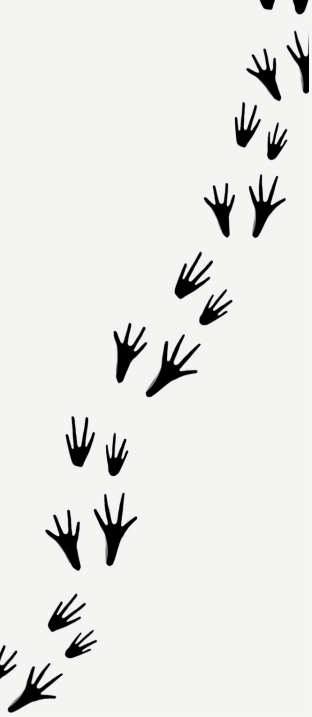
The neurological and behavioral consequences of dystrophin deficiency in Duchenne muscular dystrophy: insights from mouse models

Verhaeg, M.A.T.



CHAPTER 1

Introduction

Duchenne muscular dystrophy

Approximately 1 in 5,000 newborn males are affected by Duchenne muscular dystrophy (DMD), a progressive X-linked disorder (Mendell et al. 2012). The disease is primarily defined by severe muscle wasting, which is caused by mutations in the *DMD* gene, resulting in a lack of the dystrophin protein. The first symptoms become apparent at approximately two to three years of age, starting with reduced function of the lower extremities (Ryder et al. 2017). Patients become non-ambulant between 10 to 12 years of age and eventually die, in the Western World, in the 3rd or 4th decade of their lives due to cardiac or respiratory failure (Wein et al. 2015, Yiu and Kornberg 2015).In addition to the muscle deficits, DMD patients often exhibit cognitive and behavioral problems. Their intelligence quotient is approximately 1 standard deviation below the population average (Hinton et al. 2007, Banihani et al. 2015) and a subset of DMD patients suffers from additional cognitive impairments and behavioral problems, including autism spectrum disorder (15%), obsessive-compulsive disorder (4.8%), attention-deficit hyperactivity disorder (32%), depression (19%)**,** anxiety (27%), dyslexia (40%), dyscalculia and/or epilepsy (6.3%) (Hinton et al. 2006, Hendriksen and Vles 2008, Pane et al. 2012, Waite et al. 2012, Banihani et al. 2015, Pangalila et al. 2015, Ricotti et al. 2016). Furthermore they exhibit problems with working memory, attention and automatization (Bouquillon et al. 2024). These cognitive and behavioral deficits significantly increase the healthcare burden and reduce the quality of life of DMD patients (Webb 2005, Bendixen et al. 2012, Ueda 2019).

The DMD gene and dystrophin isoforms

DMD gene and mutations

The *DMD* gene, located on the X-chromosome, is the largest known human gene. It consists of 79 exons and spans approximately 2.3 megabases (Mercuri et al. 2019, Hildyard and Piercy 2023). The gene contains 7 promotor regions that drive the expression of multiple tissue-specific dystrophin isoforms, each with distinct functions (we refer the reader to Chapter 2 for an overview of the promotors and dystrophin isoforms). Of these isoforms, only Dp427m is expressed in the muscle, while Dp427c, Dp427p, Dp140, Dp71 and Dp40 are expressed in the brain (Doorenweerd et al. 2017). Additionally, Dp140 is expressed in the kidney, and Dp71 and Dp40 are ubiquitously expressed throughout the body. Dp260 is expressed in the retina and Dp116 in the peripheral nerves (Byers et al. 1993, D'Souza et al. 1995). There are many types of mutations that have been described in the *DMD* gene including deletions, duplications and point mutations. These mutations can affect the synthesis of dystrophin in different ways, depending on their influence on the reading frame. When a mutation result in the disruption of the reading frame, a premature stop codon is introduced. No functional Dp427 can then be produced

and the lack of Dp427m in muscles causes the patients to be affected by DMD. Two mutation hotspots have been identified on the *DMD* gene, around exons 2-22 (mostly duplications) and exons 45-55 (predominantly large deletions) (Takeshima et al. 2010, Aartsma-Rus et al. 2016, Kumar et al. 2020, Fortunato et al. 2024). The location of the mutation determines the number of dystrophin isoforms lacking, meaning mutations close to the 5' end of the gene impact only the full-length dystrophin isoforms (Dp427), whereas mutations closer to the 3' end also disrupt one or multiple of the shorter isoforms. For this reason, all DMD patients exhibit muscle wasting, while the cognitive phenotype is a lot more diverse. Approximately 45% of patients lack Dp427 and another 45% also lack Dp140, but only up to 10% lack all dystrophin isoforms (Desguerre et al. 2009, Taylor et al. 2010, Pane et al. 2012, Rasic et al. 2014, Ricotti et al. 2016). Correlations have been made between the amount of missing dystrophin isoforms and the incidence and severity of cognitive impairments, however, a lot of heterogeneity remains within the patient groups (Taylor et al. 2010, Chamova et al. 2013, Ricotti et al. 2016).

Dystrophin expression patterns and functions in humans

In muscles of healthy individuals, dystrophin stabilizes muscle fibers by linking the actin cytoskeleton to the extracellular matrix via the dystrophin associated protein complex (DAPC) (Ervasti 2007). This mechanical connection protects the fibers from contraction-induced damage. Dystrophin also interacts with proteins in the DAPC that are involved in signaling processes such as calcium homeostasis and cellular communication (Constantin 2014). In the absence of dystrophin, the membranes of the muscle fibers become unstable and eventually rupture. This induces necrosis of the fibers and initiates inflammatory processes (Wallace and McNally 2009). Over time, chronic damage, in the absence of efficient regeneration, leads to replacement of muscle fibers by fat and fibrotic tissue, resulting in progressive muscle deterioration (Wallace and McNally 2009, Klingler et al. 2012).

In the brain, dystrophin also forms DAPC complexes and its role has been associated with synaptic functioning (Waite et al. 2009) and $GABA_A$ receptor clustering (Waite et al. 2012). The different dystrophin isoforms each have a unique expression pattern in the brain. Dp427p has been reported to be present in the Purkinje cells of the cerebellum (Holder et al. 1996), although a more recent study could barely find detectable levels in the human brain (Doorenweerd et al. 2017). Dp427c is expressed at low levels in the neurons of the cortex and CA regions of the hippocampus throughout human development (Nudel et al. 1989, Lidov et al. 1990, Doorenweerd et al. 2017). Dp140 peaks during fetal development, in the cerebral cortex, while postnatally expression is mostly found in the cerebellum (Lidov et al. 1995, Morris et al. 1995, Doorenweerd et al. 2017). Dp71 and Dp40 are widely spread throughout the brain including in the cerebellum, cortex and the choroid (Morris et al. 1995, Lumeng et al. 1999, Doorenweerd et al. 2017).

Mouse models

Need for mouse models

Although the first clinical description of DMD already included indications of brain involvement, research has mainly focused on the muscle phenotype. Even though the focus has been adjusted over the last decade, knowledge about the human DMD brain remains limited. This is partially due to the scarcity of available tissue, as the most recent reports date back many years (Rosman and Kakulas 1966, Dubowitz and Crome 1969, Jagadha and Becker 1988). Non-invasive techniques such as magnetic resonance imaging and behavioral characterizations have aided to our understanding of the brain related pathology in DMD, however, since patients can lack different isoforms in the brain, and only a small percentage of patients lacks all dystrophin isoforms, adequate representation of all patients groups (due to the different genetic variations of the disease) remains challenging.

Animal models, such as the DMD mouse models, have made significant contributions to the field. These models are well-suited to study DMD as they enable the use of a controlled environment and can equally represent the full range of disease variation, particularly in terms of the extent of missing dystrophin isoforms. Many mouse models have been used to study DMD, including mice lacking only full-length dystrophins (*mdx*, *mdx*^{5cv} and *D2-mdx* mice), mice lacking both Dp427 and Dp140 (*mdx*^{4cv} and *mdx52*) and mice lacking all dystrophin isoforms (*DMD-null*).

Mouse model	Mutation location	Dp427	Dp260	Dp140	Dp116	Dp71/ Dp40
Mdx	Exon 23	-	+	+	+	+
Mdx^{5cv}	Exon 10	-	+	+	+	+
D2-mdx	Exon 23	-	+	+	+	+
Mdx^{4cv}	Exon 53	-	-	-	+	+
Mdx52	Exon 52	-	-	-	+	+
DMD-null	Complete deletion by Crispr-Cas	-	-	-	-	-
Dp71-null	Dp71 promotor region	+	+	+	+	-
Mdx^{3cv}	Intron 65	+/-	+/-	+/-	+/-	+/-

Table 1: Overview of dystrophin isoforms per mouse model. *+: isoform present. -: isoform absent. +/-: isoform present in low levels*.

Other mouse models that have been studied are the Dp71-null mice, which lack only Dp71 and Dp40 while retaining the longer isoforms, and the *mdx*^{3cv} mice, which express low levels of all dystrophin isoforms.

Dystrophin expression in the murine brain

Through preclinical studies, many functions of the different dystrophin isoforms expressed in the brain have become apparent. Dp427 is primarily involved in synapse structure and functioning (Blake and Kröger 2000, Miranda et al. 2011). At the excitatory synapses, Dp427 regulates synaptic plasticity, including long term potentiation (Vaillend et al. 2004), while at the inhibitory synapses it facilitates clustering of the $GABA_A$ receptors (Anderson et al. 2012, Zarrouki et al. 2022). Dp140 has been implicated in presynaptic plasticity at the excitatory synapses (Blake and Kröger 2000, Hashimoto et al. 2022). Dp71 is associated with many functions, including maintenance of blood-brain barrier permeability (Anderson et al. 2012), organization of synaptic structures (Daoud et al. 2009) and regulation of ionic and water homeostasis through potassium and water channel modulation at the astrocytic endfeet (Connors et al. 2004, Haenggi and Fritschy 2006, Fujimoto et al. 2023). Additionally, Dp71 interacts with the DAPC at both the excitatory and inhibitory synapses, where it regulates synaptic transmission and neuronal communication (Greenberg et al. 1996, Blake and Kröger 2000, Haenggi et al. 2004). Finally, Dp40 is linked to presynaptic functioning (Tozawa et al. 2012, Fujimoto et al. 2014) and neuronal differentiation and maturation (Blake and Kröger 2000, García-Cruz et al. 2022).

One of the challenges of using DMD mouse models lays in the discrepancy in expression patterns of dystrophin between mice and humans. In terms of Dp427, expression in the neurons of mice is more broadly spread throughout different cortical structures and the cerebellum (García-Cruz et al. 2023), compared to the human brain, where it seems limited to a few specific structures including the hippocampus and cortex (Doorenweerd et al. 2017). Furthermore, expression levels of Dp427p and Dp427c in the murine brain seem relatively similar, while in humans Dp427p expression levels are much lower compared to those of Dp427c (Doorenweerd et al. 2017, García-Cruz et al. 2023). Timing of Dp140 expression is similar between mice and humans, as expression is high in fetal stages and lower in adults (Doorenweerd et al. 2017, García-Cruz et al. 2023). However in mice, Dp140 expression remains more detectable in the adult brain, primarily in oligodendrocytes (Blake et al. 1994, Morris et al. 1995, Aranmolate et al. 2017). Dp71 and Dp40 expression seems relatively conserved across mice and humans, both showing a broad expression across cell types and regions.

Scope and outline of this thesis

The *mdx* mouse has by far been the most commonly used mouse model to study the DMD brain, however, it only represents less than 50% of the DMD patient population. Characterization of models lacking multiple dystrophin isoforms which represent the remainder of the DMD patient population is minimal. Moreover, direct comparisons between mouse models lacking different dystrophin isoforms are scarce, making it difficult to determine the exact consequences of the lack of the shorter isoforms. It is however vital to understand the impact that the different dystrophin isoforms have on the brain. Notably, the field has started to develop genetic therapies targeting the DMD brain. While dystrophin expression can partially be restored in mice, it is vital to understand how this restoration will impact behavior and cognition. Therefore, there is a need for a better understanding of which behavioral and neurological deficits are associated with each of the dystrophin isoforms, making it possible for the appropriate assays to be chosen when investigating the potentials of genetics therapies in rescuing cognitive deficits.

This thesis aimed to understand the role of the different dystrophin isoforms and how the lack thereof influences behavior and brain pathology in DMD mouse models. In **Chapter 2**, an overview is given of the current knowledge on the brain pathology and behavioral deficits in both DMD patients and the most commonly used DMD mouse models. Furthermore, the current therapeutic developments targeting the DMD brain in mouse models are described, focusing specifically on exon skipping approaches. **Chapter 3** provides an overview of the deficits found in *mdx* (lacking Dp427) and *mdx*^{4cv} (lacking Dp427 and Dp140) mice. This chapter emphasizes the primary role of Dp427 in learning and spontaneous behavior, as mice lacking Dp427 showed deficits in both domains. Dp140 did not seem to play a prominent role in these processes. In **Chapter 4**, an extensive behavioral study is described, phenotyping mouse models lacking one (*mdx*^{5cv}), multiple (*mdx52*) or all dystrophin isoforms (*DMD-null*). We confirmed the role of Dp427 in anxiety and fear, a possible role of Dp140 in anxiety and found that Dp71 and/or Dp40 are associated with many behavioral domains, including anxiety, fear and spontaneous behavior. In **Chapter 5,** the effects of corticosteroids on *mdx* and *mdx*^{4cv} mice were assessed. Due to technical issues with delivery of the corticosteroids, the effects of the treatment on many behavioral domains remains uncertain. However, short-term corticosteroid treatment did not seem to negatively affect anxiety or social interaction in DMD mice. **Chapter 6** focusses on the brain pathology in *mdx*^{5cv}*, mdx52* and *DMD-null* mice, providing an overview including volumetric analysis, perfusion, diffusion and AQP4 expression data. Here, no effect of the lack of Dp427 on brain pathology was seen. A role of Dp140 in brain volume was suggested and lack of all dystrophin isoforms led to reduced cerebral perfusion and disrupted APQ4 clustering. Lastly, **Chapter 7** offers a broader perspective on the implications of the findings described in this thesis and outlines future directions for the field.