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

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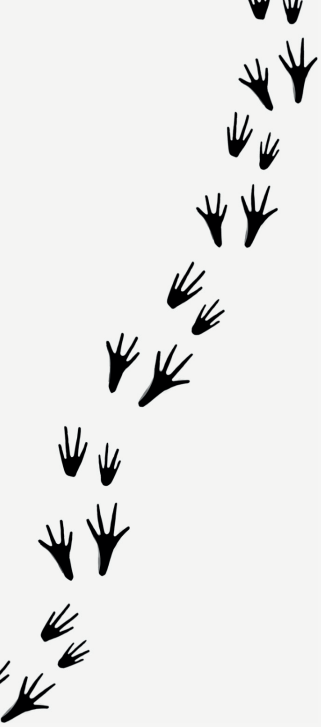

CHAPTER 7

Discussion

While muscle pathology is often the main focus in DMD research, the cognitive and behavioral problems have a significant on the lives of DMD patients and their loved ones. Patients and caregivers report that the cognitive deficits impact the quality of life and the healthcare burden significantly (Schwartz et al. 2021, Schwartz et al. 2022). There is a high need for therapeutic options to treat the DMD brain, however the remaining gaps in our knowledge regarding dystrophin and the brain hinder these advances.

This thesis aimed to contribute to the knowledge on the DMD brain by investigating the effects of the lack of dystrophin on behavior and brain pathology in DMD mice. By characterizing different DMD mouse models, this thesis provided an overview of the different behavioral deficits and brain pathology, due to the lack of different dystrophin isoforms. In short, we found that Dp427 plays an important role in working memory, anxiety, fear and blood-brain barrier permeability. Aside from an altered brain volume, the consequences of the lack of Dp140 are less clear, however there are indications that the absence this isoform might play a role in anxiety, spatial memory and spontaneous behavior. Lack of Dp71 and Dp40 widely impacts behavior and brain pathology, including anxiety, fear, spontaneous behavior, cerebral blood flow and AQP4 expression. These behavioral deficits were also studied in combination with corticosteroid treatment, however due to technical issues, it remains unclear how corticosteroids influence behavior in DMD mouse models.

Challenges in translating and standardizing behavioral research in DMD mouse models

Translatability

Translatability of results from animal models to the human situation remains a significant hurdle in preclinical research and one should always question to what extent findings in animal models can be extrapolated. In DMD specifically, the most commonly used mouse model, the *mdx* mice, is characterized as having 'mild' muscle pathology compared to DMD patients (Tanabe et al. 1986, Aartsma-Rus and van Putten 2014). In terms of brain involvement, differences are also present. For example, while the *DMD-null* mice show many deficits compared to mice retaining Dp71 and Dp40 expression, the differences between the models seem less substantial than between the corresponding patient groups (as described in **chapter 4**). Furthermore, it remains challenging to confirm if peculiar results (e.g. the lack of alterations in brain volume as described in **chapter 6**) in *DMD-null* mice are also present in patients. The overall low number of patients lacking all dystrophin isoforms and the additional recruitment difficulties of this particular patient group

due to the more severe behavioral problems hinder confirmations of these type of findings (Ricotti et al. 2015). Additionally, DMD mouse studies often omit the use of medication commonly prescribed to DMD patients, namely corticosteroids. These drugs slow down muscle degeneration but can also have a negative impact on behavior and brain pathology. Not including this factor in animal research further limits the translatability of preclinical studies, as it does not fully reflect the human condition.

There is a high need for a better understanding of the molecular pathways underlying the alterations in behavior and brain pathology. These mechanisms are almost impossible to study in humans without the availability of brain tissue, as non-invasive techniques have limited spatial resolution and specificity. Even if post-mortem tissue were available, the age of the patients is of course determined by the time of death, meaning any research performed on the tissue will be done at available ages. Animal models are a great tool to enable the study of these mechanisms in a controlled setting and at specific points in time, however the question remains to what level the information obtained from these studies will have relevance in understanding the human (DMD) brain.

Interplay between muscle function and behavior

The interplay between muscle function and behavior plays an important role in DMD research. While in humans, most cognitive and behavioral assessments are not heavily dependent of muscle performance, this does not hold true for animal experiments. Behavioral assays in mice are often activity based, introducing a confounding factor that could influence the outcome of the tests. As described in **Chapter 4**, basic movement patterns can be affected in DMD mice and velocity differences have been observed. These results emphasize the importance of the consideration of muscle performance even in basic behavioral tests. Currently, there are no mouse models available where the dystrophin depletion is isolated to only affect the brain. Results of behavioral assays should always be interpreted with caution in case of DMD, especially in high motor demanding tests or tests where speed is taken as an outcome measure.

Reproducibility and standardization

Next to challenges in translatability and interpretation, there is also a need for improvements in reproducibility and standardization. While numerous studies have been conducted in e.g. the *mdx* mice, there are many contradictory results in literature, especially in terms of memory deficits (Vaillend et al. 1995, Vaillend et

al. 1998, Vaillend and Ungerer 1999, Vaillend et al. 2004, Remmelink et al. 2016, Lewon et al. 2017, Dickson and Mittleman 2019, Bagdatlioglu et al. 2020). These discrepancies can be caused by numerous variables, including differences in genetic background (McLin and Steward 2006, Deacon et al. 2007, Flynn et al. 2021, Mortazavi et al. 2022), age of the animal (Dean et al. 1981, Kennard and Woodruff-Pak 2011, Shoji et al. 2016), housing conditions (type of cages, amount of animals per cage) (York et al. 2012, Horii et al. 2017), apparatus used (measurements, color), timing of experiments, the type of handling (Gouveia and Hurst 2019) and variations in protocols that are being used.

There is a great need to standardize practices and exclude the contribution of these conditions towards the variation in obtained results. Establishing consensus on standard operating procedures (SOPs), similar to those already developed for muscle related research in DMD (Grounds et al. 2008, Nagaraju and Willmann 2009), is a crucial first step towards increasing reproducibility. The use of SOPs could eliminate variations in habituation procedures, duration of experiments, type of handling of the animal and the type of apparatus used, thereby reducing variability and improving overall reproducibility.

Enhancing corticosteroid research in DMD: challenges and clinical relevance

Corticosteroids in DMD patient care

Corticosteroid treatment (mainly prednisolone or deflazacort) is part of the standards of care in DMD patients, and aims to slow down muscle degeneration. However, the negative consequences of the drugs on behavior and cognition are significantly impacting the lives of DMD patients and their caregivers and are among the most common reasons for patients to discontinue the treatment (Poysky 2007, Matthews et al. 2010). Despite the crucial factor of corticosteroids in DMD care, this critical component has been largely overlooked in DMD mouse model research. Only a hand full of studies have tested the effects of corticosteroid treatment on DMD mouse behavior (Guerron et al. 2010, Sali et al. 2012, Liu et al. 2024). To this date, it still remains unclear how corticosteroids exactly effect behavior and brain morphology in both DMD patients and mouse models.

Challenges in including corticosteroids in preclinical studies

A significant challenge in corticosteroid studies lays in the delivery methods. As described in **Chapter 5**, we experienced challenges with adequate and consistent corticosteroid delivery over an extended period of time. Reliable delivery strategies with minimal impact on the wellbeing and anxiety levels of the mice are essential components to successfully research the effects of corticosteroids. Current delivery strategies either induce stress (e.g. daily injections or oral gavage), to which DMD animals are highly receptive, or lead to varying levels of corticosteroid uptake (e.g. via food or water uptake), which further add to the complexity of data interpretation.

A further challenge lays in the heterogeneity of corticosteroid treatments. Guidelines for the type of drug, dosage and delivery intervals vary across countries and furthermore regimes are tailored on a patient level (Griggs et al. 2013, Van den Bergen et al. 2014, Landfeldt et al. 2015, Gloss et al. 2016, Takeuchi et al. 2016, Cowen et al. 2019), introducing additional challenges in terms of translatability. Differences in treatment regimens can influence behavioral outcomes in DMD mice (Liu et al. 2024) and also affect brain volume (Geuens et al. 2023, Geuens et al. 2024). Furthermore, the variation in clinical treatment regimens might also play a role in the heterogeneity seen in DMD patients in terms of behavior and cognition. While part of the heterogeneity is caused by variations in dystrophin isoforms expressed in patients and the progression of the disease, even within these subgroups there is substantial variation between individuals (Darmahkasih et al. 2020). Currently, it remains unclear if corticosteroids also play a role in this variability.

Recently, vamorolone has been approved as an alternative corticosteroid treatment. Studies have showed that vamorolone has less impact on bone resorption than other corticosteroids and behavior does not seem to be significantly impacted (Damsker et al. 2019, Liu et al. 2020, Guglieri et al. 2022, Liu et al. 2024). However, these effects have only been tested after short term treatment, therefore the long term effects of vamorolone on cognition and behavior remain unclear. Furthermore, in the clinic, patients still report increases of behavioral problems after vamorolone treatment, although possibly to a lesser extent compared to prednisolone or deflazacort treatment (Griggs et al. 2016, Elhalag et al. 2023). While treatment with vamorolone seems promising, the full consequences of the long term use of this drug need to be further investigated in DMD patients.

Importance of including corticosteroids in preclinical research

Despite the challenges and the added complexity of these studies by incorporating corticosteroid treatment, it is vital to include this treatment as a variable in DMD mouse research. Preclinical research should aim to mirror the different corticosteroid regimes as seen in the clinic as closely as possible and take into account delivery methods and duration of treatments. Understanding the additive effects of corticosteroids on behavior and cognition in DMD mouse models enables us to increase translatability of the preclinical research and is a necessary step in fully understanding the DMD brain.

Towards treating the DMD brain

Challenges of current genetic approaches

To date, there are no approved therapeutic approaches to treat the DMD brain, however, as described in **Chapter 2**, techniques used to restore dystrophin in the muscle are being adapted to target the DMD brain. Some of these techniques, like exon skipping, are promising, as they are able to partly restore dystrophin expression and even ameliorate behavioral deficits such as the strong fear response normally seen in DMD mice (Sekiguchi et al. 2009, Goyenvalle et al. 2015, Relizani et al. 2017, Hashimoto et al. 2022, Zarrouki et al. 2022, Saoudi et al. 2023). However, many challenges remain in regards to treating the DMD brain. For instance, translatability between mice and humans remains a significant issue. In DMD patients, skipping of exon 53 is relatively easy to achieve compared to other exons (Servais et al. 2022). This does not hold true for mice, as simultaneous treatment with multiple oligonucleotides (AONs) was required to achieve significant exon skipping and protein restoration (Doisy et al. 2023), whereas skipping exon 51 was relatively easy in mice (Saoudi et al. 2023, Saoudi et al. 2023). Another challenge lays in the delivery of the treatments into the correct brain areas where dystrophin is normally expressed. While it is possible in the mouse brain to reach subcortical structures (Saoudi et al. 2023), it remains unclear how drugs will distribute in the (larger) human brain. Lastly, it is uncertain if the timing of restoration matters. Especially in terms of Dp140 restoration, as Dp140 is mostly expressed in fetal stages (Doorenweerd et al. 2017), the exact extent to which postnatal restoration can ameliorate cognitive deficits remains unclear.

In recent years, multiple humanized DMD mouse models have been developed (‘t Hoen et al. 2008, Veltrop et al. 2018, Pickar-Oliver et al. 2021). These DMD mice have an additional, human, *DMD* gene added to their genetic code. The humanized mice also have been developed on a *mdx* mice background (‘t Hoen et al. 2008). In these models, AONs designed for the human *DMD* gene can be used

to test the restoration of human dystrophin protein without the interference of mouse dystrophin. However, with the increased necessity for treating the DMD brain, there is a need to expand on those humanized mouse models to include models that lack all mouse dystrophin isoforms. A humanized *DMD-null* mouse model could be of great significance to the field. However, before these types of mice could be used to test therapeutic approaches, it would be necessary to characterize the models, as it is unclear how the presence of human dystrophin would affect their behavior and brain pathology. Furthermore it will also be necessary to test how corticosteroid treatments impact the possible treatment of the DMD brain.

Secondary treatment strategies

While genetic approaches might seem preferable as they are directly aimed to target the cause of DMD, secondary treatment options should also be utilized (Weerkamp et al. 2023). In some cases, medications for ADHD, depression and anxiety are prescribed for DMD patients when necessary, however this is not common practice yet. The clinical practice lacks standard guidelines for DMD patients as the effects of using for example ADHD, anxiety or depression medication in DMD are not well studied. Many treatments are extrapolated from general pediatric or neuropsychiatric guidelines and much uncertainty remains about the efficacy of the treatments and the implications in DMD specifically, also in light of the co-treatment with corticosteroids (Weerkamp et al. 2023). The interaction of potential secondary treatments with other drugs often prescribed to DMD patients (including corticosteroids) and the potential exacerbation of physical problems (e.g. cardiomyopathy) caused by these treatments complicates the addition of neuropsychiatric treatments (Kenna et al. 2011, Buddhe et al. 2018). For example, while these treatments could eventually be used to ameliorate side effects of the corticosteroid treatments, many medications used to treat certain behavioral disorders could also exacerbate corticosteroid-related side effects such as mood swings and aggression (Warrington and Bostwick 2006, Taper 2016). Lastly, since DMD patients are diagnosed at a young age, and behavioral and cognitive problems also surface early on, treatments should always be adjusted to a developing brain and the long term side effects of medication on brain development should be weighted carefully. While standardized guidelines, specific to DMD patients, could significantly improve quality of live, the heterogeneity of the disease also calls for personalized treatment plans tailored to the patient.

Increased awareness to the cognitive and behavioral comorbidities of DMD is crucial for adequate psychopharmaceutical interventions. In the clinic, brain related comorbidities are still underdiagnosed in DMD patients, resulting in untreated deficits that impact the daily lives of patients and caregivers. Recently, screening

tools have been developed (Thangarajh et al. 2019, Weerkamp et al. 2023, Geuens et al. 2024, Hendriksen et al. 2024), however wide application of these tools still needs to be achieved. Early identification of cognitive and behavioral deficits in DMD patients should be a standard part of the diagnostic process. Early discovery would enable integration of the necessary personalized support into clinical follow-up and targeted treatment plans for the patients. This early recognition can also be used to address specific challenges, like reading difficulties and provide the proper help to ensure the patients can participate as much as possible in society.

Future prospects

While animal models have significantly contributed to our understanding of the DMD brain, the limitations of these models in translatability pose an ongoing challenge. Especially for the development of treatments, it will be vital to move towards DMD models carrying human *DMD* genes, as is already happening in the muscle field. The development of new humanized DMD models will of course require basic behavioral characterization to determine both the consequences of the human dystrophin presence and to choose suitable assays to measure the effect of dystrophin restoration.

Furthermore, there is a great need for studies focusing on the long term impact of treatments, both for genetic interventions and for corticosteroid administration. Safety and efficacy analyses are often performed in short time frames in preclinical studies, however, as these treatments are likely to be administered long term in patients, the preclinical field should aim to reflect the duration better in their research.

Lastly, it is vital to actively keep promoting awareness in the DMD field. Recognition of the cognitive and behavioral deficits and timely interventions can only be achieved through integration of (preclinical) findings into clinical practice. Increased screening for CNS deficits during diagnostics and personalized psychopharmaceutical interventions, in case of these deficits, could greatly enhance the quality of life of DMD patients and their caregivers.

Concluding remarks

DMD is a multidisciplinary disease and therefore requires multidisciplinary care and research approaches. Treatment of the DMD brain can be improved by learning from both the approaches in the DMD muscle and in other brain disorders. Unfortunately, the impact of the cognitive and behavioral deficits on DMD on patients' lives still remains underrecognized in the clinic.

The use of mouse models will continue to play a crucial role to better understand the DMD brain and eventually develop targeted treatments for the patients. These models make it possible to study both the DMD brain and possible therapeutic strategies in a controlled setting, however there is still a long way to go before the DMD brain can be treated. While genetic therapies targeting the brain might eventually become available for DMD patients and hopefully decrease the burden of cognitive and behavioral deficits for DMD patients and caregivers, many unanswered questions remain. The approval of these treatments could become a lengthy process, therefore in the meantime, alternative strategies like secondary treatment options should be utilized and tailored more to DMD patients.

A personalized approach will be necessary to best address the needs of the individual patients. Improving the quality of life of DMD patients is a main priority in DMD and the impact of the brain plays a significant part in this process. Only through standardized practices and protocols and a multidisciplinary approach, including the use of animal models in preclinical research, can we increase our understanding of the DMD brain and will we be able to address the full range of needs of DMD patients.