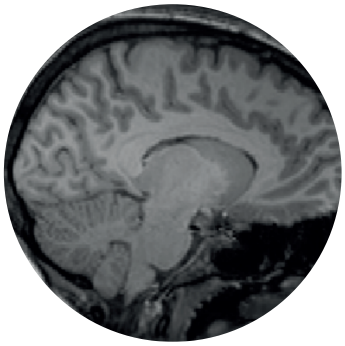
The Duchenne brain

Doorenweerd, N.



Chapter 1

General introduction

1.1 A brief background of Duchenne muscular dystrophy

Muscular dystrophy is a group of muscle diseases. They are characterized by progressive skeletal muscle weakness, defects in muscle proteins and the death of muscle cells and tissue (Emery et al. 2002). Duchenne muscular dystrophy (DMD) is a genetic, recessive and severe form of muscular dystrophy, affecting approximately one in 3,600-10,000 boys (J. K. Mah et al. 2014). It is caused by mutations in the largest human gene, the *DMD* gene, measuring 2.4Mb (Davies et al. 1988). The importance of this gene and its components will be discussed in greater detail in chapter 5.

DMD is inherited in an X-linked recessive pattern (Figure 1.1). An affected son inherits the mutation from his mother (who is a carrier of the mutation in one copy of the gene) in about two-thirds of cases. The other one-third of cases probably results from new mutations in the gene (T. Lee et al. 2014). DMD predominantly affects men, because they have only one X chromosome. A mutation in one copy of the gene is therefore enough to result in the condition. In women, who have two X chromosomes, a mutation in one X chromosome would make them a carrier. Another mutation would have to occur in the second copy of the gene to result in DMD which is extremely rare.

The first clinical symptoms are commonly seen before the child is four years of age, with a specific strategy to get up from the floor called “Gowers Sign” and calf muscle hypertrophy being the most typical. Additionally, delayed developmental milestones and the inability to run like their peers may be observed (Emery, Muntoni, and Quinlivan 2015). Blood testing will indicate elevated serum creatine kinase (CK) levels. The diagnosis can be confirmed with genetic testing for mutations in the *DMD* gene or a muscle biopsy that shows absence of dystrophin in the muscle cell membrane.

As the child is growing up, muscle weakness will worsen and loss of ambulation takes place between ten and sixteen years of age. The current average life expectancy is approximately 25-30 years of age and the main cause of death is cardiac or respiratory failure (Strehle and Straub 2015). There is no cure for DMD, although progress has been made in slowing disease progression down through the implementation of specific standards of care (Kinnett et al. 2015; Moser 1984; Zamani et al. 2016; Matthews et al. 2016; Wong et al. 2016). These include treatment with corticosteroids, respiratory care, (preventative) cardiac care and psychosocial support. Multiple therapeutic trials looking to restore, repair or prevent further damage to the muscles are currently taking place and there is hope that the life expectancy and quality will improve further (Robinson-Hamm and Gersbach 2016; J. Mah 2016; Mendell et al. 2013).

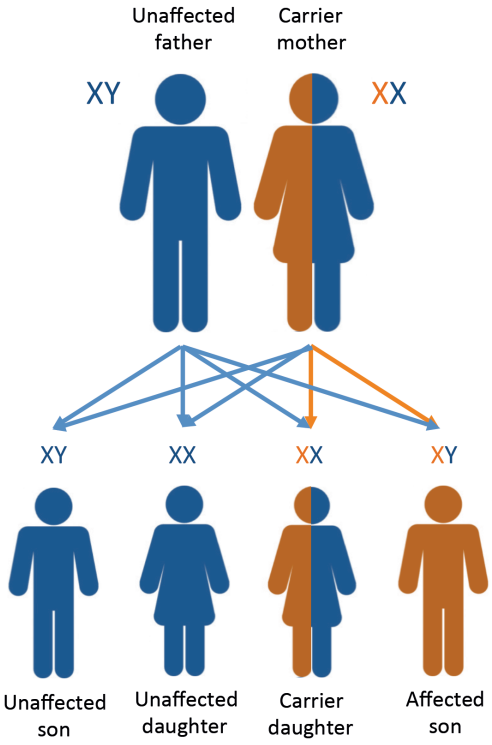


Figure 1.1 A schematic representation of X-linked recessive inheritance.

Chronic use of corticosteroid can have side-effects of which several are relevant for the interpretation of results presented in this thesis. Examples of relevant potential side-effects are rapid mood swings; increased appetite and fluid retention which can lead to weight gain; Cushings syndrome, which includes the development of a 'moon-shaped' face; weakening of the bones which can lead to osteoporosis; and finally, mental health problems such as depression and anxiety. In most cases the potential side-effects can be managed by adjusting the treatment dosage and it is common in The Netherlands to prescribe corticosteroids in a 10-day on, 10-day off dosing regimen. Potential confounding effects of chronic use of corticosteroids on the findings described in this thesis will be further discussed in chapter 2, 3 and 6.

1.2 The neurocognitive profile of Duchenne muscular dystrophy

This thesis focusses on the brain involvement in patients with DMD. When the French neurologist Guillaume-Benjamin-Amand Duchenne (1806-1875) described

the disorder in "*Paraplegie hypertrophique de l'enfance de cause cerebrale*" one of the features he included in the description was intellectual impairment. Over the years it has been shown that there is a one standard deviation shift downward in full-scale intelligence quotient (FSIQ) in patients with DMD compared the general population (Cotton, Voudouris, and Greenwood 2001; Hinton et al. 2000; Cyrulnik et al. 2007). Approximately 30% of patients with DMD have an FSIQ of below 70, which is the threshold for cognitive impairment. The cognitive profile additionally includes more specific problems such as difficulty with verbal short term memory, visuospatial long term memory and verbal fluency (Dorman *et al.*, 1988; Bresolin *et al.*, 1994; Pane *et al.*, 2013; Ricotti *et al.*, 2015). Reading problems are more prevalent (Billard et al. 1992; J. G. M. Hendriksen and Vles 2006) There is also a significantly higher incidence of neurodevelopmental disorders in patients with DMD compared to the general population, with recent studies showing the incidence of attention-deficit/hyperactivity disorder (ADHD) to be 32%, anxiety disorder to be 27%, autism spectrum disorder (ASD) to be 15%, epilepsy to be 6.3% and obsessive-compulsive disorders to be 4.8% (Banihani et al. 2015; Pane et al. 2013; J. G. Hendriksen and Vles 2008; Goodwin, Muntoni, and Dubowitz 1997; Hendriksen et al. 2016).

1.3 *DMD* gene and dystrophin protein isoforms in the brain

Within the large *DMD* gene there are multiple promotor regions from which a dystrophin protein can start to be produced. These proteins have been named to reflect their size in kilo Dalton (kDa) (Figure 1.2). The most extensively studied protein is Dp427 (dystrophin protein, 427kDa in size) which is expressed in muscle and brain (Monaco et al. 1986; Koenig et al. 1989; Nudel et al. 1989; Holder, Maeda, and Bies 1996). The shorter/smaller proteins Dp140, Dp71 and Dp40 are also expressed in the brain (Lidov et al. 1990; Lidov 1996; Austin et al. 1995; Waite, Brown, and Blake 2012). Several studies have shown a higher incidence of neurodevelopmental disorders and cognitive impairment in patients with DMD who have a distal mutation in the dystrophin gene (Taylor et al. 2010; Chamova et al. 2013; Ricotti et al. 2015; Pane et al. 2013). As a distal mutation will result in the loss of multiple dystrophin isoforms, this led to the hypothesis that a cumulative loss of CNS isoforms leads to a higher risk of cognitive and behavioural problems.

Unfortunately, very little is known of the function of the dystrophin isoforms expressed in the brain. Many different cell types are present in the brain (Figure 1.3). Neurons are electrically excitable cells that use electrical and chemical signals to process and transmit information. Oligodendrocytes wrap around the neuronal axons to form myelin for faster electrical signal transduction. Astrocytes perform many

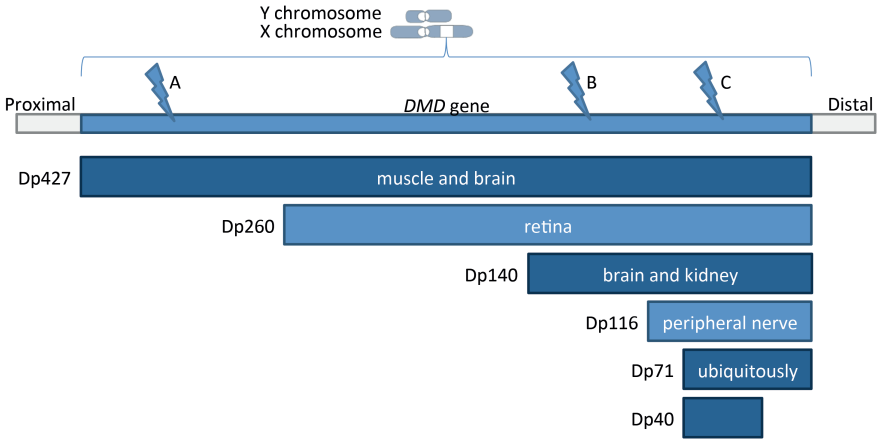


Figure 1.2. Overview of the main dystrophin isoforms and their relative read-out positions within the DMD gene. A mutation at position A, i.e. the proximal end of the gene, would only affect Dp427. At position B, the mutation would affect Dp427, Dp260 and Dp140. At the distal end of the gene, mutation C would affect all of the dystrophin isoforms.

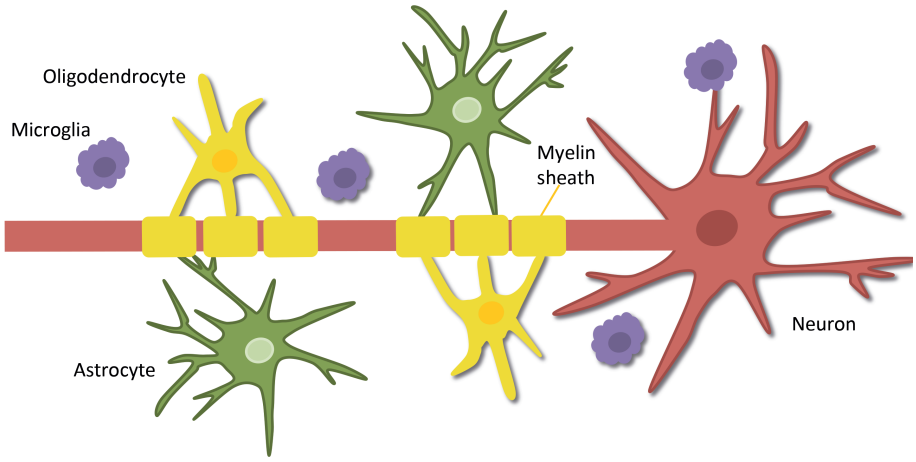


Figure 1.3. A schematic representation of the main cell types in the brain.

different functions from modulation of the blood-brain-barrier to maintenance of the extracellular ion balance. Finally, microglia present the main form of active immune defence.

In the muscle cells, dystrophin attaches to the actin cytoskeleton at one end and groups together multiple proteins at the cell membrane at the other end (Figure 1.4). Based on animal studies Dp427 is proposed to be part of a dystrophin-glycoprotein-like complex (DGC) quite similar to that in muscle (Waite, Brown, and Blake 2012). However, unlike providing structural stability to the muscle cell during muscle contraction, there is evidence that Dp427 in the brain anchors a subset of $GABA_A$-receptors at the post-synaptic membrane of neurons (Waite, Brown, and Blake 2012; R. G. F. Hendriksen et al. 2016). This may imply a function in signal transduction. Similarly, Dp71 is proposed to form a DGC to anchor aquaporin 4 receptors at the membrane of astrocyte end-feet, which wrap around the cerebral vasculature (Waite, Brown, and Blake 2012). This suggests a role in signal transduction to regulate cerebral blood flow. Little is known of Dp140. It is believed to only be expressed during the foetal stages of life and as such it is difficult to investigate (H. G. W. Lidov et al. 1990; H. G. Lidov, Selig, and Kunkel 1995).

Recent technological advances and large scale data-share projects have opened up the possibility to investigate the sites of expression and predict functions of genes and proteins in the healthy human brain across development (Hawrylycz et al. 2012; Miller et al. 2014; Chen et al. 2009; Pinero et al. 2015; Zhou et al. 2011). Such analyses, dedicated to dystrophin, are described in this thesis in Chapter 5. It has also become possible to non-invasively assess the brain *in vivo* in human beings using imaging techniques. The employment of these techniques to study the brain in DMD is the main focus of this thesis (Chapter 2, 3, and 4) for they can provide valuable information on brain structure, function and even metabolism.

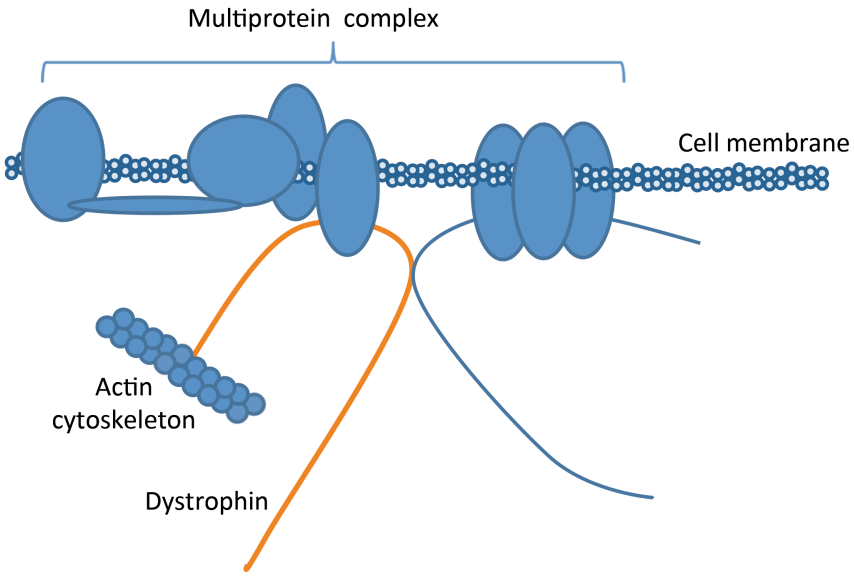


Figure 1.4. A schematic representation of the function of dystrophin (depicted in orange) as an anchoring protein holding together a multi-protein complex at the cell membrane.

1.4 Previous brain imaging and spectroscopy results in DMD

The first imaging study that investigated the brain in relation to DMD was conducted in 1980 by Yoshioka and colleagues using computer tomography (CT) (Yoshioka et al. 1980). CT uses computer-processed combinations of many X-ray images taken at different angles creating cross-sectional images. The scans indicated slight cerebral atrophy in twenty out of thirty participants with DMD. This atrophy was more pronounced in the older participants, suggesting a progressive process. However, this study predates the genetic testing requirement for confirmation of the diagnosis DMD. Therefore, there may be other muscular disorders that are clinically difficult to distinguish from DMD included in this group. Another limitation is that the scans were evaluated qualitatively (i.e. visual assessment by a radiologist) rather than quantitatively which may introduce a bias in scoring depending on who performed the evaluation. Lastly, the imaging quality at the time was more suited for the detection of gross abnormalities than for more subtle deviations from the healthy situation.

Both magnetic resonance imaging (MRI) and positron emission tomography (PET) were then performed to study the brain in patients with DMD in 1994 (n=4) and 2002 (n=10) (Bresolin et al. 1994; J. S. Lee et al. 2002). Both studies reported no

abnormalities on MRI upon visual inspection. However, the PET results showed region-specific glucose hypometabolism in the right sensorimotor cortex, lateral temporal neocortex, bilateral medial temporal structures and white matter of the cerebellum as well as variable involvement of the frontal, parietal, temporal occipital and cerebellar cortical areas. The abnormalities detected by PET may reflect local abnormalities in cell composition associated with altered neural development, which is further explored in chapter 2. PET uses a small amount of radioactive substance which is injected into the blood stream to make images. For PET scans of the brain, a radioactive atom is often applied to glucose (blood sugar). The brain uses glucose for its energy metabolism and this scan technique can indirectly provide information on brain activity. In the study performed by Bresolin and colleagues, the results from patients were compared to literature values from healthy individuals, to assess abnormalities. In the study by Lee and colleagues, a control group of 17 adults of whom eight were female (age range 21.1-38.2; mean 27.6) was scanned to compare to the ten boys with DMD (age range 5.2-16.2; mean 11.8). This enabled them to perform voxel-based analyses to pinpoint regions of interest. Lee and colleagues indicated that an ideal control group would consist of age and sex matched typically developing children. However, this was not possible due to ethical restraints with respect to the use of radioactive tracers in healthy developing children without a clinical indication. Instead, the individual datasets were first normalised to the individual mean and then compared between patients and controls for regional differences under the assumption that absolute glucose metabolism may change with age but the relative pattern should not. Nevertheless, it cannot be excluded that the glucose hypo-metabolism detected in these relatively small cohorts may be due to suboptimal methodology.

A third imaging study that investigated the brain in DMD using only MRI reported alterations in the motor cortex (Lv et al. 2011). This study differed from the previous two in that it used quantitative MRI, instead of qualitative visual assessment. Quantitative MRI can provide more objective neuroimaging biomarkers. Results showed decreased grey matter concentration and decreased synchronization of spontaneous activity when comparing ten boys with DMD (age range 6.4-14.0) to fifteen healthy controls (age range 7.9-15.1). However, because this study focussed on the motor cortex and muscles are severely affected in DMD, it is difficult to assess if these differences are secondary to muscle wasting and limited mobility, or if they are primary and linked to the cognitive profile.

Changes in metabolite composition in the cerebellum, temporo-parietal and frontal cortex were reported by three studies with conflicting results. Reduced glutamate, both elevated and reduced choline containing compounds and an increase in

N-acetylaspartate (NAA) were found with MR spectroscopy (MRS) performed at 1.5 and 3 tesla (Rae et al. 1998; Kato et al. 1997; Kreis et al. 2011). It is difficult to conclude if there is an underlying metabolic problem in the brain in DMD, because of different methodology and conflicting results (Rae et al. 1998; Kato et al. 1997; Kreis et al. 2011).

Despite the high prevalence of neurodevelopmental disorders in patients with DMD, the number of MR imaging or MR spectroscopy studies performed in DMD is very low. There is no baseline overview of whole brain structure in DMD in the context of the developing brain compared to typically developing boys from which hypotheses can be formed that can lead to a better understanding of the mechanism(s) underlying the cognitive profile in DMD.

1.5 Thesis outline

The primary 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, with the hopes of gaining a better understanding of the origin of the cognitive problems in DMD. To this end, thirty boys with DMD and twenty-two age matched control boys were recruited to undergo MRI at a magnetic field strength of 3 Tesla, MRS at a magnetic field strength of 7 Tesla and neuropsychological evaluation. The secondary aim was to assess the genetic contribution, by subdividing the patient group based on to the mutation location within the DMD gene (Figure 1.2), as well as by mapping dystrophin isoform expression in the healthy human brain across development.

1.5.1 Neuropsychological testing

The participants completed a custom designed neuropsychological test battery with a duration on one hour. The primary aim of this assessment was to describe neurocognitive functions and reading performance. It was also used to assess if the cohort was representative for the Dutch DMD population and to assess whether the control group was representative of the general population. Parents and teachers were asked to fill out questionnaires that screen for signs of behavioral difficulties.

For the neuropsychological test battery the participants were alone in a quiet room together with the researcher for approximately one hour. Subtests included in the assessment were short term auditory memory (Kaufmann number recall), visual processing (Kaufman block counting), an estimation of verbal IQ (Wechsler Intelligence scale for Children (WISC) information and digit span), an estimation of performance IQ (WISC symbol search and substitution), simple reaction times upon

visual and audio stimuli (FePsy), motor function of the dominant index finger (FePsy finger tapping), receptive language capacities (Peadbody Picture Vocabulary Test), reading performance (Continu benoemen en woord lezen, the one minute reading test and 'Het Kijkbewijs'), and finally time awareness ('Tijdsbesef' questionnaire). For comparison to the MRI outcome measures, we composed three composite scores, one for information processing, one behavior score and a reading score results of which are shown in Chapter 2.

1.5.2 Anatomical scans

The presence of brain abnormalities was first assessed by qualitative inspection performed by an experienced neuro-radiologist. Several scan sequences were evaluated; T1-weighted (T1w), T2-weighted (T2w) and fluid attenuated inversion recovery (FLAIR) (Figure 1.5), results of which are described in Chapter 2. The hallmark of T1w images is the contrast between grey matter and white matter allowing for accurate identification of anatomical structures. T2w images differentiate between fat (dark) and water (light) in the opposite way from T1w. T2w provides relatively good resolution between normal and pathologic tissue. In FLAIR images the cerebral spinal fluid (CSF) is suppressed, which allows for assessment of pathology without the markers for injury being clouded by bright CSF signal.

In addition to visually assessing potential pathologic markers, the objective of acquiring anatomical scans was to quantify brain structure and volume. The T1w scan lends itself for the automated segmentation of grey matter, white matter and cerebrospinal fluid. It can then calculate the exact volume of the structure of interest for statistical analyses. Results of these analyses are also described in chapter 2. A diffusion tensor imaging (DTI) sequence was obtained after the T1w sequence. DTI is predominantly used to assess white matter structural integrity within the brain and to visualise white matter pathways (Figure 1.6). The technique takes advantage of the natural phenomenon of diffusion. Diffusion is the random movement of molecules. Without restrictions, this movement is isotropic (equal in all directions). However, the brain contains many cells with different shapes and sizes that restrict the movement of molecules (Figure 1.4). Especially neurons, with long axons that are insulated by myelin, provide a unique environment in which it is relatively easy for molecules to move along the axon, but difficult to go across the membranes making the diffusion anisotropic. By probing this motion with a DTI MRI sequence, we can deduce the underlying anatomy of the white matter pathways. As main output measures, fractional anisotropy (FA) and mean diffusivity (MD) are used for quantification and comparison between individuals which can be computed per voxel. FA is a number between 0 and 1, wherein 0 is isotropic and 1 is very anisotropic. MD is the mean speed at which

the molecules have moved around within the time of the measurement. Results from whole brain voxel-based analyses of FA and MD are described in chapter 2.

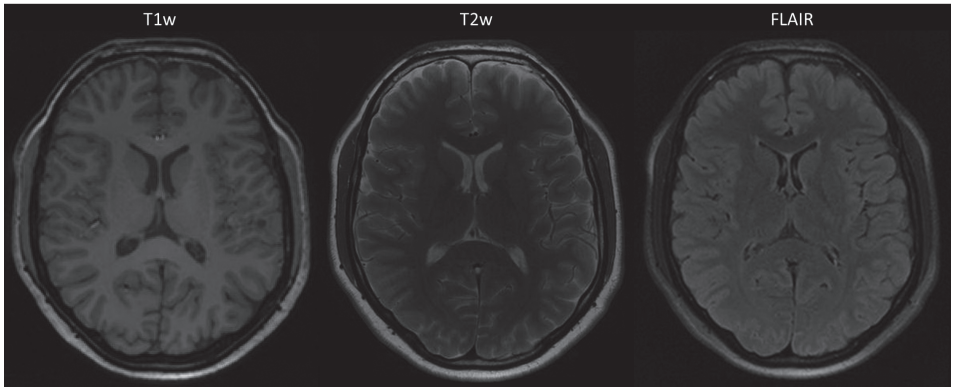


Figure 1.5. The different contrast obtained with T1w, T2w and FLAIR MRI scan sequences shown in a participant with DMD.

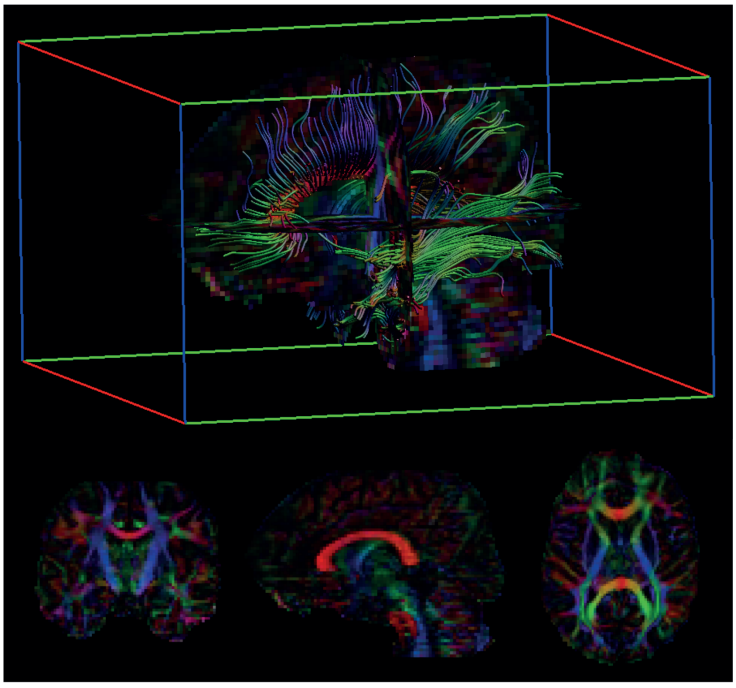


Figure 1.6. Visualization of white matter pathways in the brain. The colours reflect the orientation of the bundles: green=anterior-posterior; red=left-right and blue=top-down.

1.5.3 Perfusion scan

Next to structural integrity, an important component for brain function is the supply of oxygenated blood, glucose and other nutrients through the cerebral blood flow, also called cerebral perfusion. As mentioned in section 1.4, a PET study indicated reduced glucose uptake in the brain in DMD which can be the results of reduced perfusion. Therefore, in our study, we measured cerebral perfusion using a pseudo-continuous arterial spin labeling MR sequence. This is a quantitative method which allows detection of global hypo- of hyper-perfusion. The main advantage of this sequence is that it is a technique that uses magnetically labelled arterial blood water protons as an endogenous tracer. So in contrast to the PET study, there were no ethical constraints to acquiring this dataset in both boys with DMD and healthy age-matched controls. More details about this method and the results are described in Chapter 3.

1.5.4 Metabolic scans

Complementary to the structural and perfusion sequences, MRS can provide information of the biochemical composition of the tissue under investigation. In contrast to the imaging techniques which look at water, MRS looks at specific metabolites that have a much lower abundance and therefore much smaller signal. In order to be able to detect these metabolite a relatively large volume of interest is needed. Therefore, a region needs to be selected beforehand in which the measurement will take place. We selected three regions that were considered to normally express dystrophin and possibly relate to the neurocognitive profile; frontal cortex, hippocampus and cerebellum. We also chose to perform the measurements at a high field strength of 7 Tesla because this can give better separation between the metabolite signals and an increased signal to noise ratio compared to previous studies. The results are described in Chapter 4.

1.5.5 A healthy human brain atlas for dystrophin

Genome-wide gene expression maps of the healthy human brain have been made freely accessible (Hawrylycz et al. 2012; Miller et al. 2014). If one knows how to navigate these databases they provide an invaluable source of information on protein expression, co-expression and function. To date, our understanding of the expression of dystrophin in the brain stems from data gathered from animal models, cell cultures and a very limited number of post-mortem cases studies in human brain tissue (Nudel et al. 1989; H. G. W. Lidov et al. 1990; Holder, Maeda, and Bies 1996; Dsouza et al. 1995; Byers, Lidov, and Kunkel 1993; Morris, Simmons, and Man 1995; Lederfein et al. 1992; Austin et al. 1995; R. G. F. Hendriksen et al. 2016). Because knowing where dystrophin is expressed in the healthy human brain and during which developmental stage can provide important insight in our understanding of the brain in DMD we

used the most comprehensive atlases to create a human brain atlas for dystrophin. We could differentiate between the different isoforms in a proportion of the datasets, results of which are described in chapter 5. Secondary to describing where dystrophin is expressed, we gathered more information on potential functions of dystrophin in the brain. To this end we performed two additional analyses. We assessed which genes are expressed in the same brain region at the same developmental stage. This resulted in a ranked list of all the genes (~20.000) according to their temporal and spatial correlation to the *DMD* gene. This list was then queried to see if genes known to be associated to neurodevelopmental disorders have a significantly high correlation to *DMD*. In others words, are these genes significantly over-represented at the top of the list or are they evenly distributed throughout? We also researched the top 200 correlated genes to a greater extent. Many of these genes have been studied before and have data available on what function they have in the brain. This information can then be used to form hypotheses for the function of dystrophin isoforms through a guilt-by-association principle. Results of these analyses are also described in chapter 5.