. 2016, Finkel et al. 2017). This exemplifies that the exon skipping approach could also hold promise for the treatment of the DMD brain.

The development of the exon skip therapy to treat the DMD brain is however in its infancy. There is little known about the pharmacokinetics and dynamics of AONs in the brain and their potential to postnatally restore CNS-related impairments. In the *mdx*/bl10 mouse, which carries a stop mutation in exon 23, skipping of exon 23 restores the expression of Dp427. In the *mdx52* mouse, which has a deletion of exon 52, skipping of exon 51 restores the expression of Dp427, while skipping of exon 53 restores Dp140 in addition (Figure 2). An overview of exon skipping approaches in the murine DMD brain can be found in Table 2.

Restoration of Dp427 expression

The first study investigating the effects of AON delivery in the brain used an intravenous treatment with 200 mg/kg tricyclo-DNA AON (tc-DNA; 15-mer) on a weekly basis for 12 weeks in *mdx*/bl10 mice (Goyenvalle et al. 2015). Since tc-DNA AONs can cross the blood-brain-barrier, systemic treatment resulted in exon 23 skipping and very low amounts of full-length dystrophin, which led to improvements in the restraint-induced fear response. This was confirmed when shorter 13-mer tc-DNA AONs were used (Relizani et al. 2017).

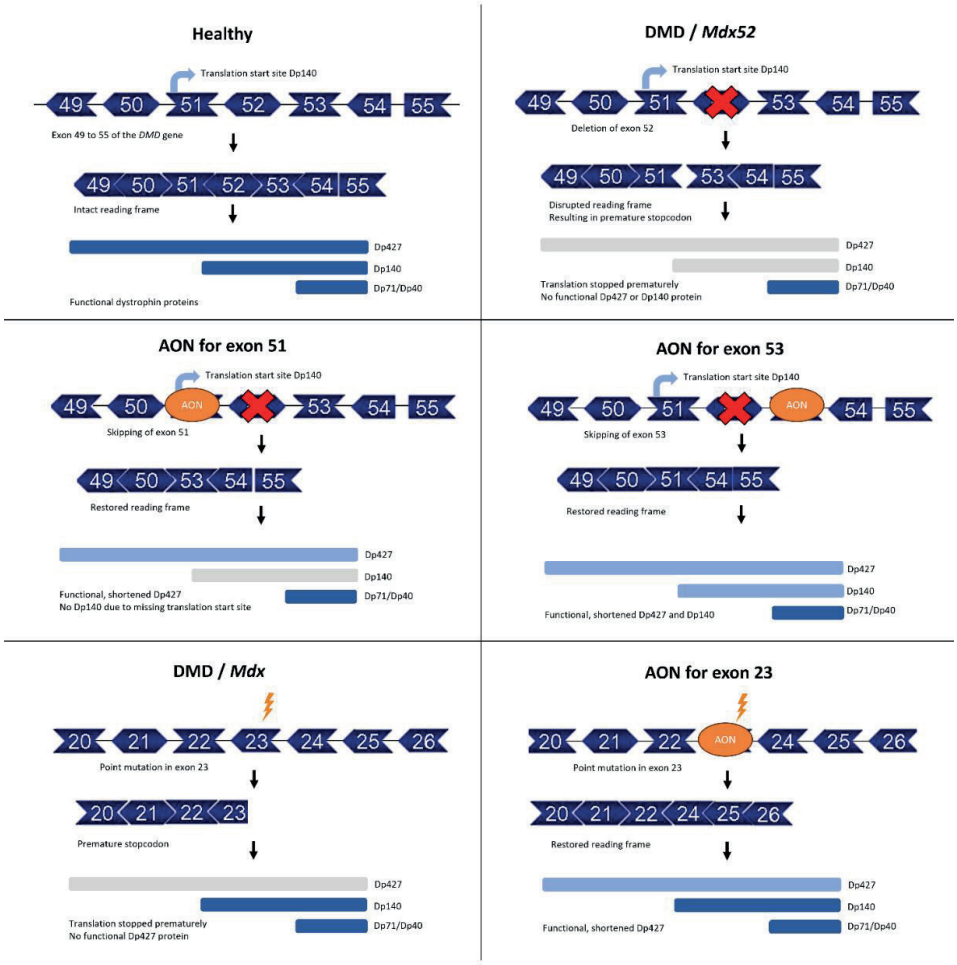

Figure 2. Exon skipping therapy in DMD. *In a healthy situation, all exons are translated and dystrophin isoforms are synthesized. In the* mdx52 *model, exon 52 of the* Dmd *gene is deleted, which disrupts the reading frame, thereby preventing the synthesis of Dp427 and Dp140, while that of Dp71/Dp40 remains unaffected. Skipping of exon 51 restores the disrupted reading frame, thereby allowing the expression of shortened but partially functional Dp427 isoforms. Since exon 51 contains the translational start site of Dp140, this isoform is not expressed. When exon 53 is skipped instead, shortened Dp427 and Dp140 proteins are produced. In the* mdx*/bl10* and mdx*/bl6 models, a point mutation in exon 23 of the* Dmd gene *leads to a disruption of the reading frame and a premature stop codon, thereby preventing synthesis of Dp427, while the other isoforms remain unaffected. Skipping of exon 23 restores the reading frame and enables the synthesis of a shortened Dp427.*

Strain	Chemistry	Target	Dose	Route	Duration	Restored isoforms	%skip	%Dystrophin	Behavioural improvement?
Mdx/ bl10	Tc-DNA	Exon 23	200 mg/kg weekly	Intra venously	12 weeks	Dp427	2-4%	Low, not quantified	Yes (fear response)
Mdx/ bl10	Tc-DNA	Exon 23	200 mg/kg weekly	Intra venously	12 weeks	Dp427	2-5%	5%	Yes (fear response)
Mdx/ bl10	Tc-DNA	Exon 23	400 µg	ICV	Singular injection	Dp427	15-35%	5-25%	Yes (7 but not 10 weeks after treatment)
Mdx/ bl10	PMO	Exon 23	1 mg AON total	ICV infusion with osmotic pump	1 week	Dp427	25%	25%	Yes (between 5 and 7 weeks after treatment)
Mdx52	Tc-DNA	Exon 51	400 µg	ICV	Singular injection	Dp427	20-30%	Not measured	-
Mdx52	PMO	Exon 51	900 µg each	ICV+intra-cisterna magna injections	Every 72h (4x total)	Dp427	16-22%	Not measured	-
Mdx52	Tc-DNA	Exon 51	400 µg	ICV	Singular injection	Dp427	10-15%	5-15%	Yes (anxiety, fear and fear learning)
Mdx/ bl10	U7-Sd23/BP22 AAV2 vector	Exon 23	4.2×10^9 vg	Intrahippo-campal	Singular injection	Dp427	15-25%	25-30%	-
Mdx/ bl10	U7-SD23/BP22 AAV2	Exon 23	6.4×10^9 vg	Intrahippo-campal	Singular injection	Dp427	Not quantified	15-25%	GABAa receptor clustering restored
Mdx52	AAV9-U7snRNA	Exon 51	3E+13 vg	Intra-venously	Singular injection	Dp427	5%	0%	No
Mdx52	PMO	Exon 53	30 mg/kg	ICV	8 injections over 4 weeks	Dp427 + Dp140	5-10%	Dp427: 1% Dp140: 5%	Yes (ASD-like behavior and E/I balance)
Mdx52	PMO (3 AONs simultaneous delivered all targeting exon 53)	Exon 53	400-900 µg	ICV	Singular injection	Dp427 + Dp140	Up to 25%	Undetect-able	-

Table 1. Exon skipping approaches in the DMD mouse brain. *Exon skipping efficiency has been tested in mdx/bl10 and mdx52 mice, targeting exon 23 and exon 51 or 53 respectively. Tc-DNA: tricyclo-DNA, ICV: intracerebroventricular, PMO: phosphorodiamidate morpholino oligomers, AAV: adeno-associated viral, vg: vector genome, AON: antisense oligonucleotide, E/I: excitatory/inhibitory.*

To increase therapeutic efficacy and rule out possible improvements due to treatment effects of dystrophin restoration in the periphery, the efficacy of a single bilateral intracerebroventricular (ICV) bolus injection of tc-DNA AONs was assessed (Zarrouki et al. 2022). A dose dependent increase in exon 23 skipping levels of up to 35% upon treatment with 400 µg tc-DNA AONs was found. This resulted in up to ~25% of dystrophin and improvements in the fear response. To assess the duration of treatment effects, mice were sacrificed at different time points post-treatment.

Dystrophin levels were highest after 6-7 weeks (up to 15%) and gradually decreased at 10 weeks. Despite a prominent restoration of the fear-response at 7 weeks, this was lost 3 weeks later. Long-term memory retention was restored, whereas treatment had minor effects on cued fear conditioning.

Applicability of the morpholino (PMO) chemistry was assessed in *mdx*/bl10 mice, aged 30 days, receiving 1 mg of AON via ICV infusion with an osmotic pump for one week (Sekiguchi et al. 2009). This led to restoration of 25% dystrophin. The time frame of dystrophin restoration was similar to that of ICV injections with tc-DNA AON, showing optimal restoration between 5-7 weeks after treatment initiation, and a significant drop 11 weeks after treatment. Also here, the freezing response was partially rescued during this 5 to 7 week post-treatment window.

To optimize treatment efficacy, several delivery routes have been tested for tc-DNA and PMOs in the *mdx52* model (Saoudi et al. 2023). ICV injections of tc-DNA resulted in the highest skipping levels (20-30%), while a combination of ICV and intra-cisterna magna injections yielded the best results for PMOs (16-22% skipping). Furthermore, singular bilateral ICV was more efficient than unilateral injections while slow delivery increased distribution compared to rapid injection, although skipping levels were similar in the later comparison. Repeated ICV injections did not increase skipping levels.

Although dystrophin levels remained relatively loaw, studies have shown promise with regards to therapeutic effects even under these conditions. Saoudi *et al* showed that 10-15% of skipping via a singular ICV injection of tc-DNA (400 µg) led to 5-15% of Dp427 restoration (Saoudi et al. 2023). Treated *mdx52* mice showed reductions in anxiety and unconditioned fear, improvements in fear memory and a complete rescue of fear conditioning.

Instead of utilization of AONs, exon skipping can also be induced upon treatment with U7 small nuclear RNAs (snRNA) that encode antisense sequences that can target a particular exon and are expressed from recombinant adeno-associated viral (rAAV) vectors (Goyenvalle et al. 2004). Single intra-hippocampal injections of U7-Sd23/BP22 AAV2 vector can partially restore dystrophin expression in the hippocampus (~15-25%) of *mdx*/bl10 mice, which lasts for at least two months. Dp427 expression completely recovered $GABA_a$-receptor clustering and hippocampal synaptic plasticity in these mice (Dallérac et al. 2011). This has been replicated in another study, where the recovery of $GABA_A$-receptor clustering was proven to last up to at least four months (Vaillend et al. 2010). Systemic treatment of *mdx52* mice with an AAV serotype 9 (AAV9)-U7 targeting exon 51 (U7ex51) vector induced ~5% exon 51 skipping in the cortex, hippocampus and cerebellum while no effect on dystrophin restoration or freezing response was observed (Aupy et al. 2020).

Restoration of Dp140 expression

With Dp140 being primarily expressed in the developing human brain, it is unclear whether postnatal restoration of this isoform would have any therapeutic effect. Using PMOs targeting exon 53, 8 ICV injections over 4 weeks of 30 mg/kg lead to approximately 5-10% skipping, 1% Dp427 and 5% Dp140. Although dystrophin restoration was very low, ASD-like behaviour and the alterations in E/I balance were ameliorated in treated animals. No differences were found in anxiety or fear (Hashimoto et al. 2022). Multiple sites have since tried to improve exon skipping and dystrophin restoration levels. A multicentre study using different AON chemistries highlighted the challenges of exon 53 skipping. A combination of multiple AONs was necessary to improve skipping to 25% (in the hippocampus) regardless of the chemistry used (Doisy et al. 2023). The authors press the increased challenges of skipping exon 53 compared to exon 51 in *mdx52* mice.

Section 5: Challenges and future directions

Cognitive problems severely limit DMD patients' ability to function in society and are often referred to as very burdensome. With increased life expectancy, improved standards of physical care, and recent advancements in the development of therapies aimed at the conservation of muscle function, this cognitive burden will likely play a more prominent role in the near future. As such, brain deficits largely impact overall quality of life of patients and substantially increase costs of the condition in terms of health economics.

Mouse models have aided the field in unravelling the pathomechanisms of dystrophinopathy and enabled preclinical studies aimed at dystrophin restoration in the brain. Despite the knowledge gained in the last decade, there is still a lot to learn about the role of brain-specific dystrophin isoforms and how dystrophinopathy affects the brain. An important difference between clinical and preclinical studies is the use of corticosteroids. Whereas the majority of DMD patients receive corticosteroids as part of the standards of care to slow down disease progression, preclinical studies have been predominantly executed in steroid naïve mice. Corticosteroids increase behavioural problems in DMD patients (Angelini 2007, Counterman et al. 2022), which is the most common reason to discontinue treatment (Poysky 2007, Matthews et al. 2010). Furthermore, corticosteroid treatment regimens seem to affect grey matter volume (Geuens et al. 2023). Although the use of steroid naïve animals allows to study the consequences of dystrophinopathy in isolation, it neglects the additive negative effects on the brain. To unravel the contribution of chronic steroid treatment in DMD on brain pathology, future preclinical studies should consider including a chronically treated corticosteroid study arm.

Preclinical studies revealed that dystrophin restoration in the DMD brain holds promise, however, there are several challenges to overcome and outstanding ques-

tions that hold back further clinical development. Firstly, treatment of the lowly abundant *Dmd* transcript in the brain is challenging and some exons appear more difficult to skip than others. Notably, skipping of exon 53 in the *mdx52* mouse required three AONs to obtain efficient skipping. This is in sharp contrast to the human *DMD* gene, in which exon 53 seems more easy to skip than exon 51. The underlying mechanism remains to be elucidated, but it may be due to differences in transcript processing dynamics (Gazzoli et al. 2016). Furthermore, protein restoration seems challenging in the brain of *mdx52* mice. Whereas the discrepancy between skipping levels and dystrophin expression is ~2-4 fold for exon 51 (Aupy et al. 2020, Saoudi et al. 2023), due to transcript imbalance (Spitali et al. 2013, García-Rodríguez et al. 2020), it is unclear why 25% of exon 53 skipping translates to only <2% protein. Utilization of the hDMDdel52/*mdx* mouse, which allows targeting exon 51 and 53 in the human *DMD* gene, could be instrumental in future investigations (Veltrop et al. 2018, Yavas et al. 2020), especially when crossed with *DMD-null* mice to eliminate expression of murine dystrophin.

Secondly, since AONs target the pre-mRNA, repeated treatment is required to maintain dystrophin expression. Since the protein is only stably expressed for a short duration, its effects on behavioural and cognitive impairments have so far been assessed with tests that offer a large therapeutic window and can be executed in a short amount of time, such as the unconditioned fear test. As such, the field still lacks a complete picture of which of the deficits can and which ones cannot be restored.

Thirdly, in the light of the different spatio-temporal expression profiles of the brain specific dystrophin isoforms (Doorenweerd et al. 2017), restoration of specific isoforms could have a larger therapeutic effect than others. Since Dp427 is expressed throughout life, postnatal restoration is likely to be beneficial, whereas postnatal restoration of Dp140 could be less effective since this isoform is primarily expressed in the developing human brain. Lastly, since expression profiles of dystrophin also seem to differ between men and mice, it is hard to predict how well findings in model systems will translate to the clinic.

The recent discoveries and developments in the DMD field have much aided our understanding of the disease and brain pathology in general. Notably, the impact of this research is not limited to DMD but is widely applicable for other neurological conditions. The knowledge gained on synaptic functioning and gene expression in relation to certain behaviours (such as social deficits and repetitive behaviours) can be used as a gateway to broaden the understanding of disorders like ASD and ADHD. Furthermore, by investigating the altered molecular and cellular pathways and their influence on brain plasticity and function, new potential therapeutic targets could come to light that might benefit not only DMD patients but a broader spectrum of neurodevelopmental disorders.