The Duchenne brain

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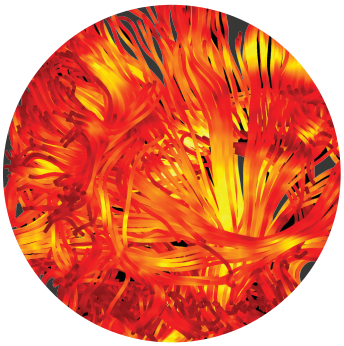

Chapter 6

Summary and general discussion

The aim of this thesis was to provide a detailed description of the structural, perfusion and metabolic differences in the brain between patients with DMD and healthy age-matched controls and to assess the role of dystrophin isoforms. All in the hopes of gaining a better understanding of the origin of the cognitive problems in DMD. The main results are summarized in Figure 6.1.

In chapter two we demonstrated structural alterations in the brain in boys with DMD compared to age matched healthy controls. Total brain volume was reduced in patients with reduced grey matter volume, but the white matter volume and cerebrospinal fluid were of similar size. However, despite the lack of a difference in volume of the white matter, the white matter structural integrity was affected as demonstrated by reduced fractional anisotropy and increased mean diffusivity. There was no specific region identified, but rather the differences were found throughout the brain. In a subgroup of patients with a mutation predicted to affect the Dp140 dystrophin isoform in addition to the Dp427 isoform the differences were more profound compared to controls. This subgroup of patients also performed worse on specific neuropsychological tests, but the exact relationship between the structural alterations in the brain and cognitive performance in boys with DMD remains to be elucidated.

In chapter three we show that there are not only structural alterations in the brain, but that the cerebral blood flow (CBF) is also reduced as compared to controls. We evaluated if this was related to the reduced grey matter volume, but even after correcting for partial volume effects the difference between patients and controls remained significant and showed that the reduced cerebral blood flow was independent finding. This may suggest a different underlying mechanism. The reduced cerebral blood flow was again most profound in patients missing Dp140 compared to controls.

Biochemical changes as assessed by magnetic resonance spectroscopy (MRS) had previously been shown in DMD. In chapter four we performed MRS at the high field strength of 7 Tesla which enables increased signal to noise ratio and spectral resolution compared to these previous studies. In contrast to earlier findings, we demonstrate preserved biochemical composition at rest. Whether the brain metabolism is equally capable of responding to functional challenges remains to be addressed.

In chapter five we addressed the limited knowledge on where dystrophin isoforms are expressed in the human brain and at which developmental stage of life. Dystrophin isoforms show large changes in expression through life with pronounced differences between the foetal and adult human brain. The Dp140 isoform was expressed in the cerebral cortex only in fetal life stages, while in the cerebellum it was also

expressed postnatally. The Purkinje isoform Dp427p was virtually absent, which contrasts sharply to mouse models previously used to assess dystrophin expression in the brain. The expression of dystrophin isoforms was significantly associated with genes implicated in neurodevelopmental disorders, like autism spectrum disorders or attention-deficit hyper-activity disorders, which may explain the high co-morbidity of these disorders with DMD. We also identified relevant functional associations of the different isoforms, like an association with axon guidance or neuron differentiation during early development. Our results point to the crucial role of several dystrophin isoforms in the development and function of the human brain.

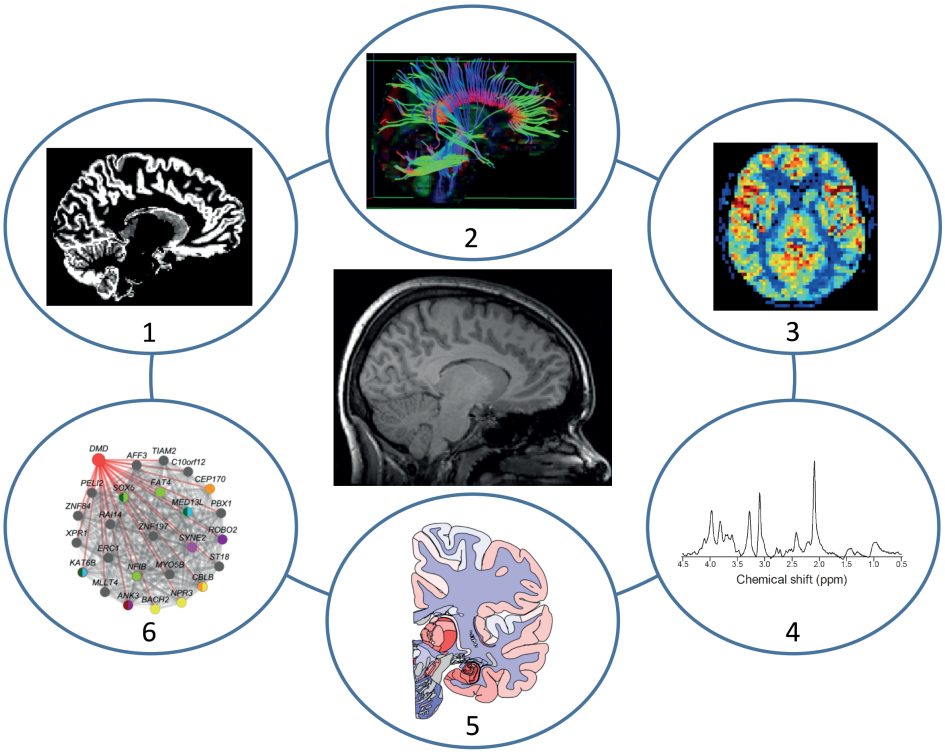

Figure 6.1. An overview of the different components of the brain of Duchenne patients that we evaluated within this thesis is shown. Panel one represents the reduced total brain and grey matter volume from chapter two. Panel two, the altered white matter microstructure from chapter two. Panel three, the reduced cerebral blood flow from chapter three. Panel four the preserved bio-chemical composition from chapter four. Panel five the expression of dystrophin in the healthy human brain from chapter five. Panel six a network of genes co-expressed with DMD from chapter five.

The different chapters combine to provide a framework towards understanding the origin of the neuropsychological profile in DMD. Previously there was little evidence to support that the cognitive profile in DMD has a tangible foundation in the brain. Our results extend and refine what was previously hypothesized. In agreement with Dubowitz and Crome (Dubowitz and Crome 1969) we did not find gross abnormalities upon visual evaluation of the MR data. We demonstrate instead, the need to use quantitative measures to capture the more subtle, yet significant differences between DMD and controls. The reduced grey matter volume is in line with the CT findings described in the introduction chapter of this thesis (Yoshioka et al. 1980). However it is not yet clear if there is cerebral atrophy (i.e. that the brain volume was initially larger, but became smaller), or if it reflects a developmental disorder (i.e. that the brain does not grow as much or at a different rate). It is important to note that the brain is undergoing normal development within the age range of the participants of our study. Grey matter is gradually pruned in favour of higher white matter density. Therefore, the lower grey matter volume could also indicate that boys with DMD are ahead of their peers in this respect. For example, a change in the time course of cortical pruning has previously been reported for ASD. In ASD children have been shown to have a thicker cortex than controls in areas of the brain that related to ASD social difficulties and executive functioning up to early adolescence (Mensen et al. 2017). From adolescence into their early twenties there was faster and longer lasting pruning of the cortex in these areas in ASD compared to controls (Wallace et al. 2015). This has led to hypothesis of early overgrowth followed by overcorrection consisting of selective elimination of synapses in ASD. The best way to address the question of the time course of cerebral development in DMD would be to perform a longitudinal study. By monitoring the cerebral volume distribution over time, it may become clear whether there is delayed development, faster development, a different steady-state or progressive decline of the grey matter in DMD compared to controls.

The reduced white matter microstructural integrity is a new finding in DMD. There have been many studies using DTI to investigate neurodevelopmental and neurodegenerative disorders over the years (Dougherty et al. 2016; Thompson et al. 2014; Amlien and Fjell 2014; Griffa et al. 2013). Most of these find localized differences between patient populations and controls that relate to the respective cognitive symptoms. In contrast, we find differences throughout the white matter and were not able to determine to what extend these are related to the neurocognitive profile in DMD due to the relatively small study cohort.

One of the strengths of DTI is that it is a sensitive method. However, DTI is also a non-specific method in which many factors contribute to the signal. Any of these factors

may differ between DMD and controls, and there is no way of differentiating between them based on the DTI data alone. Our data shows that the overall streamlining within the white matter is less restricted or unidirectional in DMD compared to controls. The resolution of the scan allows us to say something about clusters of hundreds of thousands of cells within one voxel. Figure 6.2 illustrates three different hypothetical scenarios that cause FA to go down and MD to go up within one of these voxels as compared to the healthy situation (indicated by the yellow oval) and end up giving identical signal output (indicated by the red oval). In order to understand what the underlying pathology of the white matter is in DMD, the ideal scientific scenario would be to perform a post-mortem analysis of the brain of a DMD patient. However, this is a delicate issue to address within the patient community as the brain extraction procedure does need to occur relatively quickly after death and would only reflect the end stage of the disease. There is currently no DMD brain tissue available for research within The Netherlands. Alternatively, even more advanced MR methods such as DTI with multiple b-values to also extract diffusion kurtosis imaging (DKI) information for constrained spherical deconvolution (CSD) analyses could be used. This methods can help distinguish between crossing and kissing pathways and enable more pathways to be accurately extracted from the data. However, this would still be at a similar resolution as the current DTI data, so not much can be gained with respect

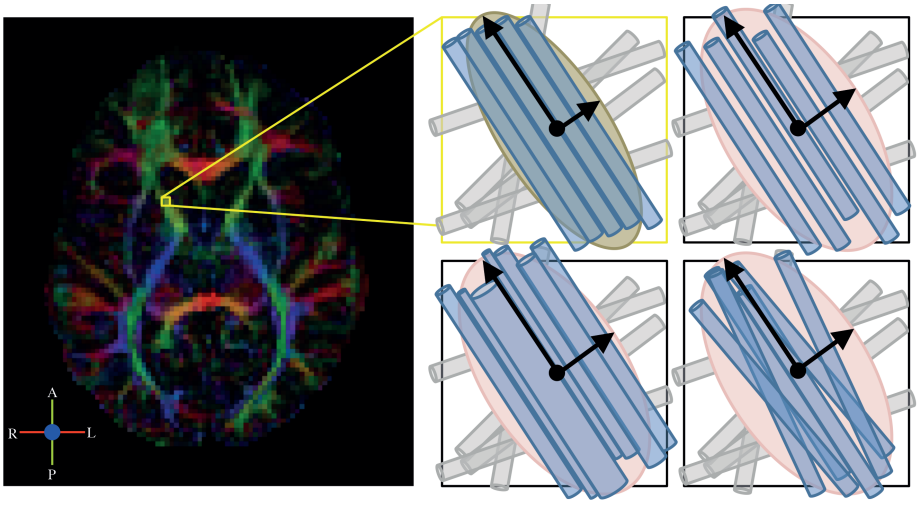

Figure 6.2. A simplified representation of several possible structural orientations that all result in the same reduced FA and increased MD within a voxel. Indicated in yellow is the healthy situation. Fewer bundles, spaced further apart are shown in the top right. In the bottom left, different and mostly larger bundle sizes are shown. In the bottom right the bundles have a less uniform direction.

to differentiating between cells or cell-types. Diffusion weighted spectroscopy may provide a solution, for it can measure the diffusion properties of metabolites rather than those of water. Because metabolites are expressed in different cell-types in different proportions, this may provide information about the cell-type specificity of the diffusion alterations. For example, should neurons be the specific cell-type to be affected in DMD, then the diffusion properties of NAA will be different, whereas those of choline will not. If all cell-types are altered, then the diffusion properties of all should differ from controls. If such a study was being designed it would be advisable to also include proton or phosphorous spectroscopy with a challenge as proposed in chapter 4. Doing so would allow us to determine if the brain can respond metabolically to cognitive challenges which will also help determine which signalling pathways may be affected. Unfortunately the voxel-size of spectroscopy measurements would need to be even larger than DTI or DKI due to the much lower signal of the metabolites compared to water, therefore reducing the potential for spatial differentiation. A third option to further investigate the origin of the reduced FA and increased MD is called magnetization transfer (MT) MRI which has been previously employed in multiple sclerosis, a disease in which myelin is broken down. Should myelin breakdown be the underlying cause for the reduced FA and increased MD in DMD, then there should be a detectable shift in MT ratios.

The reduced cerebral perfusion was also a new finding in DMD patients, although it was known that the blood-brain-barrier was affected in a mouse model for the disease. As discussed in chapter 3, potential explanations include the normal expression of dystrophin in the brain, in the cerebral vasculature and also in the heart (Figure 6.3). On top of that, it could be a secondary effect of the limited mobility or potentially increased BMI in DMD. There are several experimental set-ups that may help dissect which is the most likely cause, which is important information when considering current and future treatment options. If, for example, the blood-brain-barrier is affected in patients in a similar way as in the mouse model, then drugs may enter the brain more easily in DMD, which will affect what would be an optimal dose. Another example, should the heart function play a key role, then drugs that are prescribed to prevent or slow down cardiac function decline may also have an effect on the cerebral perfusion and which should then be monitored in the clinic. One way to assess the systemic regulation of cerebral blood flow is to perform a orthostatic challenge with a head-up-tilting table. By passively resting on the table and being tilted to a 60-80 degree angle, gravity will cause the blood to pool in the legs and the body will need to respond to keep the blood flow to the brain sufficient to avoid fainting. A slight increase of heart rate and constriction of the blood vessels in the legs make sure the CBF is unaffected. If the body is unable to meet the higher demand of the orthostatic

challenge to maintain cerebral circulation homeostasis, then the systemic regulation of CBF may be responsible for the lower CBF in DMD. To assess the function the of the cerebral vasculature, a perfusion MRI sequence (BOLD or ASL) can be used together with a cognitive task. For example, a visual stimulus will require activation of the occipital lobe, which in turn will require more sustenance to perform. This will lead to vascular dilation in this brain region. Such a test of vascular reactivity could show several responses. For example, a normal response if the blood vessels are not affected, a reduced response if the vessels have become more rigid and cannot dilate properly or a delayed response if the blood vessels are leaky. Once we have a better idea of the underlying cause for the reduced perfusion it will be more feasible to design a study that can assess to what extent the reduced perfusion is related to

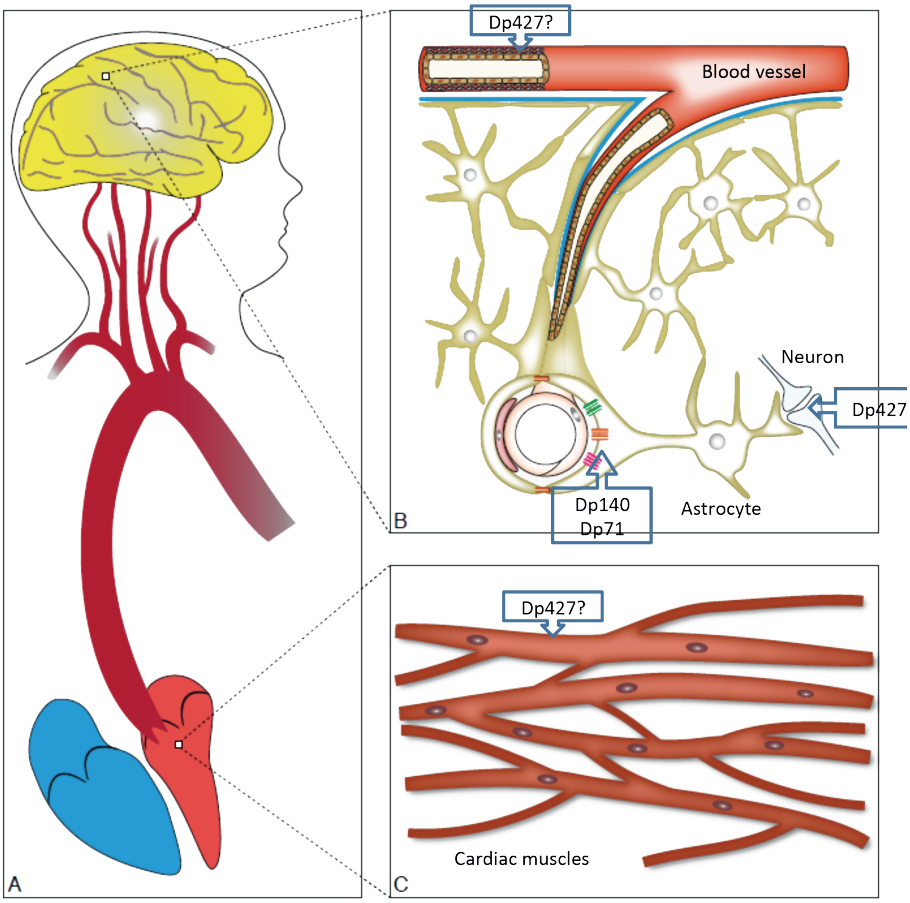

Figure 6.3. The heart-brain dystrophin connection. Panel B shows dystrophin in post-synaptic neurons, astrocyte end-feet where they wrap around the cerebral vasculature and within the blood vessels. Panel C shows dystrophin within the muscle cells of the heart.

the neurocognitive profile in DMD. Theoretically, if the brain chronically deprived of nutrients, this could affect brain function. However, there are no other reports of a paediatric cohort with global reduced perfusion to a similar extent as we have found. What effect the 17% reduced CBF may have on brain function in DMD is open to speculation.

Having a better understanding of the location and timing of expression of dystrophin isoforms in the human brain was greatly needed. However, there is much more information to be distilled from the vast amount of transcriptome data. New DNA/RNA sequencing projects have focussed on providing expression data of the different cell types within the brain. This is of special interest in DMD as studies have suggested that the expression of dystrophin isoforms is cell-type specific in the brain (Waite 2011; Hendriksen 2016). As most of the differentiation between types of neurons and glia cells is by definition based on our understanding of their function, we can gain more insight into the function of dystrophin by learning which cell-types express the protein. There is also potential for a better translation between animal models and humans, as more research is aimed at providing detailed cerebral transcriptome data of various species among which mouse and non-human primate (Allen Brain Atlas). We have demonstrated important overlap as well as inconsistencies between man and mouse with respect to the expression of DMD. Animal models provide an invaluable source of information to help us understand mechanistic biological pathways involved in DMD brain pathology. And, therapeutic compounds would need to be tested in animals before going to people. Needless to say, it is important to know to what extent a mouse model actually models the human pathology and what the limitations are.

As mentioned in chapter 1, the implementation of improved standards of care for DMD, which in itself is a positive development, also results in potential confounding effects for the findings described in this thesis. Specifically, the treatment with corticosteroids is important to address. Corticosteroids are a class of steroid hormones that are naturally produced by the body. They are involved in a wide range of physiological processes, including stress response, immune response and regulation of inflammation. As a therapy, corticosteroids have anti-inflammatory, immunosuppressive, anti-proliferative and vasoconstrictive effects. In animals, stress and corticosteroids can be associated with both reversible and irreversible changes in the brain such as cortical atrophy and reduced hippocampus volume. Within our study population we had only five boys who did not receive corticosteroids. The MR findings in these five boys, were consistently distributed throughout the DMD group giving no indication that the steroid treatment is a likely cause for the results. There are only

a few MRI studies investigating the effect of corticosteroids in humans, but those that do could not find a significant association between corticosteroids and cerebral abnormalities[Chinn et al 1997; Steens et al 2005; Zavidanov et al 2001; Brown et al 2006]. We could not find any reports investigating cerebral blood flow with and without corticosteroids with respect to their vasoconstrictive effect. However, between January 2004 and October 2012 there were 80,774 reported adverse events for prednisone, of which 282 were of cortical atrophy. The percentage of patients receiving corticosteroids with cerebral atrophy as a side-effect was 0.35% (factmed. Inc). Thus, to ascertain the potential role of corticosteroids in more detail, a placebo-controlled trial would need to be conducted.

There is still a great heterogeneity in both the extent of the MR abnormalities as well as the severity of the symptoms related to the neurocognitive profile in DMD. We can state that the relationship between grey matter volume, white matter microstructure and cerebral perfusion as measured with MR and specific brain functions known to be affected in DMD is not one on one. There is a lot of work to be done to understand the origin of the neuropsychological profile in DMD and the biological mechanisms involved. This thesis provides a solid foundation to perform hypothesis driven studies in the future, rather than exploratory ones. In other words, we now have a better understanding of where to look and what to look for.

Nederlandse samenvatting

Duchenne spierdystrofie (DMD) wordt gekarakteriseerd door progressieve spierzwakte. Het wordt veroorzaakt door mutaties in het *DMD* gen, die leiden tot afwezigheid van functioneel dystrofine eiwit. In de hersenen komen meerdere varianten van het dystrofine eiwit voor, maar in tegenstelling tot dystrofine in de spieren, is er weinig bekend over de functie van dystrofine in de hersenen. Wel komen leer- en gedragsproblemen vaak voor bij patiënten met DMD. Patiënten met mutaties in het DMD gen waarbij meerdere dystrofine varianten zijn aangedaan lopen hier een hoger risico op. Om goede hypotheses te kunnen vormen over de oorsprong van de cognitieve problemen bij DMD moet eerst in kaart gebracht worden of er structurele, doorbloedings- of metabolische verschillen zijn in de hersenen bij patiënten met DMD vergeleken met typisch ontwikkelende controles. Hiertoe hebben we een exploratieve studie uitgevoerd met gebruik van MRI en MR spectroscopie.

In hoofdstuk twee hebben we structurele veranderingen in de hersenen van jongens met DMD vergeleken met gezonde leeftijdsgenoten beschreven. Het totale hersenvolume en grijze stof volume waren kleiner, maar het witte stof volume evenals het volume van de hersenvloeistof waren vergelijkbaar. Ondanks het vergelijkbare volume binnen de witte stof bleek echter dat de microstructurele integriteit aangedaan was. Deze bevindingen hebben we door de hersenen verspreid gevonden. Ook waren de bevindingen sterker in een subgroep van patiënten die een tweede isoform van dystrofine (Dp140) missen. Dezelfde subgroep had ook slechtere scores bij het neuropsychologisch onderzoek. Vooralsnog is er geen één-op-één relatie aangetoond tussen de structurele veranderingen en het cognitief functioneren.

In hoofdstuk drie hebben we aangetoond dat er niet alleen structurele veranderingen zijn in de hersenen, maar dat ook de doorbloeding verminderd was met gemiddeld 17%. We hebben getoetst of de verminderde doorbloeding verband hield met de verminderde grijze stof, maar het bleek een onafhankelijk bevinding. Mogelijk duidt dit op twee verschillende onderliggende mechanismes. Wederom was de doorbloeding het sterkst verminderd in de subgroep patiënten zonder Dp140.

Eerdere studies hebben gekeken naar biochemische veranderingen in de hersenen van jongens met DMD met behulp van MR spectroscopie. In hoofdstuk vier hebben we deze techniek toegepast op een hoge veldsterkte van 7 Tesla wat een betere signaal-ruis verhouding en spectrale resolutie oplevert vergeleken met de eerdere studies. In tegenstelling tot eerdere bevindingen, was de biochemische compositie binnen ons cohort vergelijkbaar tussen patiënten en gezonde controles. Mogelijk is dit een

weergave van een stabiele metabolische balans in rust. Of het metabolisme ook in staat is adequaat te reageren op een functionele taak is onbekend.

In hoofdstuk vijf gingen we in op de beperkte kennis over waar en tijdens welke fase van de ontwikkeling de dystrofine eiwitten tot expressie worden gebracht in het gezonde humane brein. We hebben relatief hoge expressie gevonden in de amygdala en hippocampus. Beide gebieden zijn betrokken bij complex gedrag en de werking van het geheugen. Deze functies zijn een belangrijk onderdeel van het cognitief profiel in DMD. We hebben ook gevonden dat Dp140 hoofdzakelijk tijdens de foetale ontwikkeling tot expressie wordt gebracht, maar in het volwassen brein nog in het cerebellum aanwezig is. Verder konden we de Purkinje variant, die zijn naam te danken heeft aan de expressie in Purkinje cellen van het cerebellum in muizen, niet terug vinden in het humane brein. Met behulp van co-expressie analyse hebben we vervolgens gekeken welke genen op dezelfde plekken binnen het brein tijdens dezelfde ontwikkelingsfasen tot expressie werden gebracht. Omdat er over deze andere genen veel meer bekend is wat betreft de functie in het brein, kunnen we dat als basis gebruiken voor toekomstige studies die toetsen welke functie dystrofine heeft in de hersenen. Tot slot vonden we een hoge co-expressie met genen die bekend zijn van autisme, ADHD, OCD en dyslexie, wat een mogelijke verklaring biedt voor het vaker voorkomen van deze ziektebeelden bij DMD.

De verschillende hoofdstukken vormen samen een sterke basis voor verder onderzoek naar de oorsprong van de cognitieve problemen bij DMD.