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Mechanisms of brain dysfunction in myotonic dystrophy type 1: impact of the CTG expansion on oligodendrocytes and myelin

Sandra Oliveira Braz

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Université de Paris

École doctorale Bio Sorbonne Paris Cité - 562

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Mechanisms of brain dysfunction in myotonic dystrophy type 1:

*Impact of the CTG expansion on oligodendrocytes and
myelin*

Par Sandra BRAZ

Thèse de doctorat de Neurobiologie et Génétique Humaine

Dirigée par Mário GOMES-PEREIRA

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Titre :

Dysfonction des cellules oligodendriales et anomalies de la myéline dans un modèle de souris transgénique de la Dystrophie Myotonique de type 1.

Résumé :

La dystrophie myotonique de type 1 (DM1) est une maladie neuromusculaire multisystémique. L'atteinte cérébrale se manifeste par des symptômes neurologiques handicapants et des modifications structurales du cerveau, telles que des lésions prédominantes de la substance blanche. Les manifestations neurologiques de la DM1 impactent sévèrement le quotidien des patients et de leurs familles.

La DM1 est due à l'amplification anormale de répétitions CTG dans la partie 3'UTR du gène *DMPK*. Les transcrits *DMPK* mutants ont un effet toxique en *trans*: ils s'accumulent sous forme d'agrégats d'ARN (foci) qui dérèglent des protéines de liaison à l'ARN, l'épissage alternatif ainsi que d'autres aspects du métabolisme des ARN. Aujourd'hui, les types cellulaires et voies moléculaires principalement perturbés dans le cerveau ainsi que leur contribution aux symptômes neurologiques restent méconnus. Pour étudier ces mécanismes, notre laboratoire a créé des modèles murins transgéniques DM1 dont les DMSXL. Ces souris expriment des transcrits *DMPK* mutants humains dans plusieurs tissus, y compris le cerveau, et présentent des phénotypes comportementaux, électrophysiologiques et neurochimiques pertinents. Ces souris représentent un outil unique pour l'étude du dysfonctionnement cérébral dans la DM1. Une approche protéomique réalisée sur le cerveau des DMSXL a notamment montré une dérégulation de protéines spécifiques des oligodendrocytes (OL). Cette dérégulation protéique dans les DMSXL, associée aux lésions de la substance blanche décrites chez les malades, suggère l'implication des OL dans la neuropathologie de la DM1. Mon projet de thèse avait comme objectif d'étudier l'implication des OL et de la myéline dans l'étiologie de l'atteinte cérébrale de la DM1.

J'ai d'abord caractérisé la toxicité des ARN CUG dans les OL et les phénotypes de la myéline dans les DMSXL. J'ai confirmé l'accumulation de foci d'ARN *in vivo*, associée à des défauts d'épissage dans les OL isolés de souris adultes. Pour confirmer les atteintes de la myéline, j'ai étudié l'expression de ses composants clés, et j'ai constaté une expression retardée de certaines protéines dans les souriceaux DMSXL, ainsi que dans

d'autres modèles murins de la DM1. La dérégulation de ces protéines n'est pas associée à des anomalies ultrastructurales de la myéline dans les souris DMSXL, en revanche moins d'axones y sont myélinisés, suggérant une hypomyélinisation précoce chez ces animaux. Pour rechercher si l'hypomyélinisation était due à des anomalies de maturation des OL, j'ai quantifié les sous-populations de la lignée oligodendrogliale : l'hypomyélinisation des souris DMSXL est associée à une diminution de la densité d'OL pleinement maturés.

J'ai par la suite étudié les défauts cellulaires derrière les phénotypes de la myéline. Pour cela, j'ai mis au point les conditions de culture d'OL primaires issus des souris DMSXL, ainsi que les protocoles d'analyse de leur prolifération et différenciation par vidéo-microscopie semi-automatisée. Malgré une prolifération normale en culture, les précurseurs des OL (OPC) présentent des défauts de migration et de différenciation. Afin de caractériser les anomalies d'expression et d'épissage dans les OL primaires des DMSXL j'ai fait un séquençage des ARN. L'analyse bioinformatique a mis en évidence une dérégulation du cytosquelette, ainsi que des voies de signalisation impliquées dans la motilité et la différenciation cellulaire. De plus, l'analyse a montré que les défauts d'épissage détectés dans les OL DMSXL ressemblent aux profils d'épissage d'OPC indifférenciés, appuyant l'hypothèse d'un retard de maturation de la lignée oligodendrogliale. Certains défauts de maturation cellulaire ont été validés dans des OL humains différenciés à partir de cellules iPS dérivés de patients DM1.

Mes travaux ont démontré des défauts des OL et de la myéline dans un modèle de la DM1, contribuant ainsi à une compréhension intégrative des atteintes cérébrales dans la DM1.

Mots clefs :

Dystrophie myotonique de type 1, SNC, myélinisation, oligodendrocytes, modèle murin transgénique.

Title:

Oligodendrocyte dysfunction and myelin abnormalities in a transgenic mouse model of myotonic dystrophy type 1.

Abstract :

Myotonic dystrophy type 1 (DM1) is a neuromuscular disease that affects many tissues. Brain involvement is demonstrated by debilitating neurological symptoms, as well as brain structural changes, such as predominant white matter lesions. DM1 neurological manifestations have a profound impact on the daily life of patients and their families.

DM1 is caused by the abnormal expansion of a CTG repeat in the 3'UTR of the *DMPK* gene. Expanded transcripts are toxic: they accumulate in RNA aggregates (foci), affecting RNA-binding proteins and deregulating primarily the alternative splicing of downstream targets and other aspects of RNA metabolism. Today, we still do not know which cell types and molecular pathways are primarily affected in the brain and how they contribute to DM1 neurological symptoms. To investigate this problem our laboratory has developed a transgenic mouse model of DM1. The DMSXL mice express expanded human *DMPK* transcripts in multiple tissues, notably in the brain, and display relevant behavioural, electrophysiological and neurochemical phenotypes. As a result, these mice provide a powerful tool to study DM1 brain dysfunction. A previous global proteomics study of DMSXL brain tissue revealed changes in oligodendrocyte-specific proteins, among others. Together with the prevalent white matter lesions reported in patients, the abnormalities in mouse myelin proteins suggest the implication of oligodendrocytes (OL) in DM1 neuropathology. Thus, my project aimed to investigate OL and myelin dysfunction in the aetiology of DM1 brain disease.

I first looked for signs of RNA toxicity in OLs and characterised myelin phenotypes in DMSXL mice. I confirmed RNA foci accumulation *in vivo*, associated with mild spliceopathy of candidate genes in OL isolated from adult mice. To confirm the impact on myelin biology, I studied the expression of key myelin protein components and found delayed expression in young DMSXL animals, as well as in other complementary mouse models of DM1. Although myelin protein deregulation was not accompanied by abnormalities in myelin ultrastructure, it was associated with a decrease in the number of myelinated axons, suggestive of early hypomyelination in these animals. To investigate whether hypomyelination was due to abnormalities in OL maturation, I

quantified subpopulations of the oligodendroglia cell lineage and found a decrease in fully differentiated cells.

Following the confirmation of myelin phenotypes in DMSXL mice, I investigated the underlying cellular physiology. To this end, I used DMSXL mice as a renewable source of homogenous cultures of OL. I optimised cell culture conditions and semi-automated analysis of live-cell videomicroscopy to monitor OL proliferation and differentiation in culture. Although displaying normal proliferation in culture, DMSXL OL precursor cells (OPC) display signs of impaired migration and defective differentiation. To gain insight into the molecular mechanisms behind OL defective differentiation, I used RNA sequencing to find expression and splicing events deregulated in primary DMSXL OLs. Gene ontology analysis of transcriptome abnormalities pointed to abnormal expression of structural and regulatory components of the cytoskeleton, as well as critical signalling pathways involved in cell motility and differentiation. Interestingly, the analysis revealed that the spliceopathy detected in DMSXL OLs recreates the splicing profiles of undifferentiated OPC, providing molecular support for the maturation delay of these cells. Importantly, the cell maturation defects are recreated to some extent in human OLs differentiated from DM1 iPS cells models.

All together my work has provided novel insight into the cell-specific mechanisms operating in DM1, demonstrating the implication of OL and myelin defects in a DM1 mouse model, and contributing towards an integrative understanding of brain pathology.

Keywords :

Myotonic dystrophy type I, CNS, myelination, oligodendrocytes, transgenic mouse model.

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Geneviève, have supported Mário's craziness and accepted me as part of her lab. If this was not enough, she became someone I look up to for her integrity and dedication to science, reassuring me with her contained excitement, that it is worth of continuing in this sometimes-frustrating job.

I was really lucky to have teammates that took me in as part of the team since the beginning. **Stephanie** always had kind words and gestures to me. **Aline**, besides helping me with all the mice tricks, her straightforwardness (*Aline, ce mot signifie frontalité*) allowed me to be myself (probably because we are pretty much alike) and made me feel like home, even when we could not understand each other very well. She became someone that I trust and I can count on for everything and I will always have a drawer for her to sleep on. **Hélène** arrived later but even so, we had time to share some good laughs mostly based on her funny 'French grumpiness'. Then there were my two favorite 'doctor post-docs', Dr. **Anchel** and Dr. **Chakraborty**, I appreciate all the post-lunch coffee breaks in their office wondering about silly things.

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Abbreviations and gene nomenclature

| | |
|-------------------------------------------------------------------|----------------------------------------------------------|
| FXS - Fragile X syndrome | MMR - mismatch repair |
| SBMA - Spinal and bulbar muscular atrophy | MSH2 - MutS homologue 2 |
| RNA - Ribonucleic acid | OE - overexpressing |
| FRDA - Friedreich Ataxia | KO - knock-out |
| DM1 - Myotonic dystrophy type 1 | MBNL - muscleblind-like |
| HD - Huntington disease | CELF - CUG-binding protein Elav-like family |
| SCA - Spinocerebellar Ataxia | CLCN1 - chloride channel |
| ALS/FTD - Amyotrophic lateral sclerosis & frontotemporal dementia | BIN1 - bridging integrator 1 |
| CTG - Cytosine, thymine and guanine | CACNA1S - calcium voltage-gated channel subunit alpha1 S |
| RBPs - RNA binding proteins | IR - insulin receptor |
| FRAXE - Fragile X E Syndrome | SCN5A - cardiac voltage-gated Na ⁺ channel |
| EPM1 - Progressive Myoclonus Epilepsy 1 | RBFOX - RNA Binding Fox-1 Homolog |
| DM2 - Myotonic dystrophy type 2 | APP - amyloid beta precursor protein |
| FXTAS Fragile X tremor/ataxia syndrome | NMDAR - N-methyl-D-aspartate receptor |
| FXPOI - Fragile X-associated primary ovarian insufficiency | APA - alternative polyadenylation |
| DRPLA - Dentatorubral-pallidolusyan atrophy | SP - specificity protein |
| HDL2 - Huntington's disease -like 2 | RAR γ - retinoic acid receptor gamma |
| FRA7A - CGG Expansion at fragile site 7A | STAT - signal transducer and activator of transcription |
| FRA2A - CGG Expansion at fragile site 2A | NKX2-5 - NK homeobox 5 |
| ASD - autism spectrum disorder | SHARP - SMART/HDAC1-Associated Repressor Protein |
| UTR - Untranslated region | SLITRK - NTRK like protein |
| GI - Gastrointestinal | DDX5 - DEAD-Box Helicase 5 |
| MRI - magnetic resonance imaging | AAV - adeno-associated virus |
| WM - white matter | microRNA (miRNA) |
| CDM - Congenital DM1 | GJA1 - connexin 43 gap junction protein |
| QoL - Quality of life | CACNA1C - calcium channel |
| GM - Grey matter | RNAi - RNA interference |
| WMH - white matter hyperintensity | siRNAs - small interference RNAs |
| NFT - neurofibrillary tangles | circRNAs - circular RNAs |
| MAPT - microtubule associated protein tau | PKC - protein kinase C |

GSK3 β - glycogen synthase kinase 3 beta
AKT - AKT serine/threonine kinase
SYN1 - synapsin 1
NSCs - neural stem cells
rpS6 - ribosomal protein S6
MEF2A - myocyte enhancer factor 2A
p21 - cyclin-dependent kinase inhibitor 1A
SAMD4A - sterile alpha motif domain containing 4A
RAN - repeat-associated non-ATG
ASO - antisense oligonucleotide
CRISPR - clustered regularly interspaced short palindromic repeats
Cas9 - CRISPR-associated protein 9
WT - wild-type
all-LNAs - locked nucleic acids
AMPK - AMP-activated protein kinase
mTOR - mechanistic target of rapamycin kinase
NG2-glia - nerve/glia antigen 2 - expressing glia
OPC - oligodendrocyte precursors cells
pre-OL - pre-myelinating oligodendrocytes
mOL - myelinating oligodendrocytes
PDGF - platelet-derived growth factor
PDGFR α - PDGF receptor alpha
E - embryonic day
P - postnatal day
Div - days in vitro
GF - growth factors
FGF - fibroblast growth factor
ECM - extracellular matrix
LN - laminin
NCAM - neuronal cell adhesion molecule
O4 - oligodendrocyte antigen 4
MBP - myelin basic protein

AMPA - α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CC - corpus callosum
PLP1 - proteolipid protein 1
CNP - 2',3'-Cyclic-nucleotide 3'-phosphodiesterase
MOG - myelin oligodendrocyte glycoprotein
OLIG2 - oligodendrocyte transcription factor 2
NRG1 - neuregulin-1
FAK - focal adhesion kinase
Rho GTPases - Ras homologue family of small guanosine triphosphatases
RAC1 - Ras-related C3 botulinum toxin substrate 1
CDC42 - cell division control protein 42
Arp2/3 - actin-related protein 2/3
ADF - actin depolymerizing factor
ErbB - erythroblastic leukemia viral oncogene homologue
MCT - monocarboxylate transporter
MS - multiple sclerosis
Glu - Glutamate
Glc - glucose
GLUT1 - glucose transporter 1

Chapter I

Introduction and thesis outline

Summary

Microsatellites expansions are known to be the cause of some human inherited neurological and neuromuscular disorders. These mutations can lead to expanded RNA molecules that, disrupting trans-acting factors, become toxic to the cell. RNA toxicity was first described in myotonic dystrophy type I, a multisystemic disease caused by the abnormal expansion of a non-coding CTG trinucleotide repeat in the DMPK gene. Since the discovery, multiple cellular and animal models have been developed to understand the disease mechanisms and how the mutation affects the physiology of different tissues and organs. Notably, the central nervous system has been undoubtedly implicated in the disease, although the mechanisms operating in the brain remain largely unknown. The progresses made to unveil the disease pathophysiology have come far enough to create innovative therapeutic strategies and initiate promising clinical trials. However, further research is needed to be better understanding the brain disease in order to refine and improve therapies.

“... a blood test in 1997 confirmed my inclusion with those affected with myotonic dystrophy. Looking back, my husband and I realize how long before that I have been showing the many seemingly unrelated symptoms and trying to find answers for it all and now I realize how frustrated my internist had become when he was trying to find a diagnosis for me... I've had three memory tests... the first showing a very sharp memory and the latest showing loss in executive functions. I used to be the one with the strong memory in our family, but no more... I forget more people's names and I feel more and more alone. I've had to surrender my car keys because I kept getting lost on route... I also used to keep our finances, but no more, I find it hard to keep track... So, although we do find some things to help the physical aspects of myotonic dystrophy... I haven't find much to help my memory or my sense of organization... I do wish that a therapy or medicine could be found that would slow down this loss of memory or maybe even stop it... I miss the pleasure of organizing our lives. It's hard to admit how much I mourn the loss of my old identity...”

Translation from 'Bringing the patient voice to CNS targeting drug development in DM'
at 2017 MDF Annual Conference

Until 1988, Lee Baker, a lively schoolteacher did not know what was wrong with her. A meaning for the diversity of symptoms she had been experiencing came with the knowledge that part of her mother's family had myotonic dystrophy. Even if the genetic diagnosis was only confirmed later, Lee was a 'text book' case of the disease and soon enrolled trials to study memory and cognitive decline. Lee reported on how the CNS related symptoms impact her day-to-day family life and how they have taken so much of the person she used to be. More than 30 years have passed and the knowledge about the genetic mechanisms of this devastating disease and its biological consequences has been growing quickly, although not fast enough in respect to the brain disease. Doctors, scientists, pharmaceutical industries and patients themselves have been working together towards a better understanding of the far-reaching effects of the mutation on different organs and cell types, pushing forward new and innovative treatments to help myotonic dystrophy patients to keep their hope.

I. Unstable microsatellites and neurological diseases

DNA microsatellites consist in simple sequence repeats of 1-13 nucleotides, composing about 3% of the human genome (Lander et al., 2001). These repeated sequences are distributed throughout exons, introns and intergenic regions equally across the genome, although with an increased density within chromosome 19 (Subramanian et al., 2003). The tri- and hexanucleotides are present at higher frequency in exons, while the other repeats are more frequent in non-coding regions (Toth et al., 2000).

Occurring naturally, DNA microsatellites are important for the evolution and function of the genomes, but their intrinsic instability is on the basis of severe neurological and neuromuscular diseases. Among the disease-associated microsatellites, the most common are the trinucleotide repeats. Since the discovery of the first disease-causing expanded trinucleotide in fragile X syndrome (FXS) and spinal and bulbar muscular atrophy (SBMA) in 1991, more than 20 other diseases have been associated to expanded triplet repeats. The unstable microsatellites lead to pathogenesis usually through a sequence of events triggered by the repeat expansion, which involves either (1) the transcription of expanded RNA molecules that perturb disease mediators, and/or (2) translation of expanded toxic polypeptides (Rohilla and Gagnon, 2017). In spite of this common features, the pathological mechanisms operating in each condition can vary depending on the location of the expanded repeat within the affected gene, resulting either in the loss-of-function of the gene or in the gain-of-function of the expanded sequence (Figure I.1). Expanded repeats located in the close proximity of the transcription start site, are likely to disrupt the transcription and cause gene loss of function, as observed in FXS or in Friedreich ataxia (FRDA). When transcribed, the repeats can cause a toxic gain of function of the RNA as described in myotonic dystrophy type I (DM1) and type 2 (DM2). Toxic RNA may aggregate in the nucleus and perturb the function of disease modulators and downstream RNA metabolism. In Huntington disease (HD), a toxic protein gain of function takes place due to the translation of an expanded polyglutamine tract, a mechanism shared by several spinocerebellar ataxias (SCAs). In *C9ORF72*-associated amyotrophic lateral sclerosis with frontotemporal dementia

(ALS/FTD) these mechanisms act in combination, leading to simultaneous RNA and polypeptide toxicity (Rohilla and Gagnon, 2017). The diversity of neurological disorders caused by expanded microsatellites, as well as the variability in the nature of the pantothenic repeat sequence, length and genomic location is summarized Table I.1.

The following chapters will focus on CTG trinucleotide repeat expansions and toxic gain of function of CUG RNA repeats and its consequences in DM1, a paradigm for RNA toxicity.

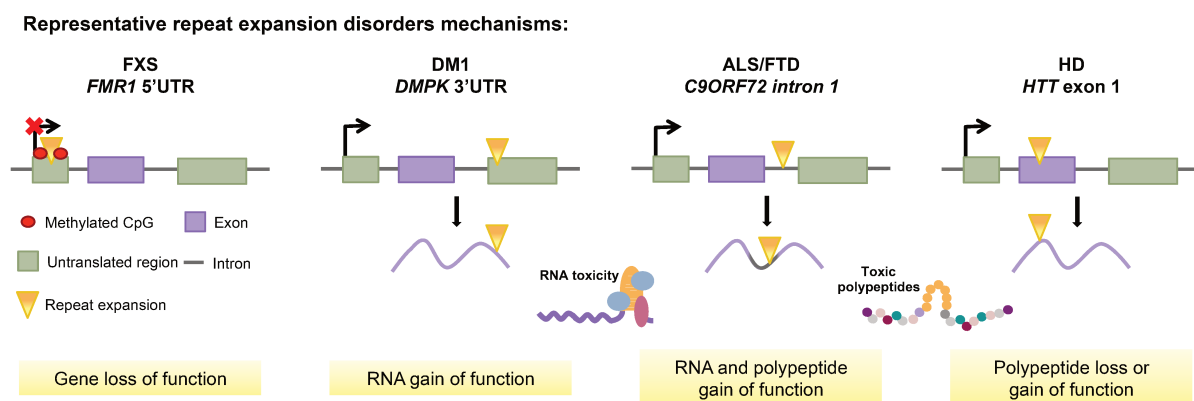


Figure I.1 – Schematic representation of the different microsatellite-associated mechanisms of neurological disease.

Some neurological diseases are linked to repeat expansions that can be located in diverse regions of the affected genes. Non-coding microsatellites, lying outside the exons, can cause gene silencing (e.g. through expansion-induced promoter methylation), leading to a loss of function of the disease-associated gene. In other cases, when the transcription of non-coding repeats takes place, the repeat-containing RNA can form aggregates, which are capable of trapping and/or perturb the function of RNA-binding proteins (RBPs). Toxic homopolypeptides can arise from the translation of repeated sequences located in exonic regions of the affected gene. These toxic products, alone or in combination, can disrupt the cell function in multiple ways (adapted from Rohilla and Gagnon 2017).

Table I.1 - Unstable microsatellite-associated neurological disorders.

| Disorder | Genomic location | Repeat unit | Normal length | Pathologic al length | Main clinical features | Reference |
|------------------------------------------------|-------------------------|------------------|---------------|----------------------|-----------------------------------------------------------------------------|-----------------------------------------------------------------|
| Loss of function | | | | | | |
| Friedreich Ataxia (FRDA) | <i>FXN</i> Intron 1 | GAA | 8-33 | 90-1000 | Progressive ataxia, muscle weakness, sensory loss, cardiomyopathy, diabetes | (Campuzano et al., 1996) |
| Fragile X syndrome (FXS) | <i>FMR1</i> 5'UTR | CGG | 6-55 | 200-4000 | Severe intellectual disability, ASD, macroorchidism | (Heitz et al., 1991, Oberle et al., 1991, Verkerk et al., 1991) |
| Fragile X E Syndrome (FRAXE) | <i>AFF2</i> 5'UTR | CCG | 4-39 | 200-900 | Mild intellectual disability | (Knight et al., 1993) |
| Progressive Myoclonus Epilepsy 1 (EPM1) | <i>CSTB</i> Promoter | CCCCGC CCCCGC | 2-3 | 30-80 | Myoclonus, progressive cognitive decline | (Laloti et al., 1997) |

RNA gain of function

| | | | | | | |
|---------------------------------------------------|----------------------------------|--------|-------|----------|----------------------------------------------------------------------------------------------------------|---------------------------------------------------------------|
| Myotonic dystrophy type 1 (DM1) | <i>DMPK</i> 3'UTR | CTG | 5-37 | 50-10000 | Myotonia and muscle weakness, cardiac, endocrine and GI defects, cognitive and behavioral deficits | (Brook et al., 1992, Fu et al., 1993, Mahadevan et al., 1992) |
| Myotonic dystrophy type 2 (DM2) | <i>CNBP</i> Intron 1 | CCTG | 10-26 | 75-11000 | Proximal myotonia and muscle weakness, multisystemic abnormalities, cognitive and behavioral dysfunction | (Liquori et al., 2001) |
| Fragile X tremor/ataxia syndrome (FXTAS) | <i>FMR1</i> 5'UTR | CGG | 6-55 | 55-200 | Intellectual disability | (Kenneson et al., 2001) |
| Spinocerebellar Ataxia 10 (SCA10) | <i>ATXN10</i> Intron 9 | ATTCT | 15-34 | 89-250 | Ataxia, dysarthria, seizures, dysphagia | (Matsuura et al., 2000) |
| Spinocerebellar Ataxia 12 (SCA12) | <i>PPP2R2B</i> 5'UTR | CAG | 7-28 | 66-78 | Tremors, mild ataxia, dementia | (Holmes et al., 1999) |
| Spinocerebellar Ataxia 31 (SCA31) | <i>TK2/BEAN</i> Shared Intron | TGGAA | 0 | >45 | Cerebellar ataxia | (Sato et al., 2009) |
| Spinocerebellar Ataxia 36 (SCA36) | <i>NOP56</i> Intron1 | GGCCTG | 3-14 | >650 | Ataxia, dysarthria, hyperreflexia, dysphagia, atrophy and motor neuron disease | (Kobayashi et al., 2011) |
| Fuchs endothelial corneal dystrophy (FECD) | <i>TCF4</i> Intron2 | CTG | <40 | >50 | corneal guttae, vision loss | (Wieben et al., 2012) |

Protein gain of function

| | | | | | | |
|-----------------------------------------------------|---------------------------|-----|-------|--------|----------------------------------------------------------------------------------|--------------------------------------------|
| Huntington disease (HD) | <i>HTT</i> Exon 1 | CAG | 10-35 | >35 | Chorea, dystonia, cognitive decline, psychiatric disease. | (MacDonald et al., 1993) |
| Spinal and bulbar muscular atrophy (SBMA) | <i>AR</i> Exon1 | CAG | 9-36 | 38-62 | Proximal muscle weakness, muscle atrophy, gynecomastia. | (La Spada et al., 1991) |
| Dentatorubral-pallidoluysian atrophy (DRPLA) | <i>ATN1</i> Exon 5 | CAG | 6-35 | 49-88 | Seizures, cognitive decline, ataxia, choreoathetosis. | (Koide et al., 1994) |
| Spinocerebellar Ataxia 1 (SCA1) | <i>ATXN1</i> Exon 8 | CAG | 6-35 | 49-88 | Ataxia, dysarthria, spasticity, ophthalmoplegia | (Orr et al., 1993) |
| Spinocerebellar Ataxia 2 (SCA2) | <i>ATXN2</i> Exon 1 | CAG | 14-32 | 33-77 | Ataxia, slow eye movement, hyporeflexia, motor disease, occasional parkinsonism. | (Imbert et al., 1996, Sanpei et al., 1996) |
| Spinocerebellar Ataxia 3 (SCA3) | <i>ATXN3</i> Exon 8 | CAG | 12-40 | 52-86 | Ataxia, dystonia, lower motor neuron disease. | (Kawaguchi et al., 1994) |
| Spinocerebellar Ataxia 6 (SCA6) | <i>CACNA1A</i> Exon 47 | CAG | 4-18 | 21-30 | Ataxia, dysarthria, sensory loss. | (Zhuchenko et al., 1997) |
| Spinocerebellar Ataxia 7 (SCA7) | <i>ATXN7</i> Exon 3 | CAG | 7-17 | 38-120 | Ataxia, dysarthria, cone-rod dystrophy retinal disease | (David et al., 1997) |
| Spinocerebellar Ataxia 17 (SCA17) | <i>TBP</i> Exon 3 | CAG | 25-42 | 47-63 | Ataxia, dementia, chorea, seizures, dystonia. | (Nakamura et al., 2001) |

Combined mechanisms

| | | | | | | |
|------------------------------------------------------------------------------|-------------------------------------|-------------|-------|-------|-------------------------------------------------------------------------|-------------------------------------------------------|
| Amyotrophic lateral sclerosis & frontotemporal dementia (ALS/FTD) | <i>C9ORF72</i> Intron1 | GGGGC C | 2-25 | >25 | Motoneuron degeneration, muscle wasting, weakness, pre-senile dementia. | (DeJesus-Hernandez et al., 2011, Renton et al., 2011) |
| Spinocerebellar Ataxia 8 (SCA8) | <i>ATXN8/OS</i> 3'UTR | CTG/ CAG | 15-50 | >71 | Ataxic dysarthria, nystagmus, limb and gait ataxia, cerebellar atrophy | (Koob et al., 1999) |
| Huntington disease -like 2 (HDL2) | <i>JPH3</i> Exon 2A 3'UTR | CTG | 6-27 | 36-57 | Chorea, dystonia, cognitive decline | (Holmes et al., 2001) |
| CGG Expansion at fragile site 7A (FRA7A) | <i>ZFN713</i> Intron1 | CGG | 5-22 | >85 | ASD | (Metsu et al., 2014a) |
| CGG Expansion at fragile site 2A (FRA2A) | <i>AFF3</i> Alternative promoter | CGG | 3-20 | >300 | Neurodevelopmental delay | (Metsu et al., 2014b) |

II. Myotonic Dystrophy Type 1 - from genetics to clinical heterogeneity

Myotonic dystrophy type 1 or Steinert disease (DM1 OMIM #160900), was first described by the German physician Hans Steinert in 1909 and by Batten and Gibb later the same year. With a worldwide prevalence of 1/8,000 individuals (Harper, 2001), DM1 is the most common adult form of muscular dystrophy nowadays. Its incidence varies between 1/5,500 and 1/200,000 depending on the geographical region (Theadom et al.). Extremely high disease prevalence has been reported in the region of Saguenay in Quebec (1/500 individuals), due to a founder effect (Mathieu and Prevost, 2012), while in contrast, the disease is almost inexistent in Sub-Saharan Africa (Goldman et al., 1996).

A. Genetic aspects of DM1 mutation

The transmission pattern of DM1 was identified some years after the recognition of DM1 as an independent disease. A mendelian autosomal dominant mode of transmission was described in 1918 by Bruno Fleischer, remaining consensually accepted since then. The same work also introduced for the first time the concept of *anticipation* in DM1, to describe the earlier onset and general increased severity of the symptoms in successive generations of a typical DM1 family. Although this idea was initially not consensual, mainly due to the possible confounding variables and lack of scientific evidence (Harper, 2001), it was later widely accepted as a DM1 feature (Howeler et al., 1989, Ashizawa et al., 1992). It was recognized that in a typical DM1 family, the first generation may show mainly premature cataracts in late adulthood; while their children may suffer from severe neuromuscular symptoms that appear at an earlier age; and their grandchildren may display severe physical and neurological impairment from their childhood onwards, or even from birth (in the most severe cases). Although marked DM1 anticipation across three generations had been reported, it was still difficult to provide a molecular explanation for it.

Almost 100 years after Steiner's clinical description, the identification of the causing mutation for DM1 shed some light on most of the unsolved questions. A decade of linkage disequilibrium studies narrowed down the DM1 locus to chromosome 19 and

allowed the mapping of an unstable CTG trinucleotide expansion in the 3' untranslated region (UTR) of the gene now known as *DM protein kinase (DMPK)*, flanked by the *DM1 Locus WD Repeat Containing (DMWD)* (upstream) and the *Sine Oculis Homeobox Homolog 5 (SIX5)* (downstream) on the chromosome 19q13.32 (Brook et al., 1992, Buxton et al., 1992, Aslanidis et al., 1992, Fu et al., 1992, Mahadevan et al., 1992). The CTG expansion is highly polymorphic: in non-affected population the repeat size varies from 5-37 repeats. Sizes between 37 and 50 CTG are sometimes called “premutation”, since the carrying individuals are asymptomatic, but at higher risk of transmitting a

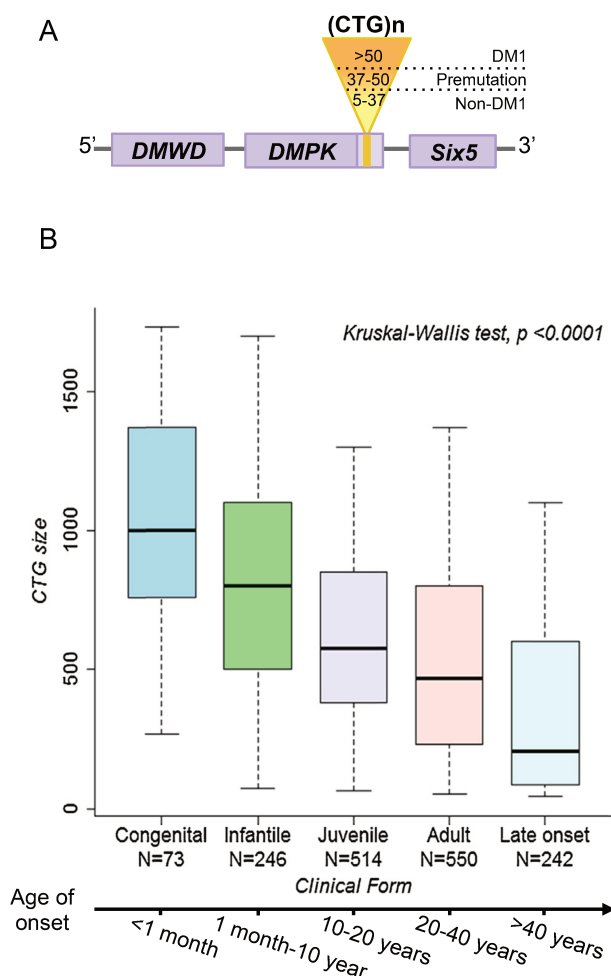


Figure II.1 – Schematic representation of the DM1 locus and correlation between CTG repeat size and age of onset.

(A) DM1 locus including *DMPK* and flanking genes with a schematic representation of the CTG expansion size spectrum. **(B)** Distribution of the number of CTG repeats across five DM1 clinical forms, and respective age of onset (adapted from De Antonio et al. 2016).

pathological allele to their children. Amplifications greater than 50 repeats are generally associated with DM1. Among the affected individuals, those carrying shorter CTG sizes (50-100) are frequently undiagnosed due to the mild symptoms and/or their late onset, while individuals with 200-500 repeats will experience the first symptoms in their adulthood. In contrast patients carrying repeats larger than 1,000 CTG will develop the disease *in utero* or at very young age. This distribution reflects a certain degree of genotype-phenotype correlation, between the CTG repeat length and the age of onset, as well as the clinical severity of the disease manifestations (Figure II.1)

(Harley et al., 1993, De Antonio et al., 2016). Thus, the mapping of the genetic cause of DM1 provided some explanation to the clinical variability of the disease symptoms among individuals. In addition it also provided a molecular basis for the phenomenon of anticipation previously mentioned. Given that larger repeats are associated with more severe and earlier clinical forms of the disease, anticipation results from the high instability of the expanded repeat, which tends to increase in size during intergenerational transmissions (Harley et al., 1993). Two major factors contribute to determine the CTG repeat size inherited by the offspring: (1) the repeat size and (2) sex of the transmitting parent (Harley, 1993 #410). Repeat tracts of ~50-100 units are usually more likely to expand during paternal transmission, resulting in larger intergenerational repeat gains. In contrast, if the affected parent carries more than ~500 repeats, 10% of paternal transmissions result in contractions, while maternal transmissions result in a dramatic increase in repeat length, responsible for the most severe congenital forms (Brunner et al., 1993, Lavedan et al., 1993, Ashizawa et al., 1994, Rakočević-Stojanović et al., 2005). Interestingly, the parent-offspring correlation for the repeat size is also observed in the premutation size range. Although associated with subclinical phenotypes, these premutations are also unstable, holding the potential to expand in the following generations, particularly by male transmission. Therefore, repeat sizes ranging from 37-50 CTG units have been considered a reservoir of new expansions in the otherwise healthy population, contributing to the persistence of DM1 (Martorell et al., 2001).

The CTG repeat is also unstable in somatic tissues. Trinucleotide repeat somatic instability generates expansion-biased somatic mosaicism, which varies between different tissues. For instance, in the same individual the level of somatic mosaicism tends to be higher in disease relevant tissues, such as the skeletal muscle, cardiac tissue and brain, relative to cell types that are mainly spared by the condition, such as leucocytes, somehow predicting the degree of dysfunction of each system (Ashizawa et al., 1993, Thornton et al., 1994, Zatz et al., 1995). Additionally, the range of the repeat size in somatic cells is age-dependent and biased towards the accumulation of larger expansions with time. Therefore, somatic repeat instability is likely to modulate the tissue-specific severity and the progressive nature of DM1 symptoms (Monckton et al., 1995, Wong et al., 1995, Martorell et al., 1998, Gomes-Pereira and Monckton, 2006,

Morales et al., 2012). The mechanisms involved in the mutation dynamics and the pathogenic *cis*- and *trans*- effects driving repeat size mutation will be addressed in section III.

B. The clinical spectrum

One of the most puzzling aspects of the DM1 is the wide clinical spectrum, which makes the clinical diagnosis difficult. As mentioned before, the genetic variability leads to a multitude of symptoms, which allow the distinction of different clinical forms. DM1 manifests through different degrees of severity and combinations of myotonia (*i.e.* delayed muscle relaxation), progressive muscle weakness, cardiac defects, posterior iridescent cataracts, insulin resistance, gastrointestinal (GI) dysfunction, excessive daytime sleepiness and cognitive and behavioral impairments (Meola, 2013, Thornton, 2014). The pleiotropy of symptoms creates an additional challenge to the establishment of standardized medical guidelines and to the definition of homogeneous clinical groups, which are usually required to set up therapeutic assays in translational research. In order to overcome this limitation, a French database (*French DM-Scope Registry*) integrates data from more than 2800 patients categorized into five major clinical categories: congenital, infantile, juvenile, adult and late onset, based on the occurrence and age of onset of the main DM1 symptoms (De Antonio et al., 2016). The principal characteristics of each DM1 clinical category are described below and summarized in Table II.1.

(a) Late onset

Patients are often asymptomatic until 40-50 years of age and remain frequently undiagnosed. The typical posterior subscapular iridescent cataract often offers the only non-genetic clinical diagnosis of the disease. Myotonia, muscle weakness and hypersomnia are rare in late onset form, which is thus considered the mildest form of DM1.

(b) Adult form

This is the most common form of DM1, previously named *classical* form. It is highly multisystemic, with the first symptoms appearing within the third decade of life. The leading manifestations are myotonia and distal muscular weakness and wasting

(Wenninger et al., 2018). Cardiac problems include conduction defects with arrhythmias that contribute to some cases of sudden death in DM1 (Dogan et al., 2016). Histopathology analysis reveals changes in muscle biopsies of skeletal and cardiac muscles, including variation, splitting and atrophy of the muscle fibers, centrally-located nuclei and fibrosis (Meola, 2013). Early-onset cataracts, GI dysfunction and endocrine abnormalities are also characterized, such as insulin resistance, and hypotestosteronism associated with testicular atrophy (in males). Neuropsychiatric deficits are reported to a variable degree: most of the patients have hypersomnia (excessive daytime sleepiness), frequent attention deficits, sub-normal intelligence quotient (IQ) scores, low performance in “*Theory of Mind*” tests (test the ability to infer other’s mental states) and behavioral changes in form of apathy and avoidant personality (Yu et al., 2011a, Winblad et al., 2006, Jean et al., 2014). Structural brain alteration can be observed in magnetic resonance imaging (MRI) scans, particularly in the white matter (WM) signal (Minnerop et al., 2011).

(c) Juvenile form






Juvenile patients experience more severe cognitive impairments than adult patients, starting from 10 years of age, and often appearing prior to the onset of muscular symptoms. Typically, this form of DM1 shows prevalent visual-spatial deficits, executive dysfunction and learning impairment, associated with greater hypersomnia, which together translate in school difficulties and poor employment (Douniol et al., 2012). While appearing later, the muscular symptoms follow the aggravated phenotype (especially for myotonia), being more severe and requiring specialized professional aid more often than in the case of adult onset patients (De Antonio et al., 2016).

(d) Infantile form

Infantile DM1 occurs in patients between the age of 1 month and 10 years, with a greater involvement of the central nervous system (CNS) than in the previous form. The cognitive, visual-spatial and attention problems are more severe; the IQ scores are lower and the communication difficulties are more pronounced and sometimes associated with autism spectrum disorder (ASD) (Ekstrom et al., 2008, Ekstrom et al., 2009). Among the muscular symptoms the myotonia develops earlier and it is followed by muscle weakness and GI troubles. Cardiac and respiratory insufficiency is more

frequently reported in DM1 infants, relative to later onset cases (De Antonio et al., 2016).

Table II.1 – Summary of the DM1 clinical forms and corresponding age of onset, repeat range and main symptoms.

| Clinical form | Age of onset | Repeat range* | Principal symptoms | CNS involvement | |
|---------------|-------------------------------------------------------------------------------------|------------------|--------------------|--------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------|
| Late onset |  | >40 years | 100-600 | Cataracts, rare myotonia | Rare hypersomnia |
| Adult |  | 20-40 years | 250-750 | Myotonia, muscle weakness and wasting, cardiac dysfunctions, GI difficulties, cataracts, male hypogonadism, insulin resistance | Hypersomnia, cognitive and behavioral challenges |
| Juvenile |  | 10-20 years | 400-800 | Similar muscular and cardiac problems as adults DM1 but more severe | Hypersomnia, cognitive impairment and autistic-like behavior |
| Infantile |  | 1 month-10 years | 500-1100 | Similar symptoms as congenital DM1 but less severe | Severe cognitive and behavioral impairments |
| Congenital |  | <1 month | 750-1400 | Generalized developmental defects, hypotonia, respiratory insufficiency, cardiac defects, facial dysmorphism, dysphagia | Severe intellectual disability |

*The CTG repeat range for each clinical form described by De Antonio *et al.* (2016), however sizes outside this range can be found elsewhere.

(e) Congenital DM1 (CDM)

With an onset occurring as early as in fetal development, CDM is the most severe form of the disease. Before birth the disease manifests itself by polyhydramnios and reduced movements of the embryo. After birth, babies show generalized weakness and hypotonia, sucking and swallowing difficulties, as well as pronounced facial dysmorphism, characterized by "fish-shaped" upper lip. Perinatal mortality is elevated in these babies, mainly due to frequent respiratory complications, which require mechanical ventilation support (Wallgren-Pettersson et al., 2004). Surviving patients will gradually improve, although the psychomotor milestones are never achieved in a normal timescale. The cognition is severely compromised, leading to the frequent need of specialized schooling (Ekstrom et al., 2009, Echenne and Bassez, 2013). CNS-related

symptoms are accompanied by both grey and white matter structural brain alterations (Zanigni et al., 2016).

C. Myotonic Dystrophy type 2

DM2, also known as proximal myotonic myopathy (PROMM) was first identified in patients presenting DM1-like symptoms that tested negative for the mutation on the *DMPK* gene. The DM2 autosomal dominant mutation was found in the intron 1 of the cellular nucleic acid-binding protein gene (*CNBP*), previously known as zinc finger protein 9 (*ZNF9*), and consists of a CCTG tetranucleotide repeat expanded in average 5000 times (Table I.1) (Liquori et al., 2001). Similar to DM1 CTG repeats, expanded CCTG repeats in DM2 are highly unstable, and they are also transcribed but not translated, causing a multitude of cellular and molecular dysfunctions. Although clinically similar to DM1, DM2 is characterized by some distinctive features: the lack of an obvious correlation between repeat length and clinical severity, the absence of visible anticipation across generations, the more pronounced involvement of proximal muscles (in contrast to distal muscle involvement in DM1), the milder nature of cognitive deficits and the absence of a congenital form (Day et al., 2003, Meola and Sansone, 2007). Nevertheless, there is sufficient overlapping between the muscular, cardiac and neurological manifestations of DM1 and DM2 to anticipate common pathological mechanisms operating in both conditions (Wenninger et al., 2018).

D. DM1 brain disease

The initial view of DM1 as a muscular disease did not take into account the cognitive and personality traits reported in patients. The list of CNS-related incapacities is long and widely documented by neuropsychological and imaging studies, in strong support of brain involvement in DM1. It is worth mentioning that for patients and their families, the neurological symptoms represent one of the heaviest burdens of the disease (Ashizawa, 1998).

(a) Cognitive and behavioural profile

Over the last decades, the neuropsychological assessment of DM1 helped dissect the cognitive and behavioral domains impaired in the disease. These studies aimed not only to understand the degree of cognitive and behavioral disability, but also to search for predictive factors of the disease severity and progression. Despite the heterogeneity of the results, the CNS impairment often correlates with the CTG repeat length, and more markedly with the age of the patient (Gourdon and Meola, 2017). Interestingly, CNS impairment can reflect the anticipation phenomenon within a family, even when no other symptom has yet developed.

All domains of cognitive function seem to be affected in DM1, although to different extents (Okkersen et al., 2017a). Intellectual impairment has been reported to follow two major patterns: (1) an age-related global decline of cognitive functions in adults or (2) a moderate to severe intellectual disability in childhood and CDM patients (Modoni et al., 2008, Douniol et al., 2012, Gallais et al., 2017). The first comprises a progressive decline of higher cognitive functions; with frequent below-the-average IQ, defective verbal and non-verbal memory and frontal dysexecutive syndrome, which affects both visuospatial and visuoconstructive skills (Rubinsztein et al., 1998, Modoni et al., 2004, Sansone et al., 2007). Adult DM1 patients also have social cognition disabilities, illustrated by their lower performances in Theory of Mind assessment of facial and emotional recognition, associated to connectivity changes between temporal and fronto-cerebellar brain regions (Kobayakawa et al., 2012, Serra et al., 2016). The inability of patients to recognize others' mental states, emotions or feelings affects their inter-individual relationships. Childhood patients are more severely impaired in their frontal and executive functions. The IQ scores are reduced along with processing speed, attention levels and short-term memory for specific tasks. Interestingly, the deficits in the visuospatial constructive skills reveal the involvement of the fronto-parietal connectivity: the process of coding, processing and transmission of complex visual representations seems inefficient, as reflected by poor results in the recalling and action planning abilities (Angeard et al., 2007, Angeard et al., 2011). More severe intellectual disabilities are observed in CDM children, with additional language and speech delays, further contributing to communication problems (Echenne and Bassez, 2013). Despite some degree of heterogeneity in intelligence levels and social cognition deficits, mostly

due to age and clinical variability, overall DM1 patients perform worse in all domains of cognition, relative to the healthy population and other neuromuscular diseases (Okkersen et al., 2017a). More recently, hypothesis such as degenerative processes or progeroid disease have been suggested to explain the correlation of the cognitive decline with age of onset and duration of the disease (Winblad et al., 2016, Gallais et al., 2017). Although controversial, the recently proposed progeroid hypothesis predicts that cognitive and motor decline in DM1 evolve with generalized accelerated physiological aging-related loss of abilities. However, in childhood forms, where the developmental cognitive milestones are often delayed or not achieved at all, it is difficult to conclude whether the worsening of the symptoms on follow-up tests is due to baseline impairments that do not allow for improvement or, if they are caused by progressive decline. In support of a neurodegenerative process, some histopathological studies (discussed in section (c)) have reported neuronal loss and other cell lesions. However the extent of these anomalies has not been fully evaluated, nor do we know how they relate with the symptoms. The evaluation of disease progression is still insufficient to provide full support for one of these two hypotheses, over the other. Extended longitudinal studies, supported by imaging and molecular data, should help characterize disease progression and distinguish disease-associated degenerative processes from accelerated aging.

Deviant behaviors and personality traits have been reported in adult DM1 patients, such as lack of initiative, apathy and a recurrent use of the emotion-focused coping mechanism, which decreases the emotional responses when facing stressful situations (Gallais et al., 2015, Minier et al., 2018). Mood disorders, such as anxiety and depressiveness, are also observed in subsets of adult patients, in association with cognitive disabilities; while avoidant, obsessive-compulsive/passive-aggressive and schizotypal personality disorders reach pathological thresholds more frequently in DM1 (Sistiaga et al., 2010, Serra et al., 2014). These features are shared with childhood and congenital patients, and even aggravated for domains of social interaction: younger patients show marked social anxiety, associated with attention deficits and hyperactivity and ASD in some cases (Douniol et al., 2012, Ekstrom et al., 2008).

Transversal to all clinical forms of the disease, the excessive daytime sleepiness, or hypersomnia, is the most frequent non-muscular symptom reported as very

incapacitating to the patients' daily-life. Both childhood and adult onset patients show an increased propensity to night- and day-time REM sleep, sleep-disturbed breathing and restless leg syndrome, reflecting a central defect of ventilation control and motor inhibition, as well as defective brainstem inputs (Laberge et al., 2009, Yu et al., 2011a, Romigi et al., 2013). However, these narcoleptic symptoms are unlikely to be related to the dysregulation of the sleep-arousal cycle regulator hypocretin, since the neurotransmitter levels and the splicing of its receptor were unchanged in DM1 patients (Ciafaloni et al., 2008). Self-reported extreme fatigue in DM1 is likely to be explained, not only by muscular impairment, but mostly by sleep disturbances, together with the apathetic and depressive neuropsychological status (Angelini and Tasca, 2012). Hence, possible bias due to muscular or mood interference should not be overlooked when interpreting both tested and self-reported CNS outcomes (Hamilton et al., 2018).

All together these data show a direct or indirect implication of the CNS-related symptoms in the quality of life of patients: nearly all aspects of the daily life of the DM1 patients are affected by the neurological manifestations of the disease. Visuospatial inability seems to be a good predictor of quality of life in younger patients. The level of education works better for adults and the fatigue is the best predictor when studying both groups simultaneously. Interestingly, the correlation between CNS symptoms and quality of life seem to be weaker in the juvenile than in adults, even if the former are more severely impaired in their cognition and psychological traits. This observation could indicate: (1) a low awareness of juvenile patients intrinsic to the disease; (2) lower internal standards, since juvenile live with the disease from earlier age, when compared to adult patients; or (3) an aggravated perception of the incapacities by adult patients, since they experience a deterioration process (Rakocevic-Stojanovic et al., 2014).

The quality of life of DM1 patients and their families would benefit from therapies targeting CNS symptoms. Therefore, research aiming to unveil the molecular and cellular mechanisms of the DM1 neuropathophysiology is imperative to achieve efficient therapeutic strategies.

(b) Neuroimaging findings

It is conceivable that the myriad of neuropsychological manifestation in DM1 may have their origin in structural and functional alterations of complex brain networks. More than two decades of imaging studies, have consistently reported widespread WM

and grey matter (GM) changes (reviewed in (Gourdon and Meola, 2017, Okkersen et al., 2017b, Minnerop et al., 2018)). The great importance of these studies resides in the possibility of simultaneously assess physical alterations, together with cognitive and behavioral impairments, which is not possible in *post-mortem* brain analysis. The findings of the structural and functional studies published are briefly described below (Figure II.2).

Structural analysis, using MRI techniques coupled with voxel-based morphometry analysis, have repeatedly reported symmetrical global and focal brain atrophy, reduced GM volumes and/or cortical thickness in all frontal, temporal, parietal and occipital lobes, hippocampus, basal ganglia and cerebellum. The data describe a progressive GM loss, which seems to be more pronounced in adult DM1, while it is more variable in younger patients (Antonini et al., 2004, Weber et al., 2010, Conforti et al., 2016). WM hyperintensity (WMH) changes and lesions have been the most consistent outcome of imaging studies (Figure II.2A) with pulled WMH changes in about 70% of DM1 individuals, against only 6% in healthy controls (Okkersen et al., 2017b). WM lesions appear scattered throughout the frontal and temporal lobes and to a lesser extent in parietal and occipital lobes. Main diffusivity alterations include decreased fractional anisotropy and increased mean diffusivity, revealed by diffusion tensor imaging with focus on the major association, projection and commissural fibers, suggesting either axonal loss or myelin damage, or a combination of both (Ota et al.,

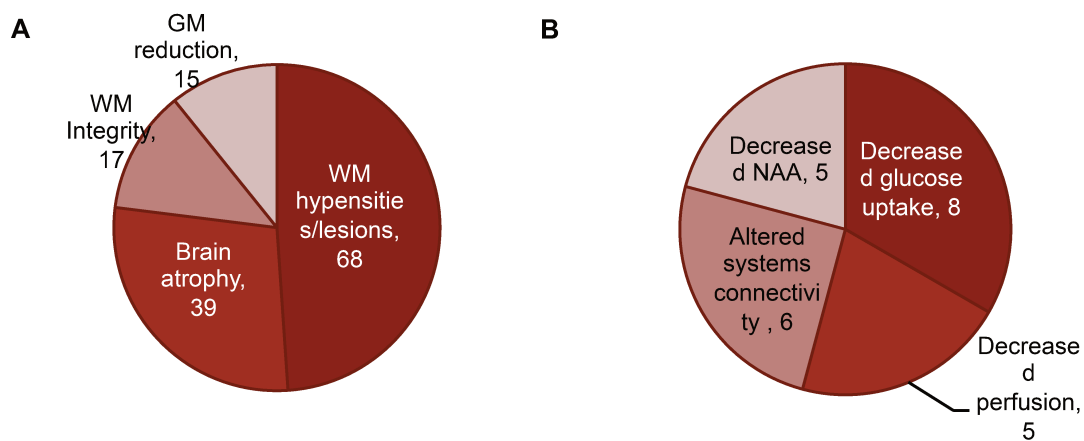


Figure II.2 - Summary of main findings of imaging studies on DM1 brains.

(A) Published studies demonstrating structural and (B) functional changes in DM1 patients, distributed by type of alteration. NAA, N-acetylaspartate. (data from (Okkersen et al., 2017b) updated with new entries from 2017-2019)

2006, Wozniak et al., 2011, Wozniak et al., 2014, Wozniak et al., 2013, Minnerop et al., 2011). Additionally, both thinning/atrophy and decreased diffusivity levels show the significant corpus callosum involvement, particularly in CDM but also in adult onset forms (Hashimoto et al., 1995a, Minnerop et al., 2011, Cabada et al., 2017). CDM patients frequently present ventricular enlargement, together with high incidence of progressive WM lesions very early in life, suggesting a developmental DM1 feature (Di Costanzo et al., 2002, Peglar et al., 2019). However, causal hypoxic-ischemic lesions cannot be discharged and further studies are required to confirm the developmental component of the imaging abnormalities.

Loss of integrity in the main fiber tracts may be predictive of connectivity defects. Indeed, defective connectivity and decreased efficiency of the brain network are revealed by functional MRI and correlate to some extent with cognitive, visuoconstructive, psychomotor and memory disabilities in patients (Romeo et al., 2010, Wozniak et al., 2011, Wozniak et al., 2014, Weber et al., 2010, van Dorst et al., 2019). Interestingly, altered connectivity is also observed both in resting- and task-induced activation states in networks associated with personality traits and social cognition (Serra et al., 2015, Serra et al., 2014, Winblad et al., 2010, Kobayakawa et al., 2012). Other abnormal functional outcomes in DM1 brains (Figure II.2B) include a global hypoperfusion and glucose hypometabolism in prefrontal, temporal and pericentral regions, which correlate with executive deficits (Renard et al., 2016, Peric et al., 2017). Among the dysregulated neurochemicals, the reduced levels of N-acetylaspartate (a neuronal integrity marker present in all brain cells, involved in glutamate metabolism) are the most consistently reported, indicating a potential neuronal pathology (Hashimoto et al., 1995b, De Stefano et al., 2005).

In spite of the progress made through imaging studies in the mapping and characterization of the most affected brain regions and networks, the nature of the structural and functional alterations in DM1 brains is not entirely clear, and the origin and biological meaning of the some imaging findings remain vague. Scarce longitudinal studies have addressed this question, but existing data point towards two divergent conclusions: (1) the brain abnormalities reflect a progressive deterioration over time (although lesions in CDM patients progress in a short period of time); (2) the lesion progression is a slow process (changes in WM is not different from age-matched healthy

individuals) (Conforti et al., 2016, Peglar et al., 2019, Gliem et al., 2019). Further follow up experiments with larger and homogeneous cohorts of patients, combining higher resolution techniques and thorough neuropsychological assessment will improve the understanding of brain changes in DM1.

(c) Histopathological data

To date, few histological data are available on DM1 brains, possibly due to the lack and poor quality of *post-mortem* material. Nonetheless, early histopathology of DM1 brains gathered evidence of brain involvement, demonstrated by neuronal cell loss and nuclear inclusions. Neuronal loss was observed in the brainstem nucleus and superficial frontal, cortical layers of the occipital and parietal lobes of patients presenting cognitive and behavioral impairment (Ono et al., 1996, Mizukami et al., 1999). Neurons showing intranuclear inclusions (later identified as ubiquitin and granulovacuolar degeneration granules, containing microtubule-associated proteins) were found in the thalamus, substantia nigra and caudate nucleus (Ono et al., 1989, Oyanagi et al., 1994, Nakamori et al., 2012). Pre-senile intracellular neurofibrillary tangles (NFT) and aggregates of modestly hyperphosphorylated microtubule associated protein tau (MAPT)/Tau have been reported (Yoshimura et al., 1990, Vermersch et al., 1996). The occurrence of NFT, associated with abnormal protein expression and missplicing of MAPT/Tau isoforms classify DM1 as a tauopathy (Sergeant et al., 2001, Leroy et al., 2006).

The most notable histological observation is the accumulation of mutant CUG RNA, as nuclear aggregates or foci in DM1 brains. The RNA foci are dispersed throughout the brain, in diverse cell types, sequestering and perturbing several proteins, in particular splicing factors, consequently dysregulating alternative splicing and other RNA processing events (Jiang et al., 2004, Hernandez-Hernandez et al., 2013a). The deleterious gain-of-function of CUG RNAs certainly contributes to the etiology of some of the CNS symptoms, acting through pathogenic mechanisms that are discussed in section III.

All together imaging and histopathological data provide solid support to CNS involvement in DM1. Still, the underlying molecular and cellular mechanisms of brain dysfunction in DM1 need further investigation.

III. Pathogenic mechanisms of DM1

The comprehension of the DM1 molecular mechanisms underlying the multisystemic symptoms has come a long way since the discovery of the mutation, however the mechanistic connections are not completely resolved, in particular those operating in the CNS. Today DM1 is the paradigm of toxic RNA disease. The molecular events operating upstream and downstream of the expression toxic CUG transcripts, as well as the *cis*- and *trans*-acting mediators, play pivotal roles in the disease pathology.

A. Dynamic of the mutation

As mentioned before, the size of the expanded repeat increases and instability accumulates in all cells of the body throughout patient's life, correlating intrinsically with the aggravation of the symptoms. The molecular mechanisms of somatic and intergenerational instability proposed so far are mainly based on the formation of hairpin-like secondary structures during DNA replication, recombination, repair and/or transcription (reviewed in (Gomes-Pereira and Monckton, 2006)). Studies in yeast and bacteria have provided evidence that the replication fork stall and double strand DNA breaks induced by the repeat during DNA replication and recombination respectively, are a source of instability in proliferative cells (Pelletier et al., 2003, Jakupciak and Wells, 2000, Mirkin, 2007). In addition, repeat secondary structures are prone to induce transcription-coupled repair errors in both mitotic and post-mitotic mammalian cells, either expanding or contracting the repeat tract (Lin and Wilson, 2009, Nakamori et al., 2011b). Similarly, DNA repair systems have been implicated in the mechanisms of repeat size mutation. Among them, the implication of the DNA mismatch repair (MMR) system is the best studied. In mouse models and mammalian cellular systems the lack or inactivation of MMR protein complexes decrease the occurrence of expansions, while their proper function leads to somatic and intergenerational instability (van Den Broek et al., 2002, Gomes-Pereira et al., 2004, Savouret et al., 2004, Foiry et al., 2006, Tome et al., 2009). The proposed MMR-dependent model consists in the recognition and binding of the MMR proteins to the alternative structures adopted by the expanded repeat. MMR proteins engage in an error prone repair reaction that results in the incorporation of additional repeats (Gomes-Pereira et al., 2004, Tome et al., 2009).

Although DNA (and possibly transcription repair) are likely to be the most prominent mechanisms promoting DM1 repeat instability in post-mitotic tissues, other factors might be involved in the formation and accumulation of expansions, such as chromatin structure and the epigenetics of the DM locus (Lopez Castel et al., 2010). Thus, the instability mechanisms and related factors are promising targets to develop therapies aiming to modulate and/or abolish repeat lengthening both in somatic tissues and during intergenerational transmission.

B. *Cis*-effects of the CTG expansion

The DM1 mutation localizes in a non-coding sequence that belongs simultaneously to the *DMPK* 3'UTR and to the promoter region of the adjacent *SIX5* gene. *DMPK* mRNA and cytoplasmic protein were found reduced in DM1 patients raising the hypothesis of a deleterious *cis* effect of the CTG expansion, which would disrupt the processing and/or nuclear export of expanded mRNAs, thereby inducing haploinsufficiency (Fu et al., 1993, Hofmann-Radvanyi et al., 1993). To test this hypothesis, mouse models deleted for the endogenous *Dmpk* gene or overexpressing (OE) the human *DMPK* gene (with 20 repeats) were generated. Surprisingly, both mouse lines showed only a partial phenotype with decreased muscular force, late onset mild myopathy and defective cardiac conduction together with some molecular abnormalities in muscle cells (Jansen et al., 1996, Reddy et al., 1996, Berul et al., 1999). A *Six5* knock-out (KO) mice was generated to study the potential contribution of SIX5 protein inactivation to DM1 pathogenesis. These animals developed cataracts (but of a different type from the characteristic cataracts reported in DM1), reduced male fertility and some cardiac defects (Klesert et al., 2000, Sarkar et al., 2004). Thus, the low penetrance and mildness of the phenotypes of these mouse lines suggested that haploinsufficiency of *DMPK* and *SIX5* alone is not sufficient to produce the multisystemic symptomatology of DM1.

C. RNA toxicity, mediators and molecular mechanisms

RNA-mediated pathology is today widely accepted to explain disease etiology in DM1. It postulates a dominant negative gain of function of the expanded CUG RNAs, transcribed from the CTG expansion (Day and Ranum, 2005, Wheeler and Thornton,

2007, Klein et al., 2011, Sicot et al., 2011). The discovery of the DM2 mutation provided critical support for this mechanistic model. The similar symptoms, resulting from common genetic features (*i.e.* non-coding expanded repeats) in genes that are apparently unrelated hinted at a common pathogenic event: the transcription of expanded C/CTG-containing mRNAs. Direct support for the toxicity of repeat-containing RNA came from mouse models expressing an expanded CTG tract within the 3'UTR of the human skeletal-specific actin gene (HSA^{LR} mice), unrelated to DM1, or in its natural human chromatin context (the *DMPK* 3'UTR; DM300/DMSXL mice) (Figure III.1). In both transgenic mice, the accumulation of toxic CUG-containing transcripts was sufficient to produce typical DM1 features, such as myotonia in HSA^{LR} mice (Mankodi et al., 2000) or a more complex multisystemic phenotype in DM300/DMSXL mice (Seznec et al., 2001, Gomes-Pereira et al., 2007, Huguet et al., 2012). Other mouse models have corroborated the correlation between the CTG expansion, toxic RNA and the onset of DM1-like phenotypes – they are summarized in Appendix I Table 1.

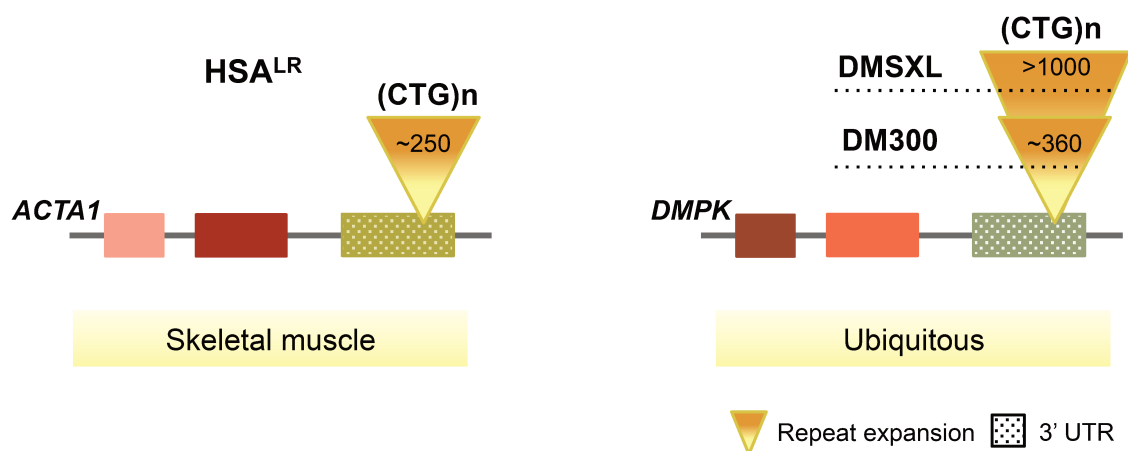


Figure III.1 – Schematic representation of the transgene structure of CUG-expressing mouse models.

DM1 mouse models carrying expanded CTG repeats in the 3'UTR of the human skeletal actin (*ACTA1*, HSA^{LR}) or *DMPK* (DM300/DMSXL), and the respective transgene expression profile.

Expanded CUG transcripts aggregate in discrete ribonuclear foci in the nucleus, where they are retained and prevented from being exported to the cytoplasm and translated (Taneja et al., 1995, Davis et al., 1997). As discussed previously for DNA, the CUG RNA expansions also form stable hairpin secondary structures, trapping and/or disrupting important RBPs such as the muscleblind-like (MBNL) or CUG-binding protein

Elav-like family (CELF) (Timchenko et al., 1996, Miller et al., 2000, Wang et al., 2015). While MBNL family of splicing factors (MBNL1, MBNL2 and MBNL3) are among the core proteins sequestered by the foci and promote their aggregation (co-localizing with nuclear RNA foci) (Fardaei et al., 2002); CELF proteins (CELF1 and CELF2) are upregulated in relevant tissues, such as skeletal muscle (Savkur et al., 2001), heart (Timchenko et al., 2001, Kuyumcu-Martinez et al., 2007) and brain (Dhaenens et al., 2011, Hernandez-Hernandez et al., 2013b), possibly as a result of (CUG)_n-induced hyperphosphorylation and consequent protein stabilization (Kuyumcu-Martinez et al., 2007). Similarly, CCUG expanded transcripts also accumulate as foci in the nucleus and co-localize with MBNL proteins. However the ribonuclear particles in DM2 are likely to contain only the expanded intronic sequence and/or abnormally spliced *CNBP* RNA, allowing the exportation of normal spliced transcripts (Margolis et al., 2006, Raheem et al., 2010). Although key players in alternative splicing, both sets of proteins have multifaceted roles in the cell. The implications of the early reported and recently unveiled functions of MBNL and CELF proteins in DM1 pathomechanisms are fully discussed in Appendix I.

In a simplified representation, the gain of function of expanded CUG RNA could be divided in two major effects: 1) the dysregulation of splicing and processing of downstream target transcripts of MBNL and CELF proteins and 2) the imbalance of protein homeostasis and cell physiology (Figure III.2). The weighted contribution of each one

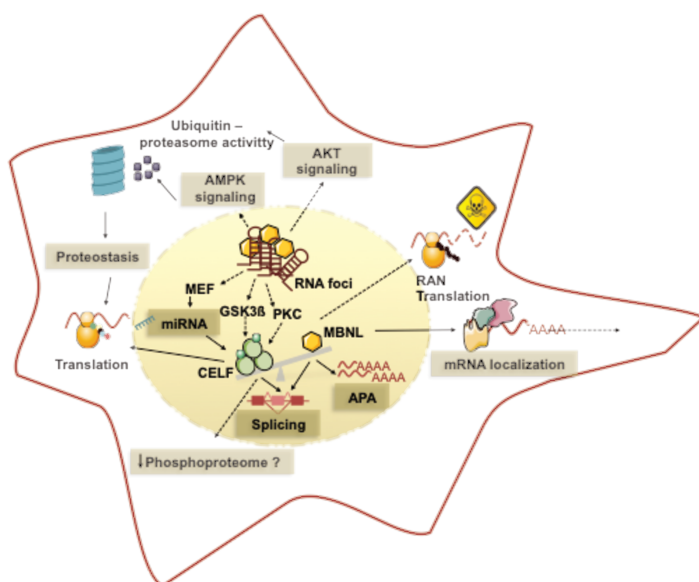


Figure III.2 - Representation of the main pathogenic molecular events in DM1.

The expanded DMPK gene is transcribed into sense and antisense (not represented) transcripts that accumulate into toxic nuclear foci, deregulating downstream mediators. MBNL family members are sequestered by the expanded transcripts, resulting downstream abnormal RNA metabolism. Upregulation of CELF proteins through hyperphosphorylation leads to perturbed splicing, mRNA decay and abnormal translation. Abnormal RAN translation also occurs from the expanded transcripts. Other transcription factors and protein kinases are also directly or indirectly affected by the CTG expansion.

of these mechanisms to the overall disease pathogenesis is not fully resolved, and it is even conceivable that it varies between cell types and developmental stages. The development of complementary animal models that recreate different features of the DM1 pathophysiology have contributed to the dissection of the disease pathways operating in DM1 (fully reviewed in Appendix I).

(a) mRNA missplicing

The demonstration of MBNL sequestration and inactivation, and CELF gain of function as key DM1 events have relied on the development of KO and OE mouse models, respectively (Appendix I Table 1). Both models reconstitute DM1-like phenotypes in association with abnormal alternative splicing in affected tissues (Gomes-Pereira et al., 2011). Interestingly, these two families of proteins have antagonist roles in the alternative splicing of DM1-related transcripts during development (Kalsotra et al., 2008). The combined dysfunction of MBNL and CELF prevents the fetal-to-adult switch in splicing profiles that happens during physiological development. As a result, abnormal fetal splicing isoforms are expressed in DM1 adult tissues, which are not compatible with proper adult tissue function (Sicot et al., 2011). Importantly, some of the MBNL- and CELF-dependent missplicing events have been directly correlated with the onset of some of the classical muscular symptoms, like myotonia (chloride channel (*CLCN1*)) (Wheeler et al., 2007), muscle weakness (bridging integrator 1 (*BIN1*) and calcium voltage-gated channel subunit alpha1 S (*CACNA1S*)) (Fugier et al., 2011, Tang et al., 2012), insulin resistance (insulin receptor (*IR*)) (Savkur et al., 2001, Guiraud-Dogan et al., 2007) or cardiac conduction defects (cardiac voltage-gated Na⁺ channel (*SCN5A*)) (Freyermuth et al., 2016). Curiously, MBNL inactivation impacts its own splicing program, and as a consequence, the expression and intracellular distribution of MBNL protein isoforms is imbalanced during *in vitro* myogenesis of DM1 cells, which could further exacerbate the severe muscle problems (André et al., 2019). Recently, Wang *et al.* showed that a subset of misspliced transcripts is shared by DM1 muscle and heart tissue (among which we find MBNL1 targets), while others are mutually exclusive dysregulations, reflecting a possible role for different RBPs (Wang et al., 2018a). Indeed, other RBP have been implicated such as the RNA Binding Fox-1 Homolog (RBFox) family, which together with MBNL (Klinck et al., 2014) and CELF (Gazzara et al., 2017) co-regulate some of the DM1 splicing events in skeletal muscle. The contribution of this

RBP to tissue-specific splicing regulation (beyond the core MBNL/CELF-dependent mechanisms) must be considered when looking for disease progression biomarkers or when developing molecular therapies.

To date, gene candidate approaches and RNA sequencing have not yet firmly connected specific missplicing events with CNS symptoms etiology. Several groups have reported splicing defects in DM1 adult brains. Splicing dysregulation in the CNS affects important neuronal genes, notably *MAPT* (Sergeant et al., 2001, Dhaenens et al., 2011, Caillet-Boudin et al., 2014), amyloid beta precursor protein (*APP*) and N-methyl-D-aspartate receptor type 1 (*NMDAR1*) (Jiang et al., 2004). Abnormal expression of these genes has been associated with other neurological disorders, such as Alzheimer disease and other tauopathies, further supporting a deleterious effect of DM1 RNA missplicing on CNS functions. The mimicking of CNS spliceopathy in a transgenic DM1 mouse model was first reported by our laboratory (Gomes-Pereira et al., 2007) (described in section I.A). Using these mice, we found that, like in muscle, RNA missplicing in the brain leads to the expression of fetal isoforms (Hernandez-Hernandez et al.). These findings were later followed by reports of additional splicing abnormalities in complementary DM1 mouse models (Charizanis et al., 2012, Suenaga et al., 2012, Goodwin et al., 2015, Wang et al., 2017). Although no direct causal connection was found between individual splicing defects and mouse CNS phenotypes, these studies have shed some light onto the mechanism behind the DM1 neurological symptoms. Importantly, they also demonstrated the valuable potential of the mouse models for the investigation of brain disease mechanisms and therapy development, as discussed later.

(b) Alternative Polyadenylation switches

Deleterious effects of MBNL inactivation on RNA processing are extended to alternative polyadenylation (APA). Batra *et al.* have demonstrated that loss of the three MBNL members disturbs the usage of alternative polyadenylation sites of a subset of transcripts. This observation was reproduced in *Mbnl* triple KO mouse embryonic fibroblast, HSA^{LR} skeletal muscle, muscle biopsies of both adult (Batra et al., 2014) and congenital DM1 patients (Thomas et al., 2017) as well as in brain tissues of *Mbnl1/Mbnl2* double KO and DM patients (Goodwin et al., 2015). A competition between MBNL proteins and the APA machinery for the binding to polyadenylation sites is necessary to the correct processing of the 3' ends of target mRNAs, affecting their stability,

localization and translation (discussed below). The sequestration/inactivation of MBNL proteins leads to a shift in the APA isoforms towards a fetal pattern, similar to alternative splicing changes in DM1 (Goodwin et al., 2015, Batra et al., 2014). It is worth noting that DM1-associated APA changes are slightly biased towards shorter isoforms, which may have particular consequences for brain cell development, in particular for neurons, where longer 3' ends are preferentially used given their role in post-transcriptional regulation (Miura et al., 2013) and transport (Braz et al., 2017). The contribution of particular APA defects to the specific disease symptoms is still to be clarified, but the mouse models available provide suitable tools to explore this question.

(c) mRNA mislocalization

In the cytoplasm, MBNL proteins are involved in mRNA trafficking and localization. Subcellular fractioning followed by RNA sequencing analysis found a shift of previously membrane-associated transcripts towards the cytoplasmic fraction, induced by knocking down *Mbnl1* and *Mbnl2* in mouse myoblasts and *Drosophila* cells. Moreover, this delocalization was more pronounced in genes with longer 3'UTR containing MBNL binding sites, suggesting that MBNL1 and MBNL2 proteins regulate the intracellular distribution of transcripts in a 3'UTR length specific manner (Wang et al., 2012). The loss of this finely regulated process in DM1 (as a result of MBNL sequestration and partial inactivation) is particularly relevant for highly polarized and ramified cells such as brain cells, since it may disrupt the correct mRNA localization in specialized cellular compartments (*e.g.* axons or glial processes) and perturb its local translation and protein function. Further investigation should elucidate whether or not mRNA mislocalization is linked with cellular dysfunction and disease manifestations.

(d) Gene expression abnormalities

The dysregulation of the transcriptional activity was first demonstrated by microarray analysis that showed altered gene expression in DM muscle biopsies (Botta et al., 2007). MBNL1 depletion is also implicated in transcriptional alterations, accounting for about 70% of gene expression changes observed in HSA^{LR} mice muscle (Osborne et al., 2009). Moreover, a recent transcriptomics study described an MBNL-associated gene expression signature in DM muscles, correlated with muscle strength levels and clustered for functional pathways important for muscle physiology (Wang et

al., 2018a). Among the altered genes is the *RBFOX2*, an MBNL splicing partner. Several transcription factors were found trapped into the foci, as it is the case for specificity protein (SP) 1, SP2, SP3, retinoic acid receptor gamma (RAR γ), signal transducer and activator of transcription (STAT) 1 and STAT3 (Ebraldize et al., 2004), in the absence of clear co-localization with the CUG RNA (Junghans, 2009). Other transcription regulators, such as NK homeobox 5 (NKX2-5) (Yadava et al., 2008) and SMART/HDAC1-Associated Repressor Protein (SHARP) (Dansithong et al., 2011) are increased or mislocalized in DM1 cells, likely contributing to muscular and cardiac symptoms. SLIT and NTRK like protein (SLITRK) factors are decreased in DM1-derived neurons and brain tissues, possibly contributing to the neuronal phenotypes detected in cellular models (Marteyn et al., 2011). Although intriguing, these changes need further investigation to decipher whether they are cell-type specific, tissue-dependent or a combination of both, before establishing cause-consequence relations. If confirmed, alterations in these transcription factors are predicted to perturb the expression of their target transcripts and play a role in disease molecular pathogenesis.

(e) *Antisense transcription*

Bidirectional transcription is a common feature among trinucleotide repeat diseases and has been implicated in a variety of regulatory mechanisms (Batra et al., 2010). In wild-type DM1 locus, an antisense *DMPK* transcript emerges from the *SIX5* regulatory region and spans towards the *DMPK* sense 3' region, flanked by CTCF-binding sites that limit the transcript to a 21-nucleotide fragment. Long CTG expansions in DM1 patients disrupt these regulatory binding sites, promote heterochromatinization and CpG methylation, leading to the generation of longer antisense transcripts (Cho et al., 2005) and abnormal sense and antisense *DMPK* relative levels (Gudde et al., 2017). Expanded CAG-containing anti-sense transcripts can also form nuclear foci in muscle and brain tissues of both adult and congenital DM1 patients, as well as in DMSXL mice (Michel et al., 2015, Huguet et al., 2012). Similarly, anti-sense foci sequester MBNL proteins, further exacerbating the functional inactivation of these proteins (Mykowska et al., 2011). Together these changes suggest a pathogenic contribution of the antisense transcripts to DM1, by mechanisms that are not fully understood.

(f) *Dysregulated mRNA decay*

MBNL and CELF proteins can also participate in the mRNA stability and decay. These proteins bind preferentially to the 3'UTR of their targets, destabilizing and facilitating the decay of a subset of transcripts, including each other's mRNAs. Thus, MBNL1 inactivation by CUG foci leads to a reduced *Cefl1* decay, potentially contributing to the protein overexpression in DM1 (Masuda et al., 2012). However, the contribution of MBNL1 depletion to CELF1 upregulation is unresolved, since *Mbnl1* KO mice do not show increased levels of CELF1 protein (Kanadia et al., 2003). On the other hand, CELF1 destabilizes several transcripts from the secretory pathway in myoblasts, perhaps contributing to changes in the extracellular matrix of DM1 muscle (Russo et al., 2017).

Another protein involved in RNA decay, the DEAD-Box Helicase 5 (DDX5), was found downregulated in biopsies from DM1 and DM2 patients. Interestingly, the restoration of DDX5 levels in DM1 and DM2 cells led to increased degradation of toxic C/CUG RNAs and consequent dispersion of foci. More importantly, in HSA^{LR} mice, adeno-associated virus (AAV)-mediated delivery of this protein rescued muscle pathology (Jones et al., 2015). These results encourage the study of mRNA decay mechanisms more extensively in additional DM1 mouse models and human tissue samples, to better understand its role in disease pathogenesis.

(g) *Dysregulated non-coding RNAs*

Alterations in microRNA (miRNA) biogenesis may induce gene expression changes and contribute to DM1 pathology. Indeed, several miRNAs appear to be dysregulated in skeletal muscle (Perbellini et al., 2011), heart (Rau et al., 2011) and plasma of DM1 patients (Perfetti et al., 2016, Koutsoulidou et al., 2015) (**Table III.1**). Curiously, miR-1 is downregulated in both DM1 and DM2 in response to MBNL depletion, which impairs the proper maturation of pre-miR-1. An expected increase in miR-1 downstream targets is observed for connexin 43 gap junction protein (*GJA1*) and *CACNA1C* calcium channel, which might subsequently contribute to heart symptoms (Rau et al., 2011). However, there are discrepancies in the reported levels of miR-1 in different tissues, perhaps reflecting a tissue-specific regulation. The prognosis and therapeutic potential of these non-coding RNAs have been recently highlighted in different studies. Muscle-specific miRNAs were found in circulating exosomes of DM1 patient serum, in levels that correlate with the progression of muscle symptoms,

opening interesting new routes for the establishment of non-invasive biomarkers of disease progression (Koutsoulidou et al., 2017). On the other hand, the silencing of miR-23b and miR-218 by “antagomiRs” is sufficient to increase MBNL levels and rescue splicing defects, histopathological changes and myotonia in a DM1 mouse model (Cerro-Herreros et al., 2018), providing windows of therapeutic intervention based on the modulation of miR species.

A second mechanism mediated by potentially pathogenic non-coding RNA involves RNA interference (RNAi). The RNAi machinery recognizes and cleaves expanded CUG RNA secondary structures, hence generating CUG-containing small interference RNAs (siRNAs) (Krol et al., 2007). Such siRNAs are capable of binding complementary sequences and silencing target transcripts, enhancing DM1 molecular phenotypes (Yu et al., 2011b)(**Table III.1**).

Finally, a third type of non-coding RNA mediators of disease was recently described: two independent studies have pointed out the implication of circular RNAs (circRNAs) in DM1 pathogenesis. The authors hypothesized that DM1-associated splicing abnormalities would have an impact on circRNA formation, since this process involves a backsplicing mechanism. Transcriptomics analysis revealed a global increase in circRNA levels in the skeletal muscle of both DM1 patients and mouse models, and in cells depleted of *Mbnl1* and *Mbnl3* (Czubak et al., 2019). Individual circular transcripts were further validated in DM1-derived myogenic cells and tissues, and their levels correlated with the clinical and molecular scores observed in the patients from which the samples originated (Voellenkle et al., 2019) (**Table III.1**).

Although the meaning of these findings is still unclear, targeting specific non-coding RNAs appear to be an alternative molecular approach to design therapies aiming to correct individual events and provide functional tissue rescuing in DM1.

(h) Post-translational modifications

As referred in a previous subsection, the phosphorylation state of CELF1 is crucial for its activity, however in DM1 tissues the balance is broken and gives rise to protein hyperphosphorylation (Savkur et al., 2001, Timchenko et al., 2001, Hernandez-Hernandez et al., 2013b).

Table III.1 – Non-coding RNAs dysregulated in DM1.

| Non-coding RNA | Function in DM1 | Alteration | Tissue studied | Targets* | Reference | |
|-----------------------|-------------------------------|-----------------------------------------------------------------------------------|-----------------------|-------------------------------------------|-------------------------------------------------------------------------------|-------------------------------------------------------------------------------|
| microRNAs | miR-1 (-a and -b) | Downregulation | Heart | <i>GJA1, CACNA1C, KCNE1, KCNJ2, CALM2</i> | (Rau et al., 2011) | |
| | | Upregulation, abnormal localization | Skeletal muscle | <i>GATA6, MEF2A, MET, HAND2</i> | (Perbellini et al., 2011) | |
| | | Upregulation | Plasma | - | (Perfetti et al., 2016, Koutsoulidou et al., 2015, Koutsoulidou et al., 2017) | |
| | miR23b | Muscle regeneration, splicing | Upregulation | Myoblasts, muscle | <i>Mbnl1, Mbnl2</i> | (Cerro-Herreros et al., 2018) |
| | miR-27b | - | Downregulation | Plasma | - | (Perfetti et al., 2016, Koutsoulidou et al., 2017) |
| | miR-29(-b and -c) | Cardiac adaption to stress | Downregulation | Skeletal muscle | <i>DAG1, DIABLO, RET, TRIM63, TGFB3</i> | (Perbellini et al., 2011) |
| | miR-33 | Cholesterol metabolism, cell proliferation | Downregulation | Skeletal muscle | - | (Perbellini et al., 2011) |
| | miR-133 (-a and -b) | Myogenesis, control of cardiac conduction, muscle hypertrophy, apoptosis | Abnormal localization | Skeletal muscle | - | (Perbellini et al., 2011) |
| | | | Upregulation | Plasma | - | (Perfetti et al., 2016, Koutsoulidou et al., 2015, Koutsoulidou et al., 2017) |
| | miR-140-3p | Myofibroblast differentiation, chondrocyte proliferation | Upregulation | Plasma | -- | (Perfetti et al., 2016) |
| Abnormal localization | | | Skeletal muscle | - | (Perbellini et al., 2011) | |
| miR-206 | Myogenesis | Upregulation | Plasma | - | (Perfetti et al., 2016, Koutsoulidou et al., 2015, Koutsoulidou et al., 2017) | |
| miR-218 | Muscle regeneration, splicing | Upregulation | Myoblasts, muscle | <i>Mbnl1, Mbnl2</i> | (Cerro-Herreros et al., 2018) | |
| miR-335 | Muscle regeneration | Upregulation | Skeletal muscle | - | (Perbellini et al., 2011) | |
| miR-454 | Tumor suppressor | Upregulation | Plasma | - | (Perfetti et al., 2016) | |
| miR-574 | Cell differentiation | Upregulation | Plasma | - | (Perfetti et al., 2016, Koutsoulidou et al., 2017) | |
| siRNAs | (CUG) _n | Silencing mutant <i>DMPK</i> | Increased | Human fibroblasts | Mutant <i>DMPK</i> | (Krol et al., 2007) |
| | (CUG/CAG) _n | Eye degenerative phenotype, silencing of CAG _n -containing transcripts | Increased | <i>Drosophyla</i> eye | atx2, tbp | (Yu et al., 2011b) |
| circRNAs | CDYL | Myogenesis [#] , splicing [#] | Upregulated | Skeletal muscle, plasma, PBMCs | - | (Voellenkle et al., 2019) |
| | HIPK3 | | | | | |
| | RTN4_03 | | | | | |
| | ZNF609 | | | | | |

*Dysregulated targets with potential involvement in DM1 symptoms

[#]Indicated by correlation analysis

In DM1 heart, this increase in CELF1 phosphorylation is likely due to an increased activity of protein kinase C (PKC) alpha and beta II (Kuyumcu-Martinez et al., 2007). However, other kinases appear to control CELF1 metabolism and activity: while glycogen synthase kinase 3 beta (GSK3 β) seems to control the translational activity of CELF1 (Jones et al., 2012), AKT serine/threonine kinase (AKT) appears to regulate the nucleus-cytoplasm distribution (Huichalaf et al., 2010). Beyond CELF1, other phosphorylation defects are likely to have a role in DM1 neuropathogenesis: synapsin 1 (SYN1) is hyperphosphorylated, possibly in response to CELF1 upregulation (Hernandez-Hernandez et al., 2013a), and hyperphosphorylated MAPT/Tau has been found in NFTs (Sergeant et al., 2001). Moreover, in addition to GSK3 β , other kinases belonging to the mTORC1 pathway appear dysregulated both in DM1-derived neural stem cells (NSCs) (Denis et al., 2013) and in transgenic mice expressing CUG repeats in skeletal muscle (Brockhoff et al., 2017), such as AKT and ribosomal protein S6 (rpS6). All together these results point to a dysregulated kinome and associated phosphoproteome in DM1 tissues. In-depth studies must be conducted to understand the underlying mechanisms and the relevance of these abnormalities for disease pathology.

(i) *Translational abnormalities*

Proteostasis imbalance in DM1 pathogenesis has been suggested to result from the deregulation of the cytoplasmic functions of CELF and MBNL proteins. CELF1 localization and activation is under control of phosphorylation events. In particular, the phosphorylation of Serine-302 regulates the role of CELF1 in translation: when Serine-302 is phosphorylated, CELF1 acts as an activator (Timchenko et al., 2005), while in the unphosphorylated form it acts as a repressor of translation (Huichalaf et al., 2010). The increased levels of CELF1 in DM1 are accounted for by both phosphorylated and unphosphorylated forms of the protein on Serine-302, which results in reprogramming of protein translation and global cell stress, with deleterious consequences on cell function. Notably, abnormal steady-state levels of CELF1 targets, such as myocyte enhancer factor 2A (MEF2A) and cyclin-dependent kinase inhibitor 1A (p21), observed in DM1 tissues and recreated in CELF1-OE mice, have a damaging impact in myogenesis (Timchenko et al., 2004). Furthermore, *Celf1* inactivation in a mouse model overexpressing a short CTG expansion corrected the expression levels of CELF targets, improved grip strength and muscle histology but failed to rescue myotonia and cardiac conduction defects (Kim et

al., 2014). On the other hand, the impact of DM1 pathogenesis on cell stress is demonstrated by the activation of polyubiquitin/proteasome stress-signaling pathways in the skeletal muscle of DM300 transgenic mice (Figure III.1), which show muscle atrophy phenotypes (Vignaud et al., 2010). This result raises the hypothesis that altered protein homeostasis may contribute to muscle wasting and weakness. In addition to CELF1, MBNL1 can also take part in the cytoplasmic response to stress, given its accumulation in stress granules (Adereth et al., 2005). The physiological impact of cytoplasmic CELF1- and MBNL1-induced cell stress and protein mistranslation to the DM1 symptoms in muscle and other tissues requires additional investigation.

(j) RAN translation

As in many other microsatellite diseases, DM expanded C/CUG RNA transcripts are substrates for non-conventional repeat-associated non-ATG (RAN) translation, which generates toxic homopolymeric proteins (Cleary and Ranum, 2013, Zu et al., 2018). The mechanisms governing RAN translation initiation are still largely unknown, however two main hypothesis have emerged, both depending on the secondary structures formed by repetitive RNA that cause (1) ribosomal stalling after 5' mRNA cap-dependent and scanning initiation (Green et al., 2016), or (2) the bypass of the canonical machinery binding to the AUG codon, with the recruitment of the initiation complex directly onto the repeat (Zu et al., 2018). RAN translation of CUG repetitive transcripts can occur in all possible frames, and it may result in the production of polyleucine, polycysteine, polyalanine species, while the CAG RNA translation originates polyglutamine, polyserine and polyalanine stretches. In DM1 polyglutamine have been observed in myoblast, blood cells and skeletal muscle from patients and, in cardiac cells from DMSXL mice (Zu et al., 2011). Curiously, in DM2 brains polypeptides derived from sense translation are preferentially located in GM, while antisense RAN products are more abundant in WM (Zu et al., 2017).

Recent studies in DM1 (Kino et al., 2014) and DM2 (Zu et al., 2017) cell models have proposed a protective role of MBNL in RNA translation: the disequilibrium caused by the sequestration of nuclear MBNL and the accumulation of RNA foci allows the escaping of expanded RNA to the cytoplasm, where it becomes available for RAN translation. The pathogenicity of RAN-generated polypeptides alone or in combination with bidirectional transcription is supported by their co-localization with apoptosis

markers (Zu et al., 2011) and increased cell death in culture (Zu et al., 2017). Although further studies are required to elucidate the cascade of events, these data hint that region/cell-specific and dose-dependent balance between MBNL and RAN translation may explain how ubiquitously expressed RNA expansions have heterogeneous pathological outcomes in distinct cell populations, even within the same tissue.

IV. Transgenic DM1 mouse models for CNS pathology

Many of the disease mechanisms known today were uncovered thanks to the modelling of DM1 in mice. A usefully toolbox of complementary mouse models was produced in the last two decades, contributing to dissect the molecular and cellular pathogenesis and to select promising therapies worth of clinical trials. Their intrinsic characteristics and contributions to the DM1 field are thoroughly reviewed in Appendix I (and elsewhere (Sicot and Gomes-Pereira, 2013)). This section will be mainly dedicated to the DMSXL model, since this was the main model used during this project.

A. DMSXL mouse – a powerful multisystemic model

The urge to understand the cellular and molecular mechanisms of DM1 led our laboratory to create a transgenic mouse model carrying a large 45-kb genomic DNA fragment from the human DM1 locus. The first version of this mouse contained the *DMPK* gene with 55 CTG repeats (DM55 mouse line), from a mildly affected patient (Gourdon et al., 1997). The expansion of the initial transgene was further elongated to 360 CTG repeats, by replacement with a *DMPK* 3'UTR from a more severely affected patient, originating the DM300 mice. At the same time, the control line DM20 was created introducing a 20 CTG repeat-long fragment within the *DMPK* locus (Seznec et al., 2000). The DM300 mice closely mimicked the expansion-biased somatic and intergenerational instability typical from DM1. Notably, in the CNS the somatic instability varies among brain regions and correlates with the age of the mice (Appendix I Figure 3). Over the years, the successive crossing of these mice originated progeny that today carries more than 1,000 CTG repeats, the DMSXL mice (Gomes-Pereira et al.,

2007). The muscle and brain phenotypes already present in the DM300 mice (Seznec et al., 2001) were aggravated by the increased CTG repeat size, showing more severe spliceopathy, myotonia and muscle weakness, accompanied by high perinatal mortality and severe growth retardation in DMSXL animals (Gomes-Pereira et al., 2007, Huguet et al., 2012).

The DM300 and DMSXL mice have been used in different lines of research in the laboratory. They have been of particular interest to study the CTG repeat instability, given the expression of the repeats in their natural context, *i.e.* with the same surrounding regulatory sequences present in the human genome. Our group has demonstrated the implication of the MMR repair machinery in the mechanisms of somatic and intergenerational repeat expansions, by crossing DM300 mice with different mouse models of deficient DNA repair (Foiry et al., 2006, Savouret et al., 2004, Savouret et al., 2003, Tome et al., 2009).

Being under the control of its own promoter, the *DMPK* transgene is ubiquitously expressed in the vast majority of mouse tissues, in clear contrast to the HSA^{LR} mice that express long CUG transcripts specifically in the skeletal muscle. Notably, the human *DMPK* transgene is expressed in tissues relevant for DM1 and follows the expression profile of the endogenous murine *Dmpk* gene, allowing the investigation of downstream pathophysiological mechanisms in multiple organs and cell types (Huguet et al., 2012). Homozygous DMSXL mice produce enough toxic CUG transcripts to reproduce the disease molecular hallmarks, such as ribonuclear foci accumulation, MBNL proteins sequestration, CELF upregulation and missplicing in several tissues, thus developing a mutisystemic phenotype (Huguet et al., 2012, Hernandez-Hernandez et al., 2013a, Michel et al., 2015, Sicot et al., 2017). Interestingly, antisense *DMPK* transcripts are also detected in DMSXL tissues and they accumulate into RNA foci, similar to the human situation (Michel et al., 2015). In the CNS *DMPK* transcripts are expressed throughout the brain, however the RNA foci accumulation shows some regional heterogeneity (Gomes-Pereira et al., 2007, Hernandez-Hernandez et al., 2013a). Both neurons and astrocytes have been reported to accumulate CUG RNA foci, associated with splicing abnormalities of candidate transcripts. A summary of the non-CNS DM1 phenotypes described in DM300/DMSXL mice is presented in **Table IV.1** and the CNS-related abnormalities are listed in **Table IV.2**.

Table IV.1 - Systemic molecular and phenotypical abnormalities (non-CNS) reported in DM300/DMSXL mice.

| Abnormality | Affected tissue | Reported defect | References |
|------------------------|-----------------------------|------------------------------------------------------------------------|------------------------------------------------------------------|
| Molecular signs | Most of the studied tissues | RNA foci | (Seznec et al., 2001, Huguet et al., 2012, Michel et al., 2015) |
| | Most of the studied tissues | CTG instability (expansion-biased) | (Seznec et al., 2000, Gomes-Pereira et al., 2007) |
| | Skeletal muscle | MBNL colocalization | (Michel et al., 2015) |
| | Liver | Splicing defects | (Huguet et al., 2012) |
| | Heart | RAN translation products | (Zu et al., 2011) |
| Metabolism | Serum | Low levels of Insulin and Insulin-like growth factor binding protein-3 | (Huguet et al., 2012) |
| Histology | Skeletal muscle | Centronuclear fibers | (Seznec et al., 2001, Huguet et al., 2012, Panaite et al., 2013) |
| | Diaphragm | Interfascicular connective tissue | |
| Phenotype | Sciatic nerve | Axonopathy of motor neurons | |
| | Phrenic nerves (diaphragm) | Reduced myelination | (Panaite et al., 2011, Panaite et al., 2013) |
| | | Reduced complexity of neuromuscular junctions | |
| | Heart | Conduction defects | (Algalarrondo et al., 2014) |
| | Diaphragm | Impaired respiratory function | (Panaite et al., 2013) |

Currently, studies are ongoing to decipher the mechanisms responsible for the disease-related phenotypes modeled in the DMSXL, in particular in the CNS, with the ultimate aim of establishing the grounds for rational therapies. In parallel, given their pre-clinical value, the DMSXL mice have been used in several therapeutic assays through multiple international collaborations, some of which are discussed later, in section A.

B. Modeling DM1 brain disease in mice

The use of DM1 mouse models, alone or in combination, has allowed the progression in the knowledge of the molecular mechanisms behind some disease phenotypes, particular in skeletal muscle and cardiac tissue. However that is not yet the case in the CNS. In spite of the progress that has been made in DM1 models, the consequences of mutant *DMPK* transcript for CNS function and related phenotypes are largely unknown. A few studies have started addressing this question using mouse

models expressing constitutive or inducible long CTG repeats in the CNS (**Table IV.2**) (discussed in detail in Appendix I).

Table IV.2 - Summary of main CNS findings in DM1 mouse models.

| Altered process | Reported defect | Tissue | Mouse model | References |
|----------------------------------|-----------------------------------------------------------------------------------------------------------------|--------------------------------------------|--------------------------------------|-----------------------------------------------------------------|
| Metabolism of RNA and RBP | Sense CUG foci | Most of brain regions | DMSXL EpA960/CaMKII-Cre | (Hernandez-Hernandez et al., 2013a, Wang et al., 2017) |
| | Anti-sense CAG foci | Frontal cortex | DMSXL | (Michel et al., 2015) |
| | MBNL colocalization | Frontal cortex Forebrain | DMSXL EpA960CaMKII-Cre | (Hernandez-Hernandez et al., 2013a, Wang et al., 2017) |
| | CELF upregulation | Frontal cortex | DMSXL | (Hernandez-Hernandez et al., 2013a) |
| Missplissing | <i>App, Mapt/Tau, Grin1/Nmdar1</i> | Frontal cortex Brainstem Spina cord | DMSXL | (Gomes-Pereira et al., 2007, Hernandez-Hernandez et al., 2013a) |
| | <i>App, Mapt/Tau, Tanc2, Kcnma1, Limch1, Spna2, St3gal3, Ndr4, Csnk1d, Ppp1r12a, Cacna1d, Add1, Dlg2, Grin1</i> | Hippocampus Forebrain | <i>Mbnl2</i> KO | (Charizanis et al., 2012, Goodwin et al., 2015) |
| | <i>Sorbs1, Spag9, Dclk1, Mprrip, Camk2d, Cacna1d,</i> | Hippocampus Cerebellum Forebrain | <i>Mbnl1</i> KO EpA960/CaMKII-Cre | (Suenaga et al., 2012) (Wang et al., 2017) |
| APA | <i>Rgs9, Sptb, Fzr1</i> | Brain | <i>Mbnl1/2</i> DKO | (Goodwin et al., 2015) |
| Protein dysregulation | Altered Tau isoforms | Brain | DM300 | (Seznec et al., 2001) (Vermersch et al., 1996) |
| | RAB3A upregulation SYN1 hyperphosphorylation | Frontal cortex Hippocampus Brainstem | DMSXL | (Hernandez-Hernandez et al., 2013b) |
| | GLT1 downregulation | Frontal cortex Cerebellum Brainstem | DMSXL | (Sicot et al., 2017) |
| Electrophysiology | Reduced paired-pulse facilitation | Hippocampus | DMSXL | (Hernandez-Hernandez et al., 2013a) |
| | Hyperexcitability of Purkinje neurons | Cerebellum | DMSXL | (Sicot et al., 2017) |
| | Reduced field excitatory postsynaptic potentials | Hippocampus | EpA960/CaMKII-Cre | (Wang et al., 2017) |
| | Reduced field excitatory postsynaptic potentials | Hippocampus | <i>Mbnl2</i> KO | (Charizanis et al., 2012) |
| Metabolism | Dopamine Reduction Increased Serotonine metabolites | Frontal cortex Brainstem | DMSXL | (Hernandez-Hernandez et al., 2013a) |

The DMSXL mice were for a while the only mouse model expressing long CTG expansion in the CNS. Meanwhile, another model (EpA960/CaMKII-Cre, see, Appendix I, Table 1-2) has been developed. These mice express expanded and interrupted CTG repeats postnatally in an inducible- and neuron-specific way. Hence, DMSXL mice represent the only model to express pure CTG repeats in all brain cells, from early embryogenesis, representing thus a unique tool to study the disease mechanisms operating in the brain throughout development. Indeed these animals show certain cognitive and behavior abnormalities comparable to those observed in DM patients: low exploratory activity associated with increased anxiety, anhedonic-like behavior, working memory deficits and cerebellum-dependent motor incoordination (Hernandez-Hernandez et al., 2013a, Sicot et al., 2017). Electrophysiological changes were also observed in DMSXL hippocampus and cerebellum in association with dysregulation of synaptic and astroglial proteins (Hernandez-Hernandez et al., 2013b, Hernandez-Hernandez et al., 2013a, Sicot et al., 2017). Previous proteomics studies on DMSXL brains revealed alteration of both expression levels and phosphorylation states of proteins specifically expressed in neurons, astrocytes and oligodendrocytes, further reflecting an ongoing toxicity in all three brain-cell types, due to the CTG repeat expansion. These findings indicate that both neurons and neuroglial interactions might be affected in DM1 brains.

DM1 features such as WM abnormalities still lack a cellular explanation, however it seems reasonable to assume that multiple brain cell types may contribute to the complex manifestations. Future studies should continue to explore which disease intermediates, cell populations and brain regions are more sensitive to RNA toxicity and when, during the disease time course, are the defects more pronounced.

V. Therapeutic prospects

Even though translational research has progressed in the last decade, there is no curative therapy for DM1 (or DM2) yet. The clinical care remains palliative, managing symptomatology and minimizing disability. However in the long run, these measures are inefficient to relieve the burden of the symptoms given the progressive nature of the

disease. Nevertheless, collaborative efforts of the DM community have contributed to the remarkable progress in the understanding of the disease molecular events that can be therapeutically targeted, establishing solid grounds for rational therapies towards pre-clinical studies and clinical trials.

A. Molecular strategies

One can imagine that targeting the root cause of DM1 would have better therapeutic outcomes, as the downstream cascade of deleterious events would be stopped. Indeed, correcting strategies at DNA level have recently emerged, taking advantage of powerful gene-editing tools currently available, with a high degree of efficacy and precision (Cinesi et al., 2016, Dastidar et al., 2018, Provenzano et al., 2017, van Agtmaal et al., 2017, Wang et al., 2018b). Nevertheless, promising pre-clinical studies aiming to reduce toxic RNA levels and/or modulate downstream intermediates of the DM1 molecular cascade, such as MBNL or CELF proteins, are in a more advanced stage, possibly due to their easier feasibility (Klein et al., 2015, Thornton et al., 2017, Overby et al., 2018). Strategies such as transcriptional or post-transcriptional silencing of toxic RNA, inhibition of MBNL-CUG RNA binding or targeting downstream pathways have been developed using antisense oligonucleotide (ASO), small interfering molecules or pharmacological chemicals (Figure V.1). Worth of note is the role of the DM1 animal models in the development, testing and validation of these therapies. Poly-CUG expressing mice routinely used for such studies and applied strategies are summarized in Appendix I, Table 3. Some of the pre- and clinical studies with most promising therapeutic potential are outlined below.

(a) Genomic deletion of CTG repeats

In line with the idea of acting on the primary cause of DM1, research groups have attempted the genetic deletion of the DNA repeat expansion (Figure V.1-1). These ambitious strategies are based in the guided activity of some nucleases coupled with DNA repair mechanisms (Richard, 2015). Initial studies demonstrated the potential of meganucleases and zinc-finger nucleases to induce double-strand breaks into CAG•CTG repeated DNA, producing mostly contracted repair products in yeast (Richard et al., 1999) and human cells (Mittelman et al., 2009). In spite of the satisfying efficiency of

these methods, the specificity for the repeated sequences is not always fulfilled, leaving room for the development of improved systems. Subsequent work, using the transcription activator-like effector nucleases (TALENs), achieved good efficiency with minimal off targets (Richard et al., 2014). Another approach using TALENs showed a successful insertion of a polyA signal into the *DMPK* intron 9, resulting in the expression of a truncated transcript that lacks the pathogenic CUG expansion and the rescuing of the molecular phenotypes in DM1 NSCs, such as foci accumulation and missplicing (Xia et al., 2015).

Later, clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) emerged as a powerful gene-editing tool. Initially, CRISPR-Cas9 methodology had some technical setbacks when applied to trinucleotide repeats, given the difficulties introduced by the sequence and structures of the guide RNA and the target repeats themselves, which may limit their recognition by Cas9. However, these limitations were recently surpassed and the system has been successfully used to remove the CTG expansion from the DM1 locus. The CRISPR-Cas9 efficacy has been demonstrated by several research groups that managed to completely and precisely excise large CTG repeats from the *DMPK* gene in DM1 myogenic cells (Provenzano et al., 2017), myoblasts from both human and DMSXL mouse (van Agtmaal et al., 2017, Lo Scudato et al., 2019), as well as in DM1 iPSCs and myoblasts derived thereof (Dastidar et al., 2018). *DMPK* gene editing resulted in the disappearance of ribonuclear foci, normal nucleo-cytoplasmic dispersion of MBNL proteins and

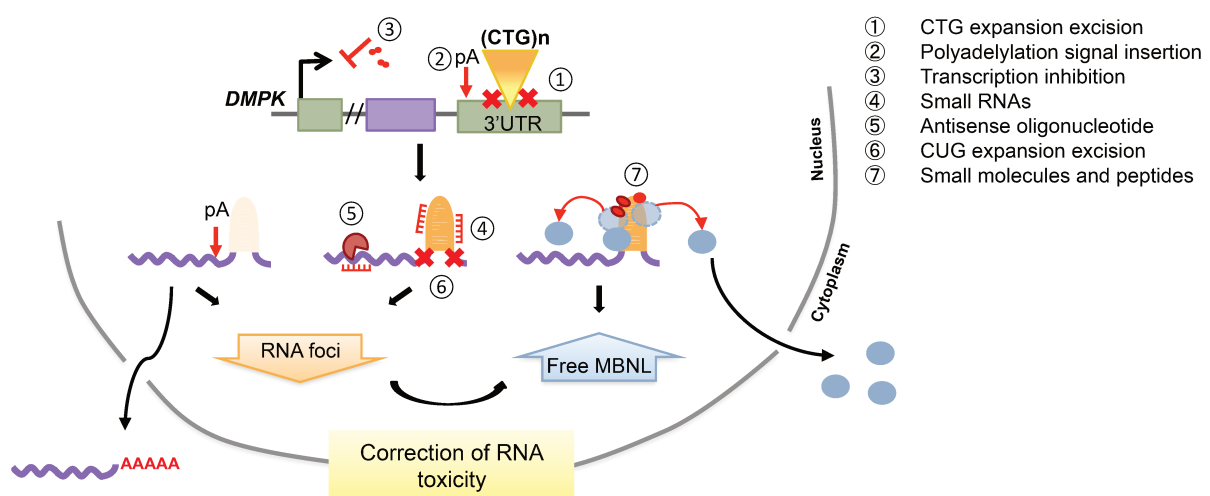


Figure V.1 – Therapeutic strategies targeting the DM1 mutation and expanded CUG RNA.

consequent reversal of the splicing pattern of target genes. Moreover, a locally-delivered single dose of CRISPR-Cas9 into skeletal muscle of DMSXL mice was sufficient to reduce toxic RNA foci in myonuclei (Lo Scrudato et al., 2019), demonstrating the suitability of the system *in vivo*. Wang *et al* obtained similar results using the CRISPR-Cas9 to knock in a polyA signal in the *DMPK* 3'UTR (Figure V.1-2), upstream of the expanded tract, which reverted the molecular phenotypes in DM1 neurons, cardiomyocytes and myoblasts (Wang et al., 2018b), confirming the advantage of this sort of approach to target the multiple cell types and the multisystemic symptoms of the disease. Although the deletion of the CTG repeats does not seem to have a negative impact in the expression of the genes of the DM1 locus (van Agtmaal et al., 2017, Dastidar et al., 2018), it presents some challenges: (1) frequent inversions of DNA are observed after double cleavage (van Agtmaal et al., 2017) and (2) there is a high risk of 'off target' effects, which should be carefully addressed before taking this gene editing therapies to clinical tests.

A theoretically safer re-engineering of CRISPR/Cas9 was developed to obtain a RNA-targeting Cas9 that eliminates the repeat from the RNA transcript, rather than the DM1 locus (Figure V.1-6). Targeting the RNA would potentially avoid the introduction of permanent off target damage in patient's DNA. The efficacy of these constructs was demonstrated: CUG-containing mRNAs were selectively reduced and the main molecular defects rescued in DM1 cells (Pinto et al., 2017, Batra et al., 2017). Moreover, systemic treatment of HSA^{LR} mouse model with the deactivated Cas9 reverted *Clcn1* missplicing and consequent myotonia (Pinto et al., 2017).

Despite some variation in success rates, these results have shown the applicability of TALENs or CRISPR-Cas9 methods to permanently inhibit and/or degrade the toxic transcripts in DM1 cells and muscle tissues. CRISPR-Cas9 strategies have marked advantages: they are more versatile (targeting both DNA and RNA), easy manipulated, allow for AAV-packaging and delivery, and they result in sustained phenotype reversal. This system is a promising new therapeutic strategy, not only for DM1 but also for other microsatellites diseases. Further *in vivo* studies must be conducted to validate the efficacy of gene editing approaches to correct DM1 mutation in multiple tissues and evaluate the correction of multisystemic phenotypes.

(b) Toxic RNA neutralization

Expanded CUG transcripts are now established as the major pathological mediator in DM1. Therapeutic strategies designed to inhibit, neutralize or degrade RNA foci should revert DM1 phenotypes and improve symptoms. However, the application of such strategies raises questions about the extent of phenotypes reversion and whether cells can tolerate collateral loss of wild-type (WT) *DMPK* RNA. The first question was answered by the inducible DM5 mouse model (Appendix I Table 1), which expresses five CTG repeats in the *DMPK* 3'UTR context (Mahadevan et al., 2006). Although short, the repeat is expressed at high levels, triggering myotonia, myopathy and cardiac defects, associated with mild missplicing but without nuclear RNA foci accumulation. Importantly, withdrawing the inducing agent and shutting down the transgene expression is enough to revert the mouse phenotypes, showing the therapeutic potential of silencing toxic transcripts. The second point was addressed in *DMPK* KO models. Initial reports on *Dmpk*-deficient mice described cardiac conduction defects (Berul et al., 1999) and myopathy (Reddy et al., 1996). However, a recent study revisited the effects of gene deletion and post-development knockdown. The authors found no DM1-related muscle or cardiac impact (Carrell et al., 2016). Moreover, reduced DMPK levels in non-human primates had no remarkable detrimental effect either. Overall these results indicate that collateral reduction of DMPK protein by strategies targeting the mutant allele is relatively safe, establishing grounds for the development of therapeutics directed to the expanded *DMPK* RNA.

Unconventional inhibition of transcription by chemical compounds has been revealed beneficial to DM1 when CTG/CAG-specific transcription is targeted (Figure V.1-3). Administration of pentamidine (an FDA-approved small molecule) (Warf et al., 2009) or its analogues (heptamidine (Coonrod et al., 2013) and furamidine (Jenquin et al., 2018)) successfully reverted the molecular and muscular phenotypes in HSA^{LR} mice, as well as the cardiac defects in CUG-expressing *Drosophila* (Chakraborty et al., 2015). Similarly, Actinomycin D, a ubiquitous transcription inhibitor with preferential affinity to CTG-rich sequences, is able to specifically reduce the expression of mutant transcripts and restore splicing, at doses suitable for human treatment in HSA^{LR} mouse model (Siboni et al., 2015b).

Small RNAs have been engineered to silencing *DMPK* transcripts by means of RNAi machinery (Figure V.1-4), decreasing both WT and mutant transcripts in DM1 patient-derived myoblasts. (Langlois et al., 2005). To avoid disturbing the expression of the WT *DMPK* gene, others have designed iRNA targeting specifically the CUG expansion. These molecules were capable of mitigating the molecular and muscle phenotypes of HAS^{LR} mice, when administrated locally (Sobczak et al., 2012) or systemically (Bisset et al., 2015).

Currently, ASO are the most well-developed therapeutic approach showing the most promising results in neutralizing RNA toxicity. ASOs are engineered (1) to block CUG hairpins by steric hindrance, while displacing sequestered MBNL proteins, hence allowing their correct distribution in the cell; and/or (2) to induce preferential degradation of expanded CUG RNAs through RNase H activation (Figure V.1-5). Early independent studies using morpholino- or 2'-O-methyl phosphorothioate-type ASOs, directly injected via intramuscular, succeeded in reducing RNA foci, redistributing MBNL1 proteins and correcting muscle spliceopathy in two distinct CUG-expressing mouse models (Wheeler et al., 2009, Mulders et al., 2009). However, only the morpholino ASO reached physiological reversion of myotonia (Wheeler et al., 2009). Curiously, both chemistries reduced toxic mRNA levels, which was unexpected since the two are RNase H-inactive, blocking-type ASOs. Another RNA blocker with good molecular outcomes in human DM1 myoblasts consists in a modified human U7-small nuclear RNA, containing a complementary antisense sequence with 15 CAG, which does not activate RNase H or the RNAi machinery (Francois et al., 2011).

Other approaches used locked nucleic acids (all-LNAs) to prevent the CUG/MBNL complexes in myoblasts (Wojtkowiak-Szlachcic et al., 2015), while also reducing the CTG repeat instability (Nakamori et al., 2011a).

A second wave of ASOs with a gap in between CAG sequences (known as gapmers) use RNase H activation to induce degradation of the target sequences (Wheeler et al., 2012, Lee et al., 2012a). In HAS^{LR} mice, the systemic delivery of 2'-O-methoxyethyl (MOE) gapmers resulted in the long-lasting reduction of expanded CUG transcripts, correction of transcriptomic abnormalities, as well as amelioration of histopathology and myotonia (Wheeler et al., 2012). Similar benefits were obtained with

peptide-linked morpholino (PPMO) ASO (Leger et al., 2013) and in 2'-4' -constrained-ethyl (cEt) ASO-treated DMSXL mice (Jauvin et al., 2017).

The exciting results obtained with ASOs, together with the low toxicity of these compounds in DM1 mouse models and non-human primates, paved the way to the first ever gene therapy-based clinical trial for DM1 (Ionis Pharmaceuticals; USA). The trial tested a gapmer-ASO aiming to abrogate the *DMPK* expanded transcripts in muscle. Despite a modest reversion of the splicing defects, the biodistribution of the therapeutic agent in the muscle was modest, which limited the therapeutic outcomes and dictated the interruption of the tests at phase 1/2a. Nevertheless the pharmaceutical research continues to improve the delivery strategies and chemical design of future ASO-based drugs for DM1.

(c) *RNA foci dispersion by small molecules*

Small molecules have been proposed as an alternative to ASOs and siRNAs, to overcome their delivery and biodistribution limitations. Such chemicals can act by inhibiting the transcription of expanded *DMPK* transcripts, as mentioned in the previous section, or by blocking MBNL: CUG interactions and/or promoting the dispersion of toxic RNA foci (Figure V.1-7). Several groups have used high-throughput chemical screenings and “drug repositioning” strategies to look for CUG ligands that efficiently release MBNL proteins from RNA foci (Garcia-Lopez et al., 2011, Gareiss et al., 2008, Siboni et al., 2015a, Nakamori et al., 2016). In one of those studies, the D-amino acid hexapeptide rescued the CUG toxicity in *Drosophila* and HSA^{LR} DM1 models, (Garcia-Lopez et al., 2011), while administration of human-suitable doses of erythromycin (an FDA-approved antibiotic) reverted the missplicing and myotonia to some extent in the same DM1 mouse model (Nakamori et al., 2016). Another study revisited the pentamidine derivatives and found that furamidine provided a dose-dependent correction of missplicing and foci dispersion in DM1 myotubes (Siboni et al., 2015a), while preserving *DMPK* mRNA levels, suggesting the blocking of RNA aggregation without degradation (Jenquin et al., 2018).

The Disney laboratory, in turn, has followed a rational pipeline to design selective and efficient bioactive molecules. Using approaches based on chemical similarity, bis-benzimidazole was identified as a compound that binds 5'CUG/3'GUC and corrects DM1-associated missplicing in cellular and mouse models (Parkesh et al., 2012). Recently,

pre-clinical tests of cugamycin in DM1-derived myoblasts and HSA^{LR} mice obtained positive results. The reduction of toxic RNA in mouse muscles was associated with an overt rescue of splicing and transcriptomics defects, leading to improvements in myotonia (Angelbello et al., 2019). Moreover, improved selectivity caused fewer off-target effects than CAG gapmers. These studies have served as scaffold for a phase 1 clinical trial (NCT03959189), sponsored by Expansion Therapeutics, to evaluate the benefits of the small RNA inhibitor *ERX-963*, derived from cugamycin and that should disperse RNA foci and neutralize the associated toxicity.

(d) Modulation of intermediators

Downstream effects of the CUG expansion such as dysregulation of MBNL and CELF proteins can also be therapeutically targeted. The therapeutic potential of MBNL1 upregulation was investigated in HSA^{LR} mice through AAV-induced overexpression in muscle, which reverted *Clcn1* splicing to the adult form, contributing to long-term correction of myotonia (Kanadia et al., 2006). Similarly, increasing levels of *Mbnl* *Drosophila* homologue in cardiomyocytes rescued DM1 heart phenotypes in a fly model of the disease (Chakraborty et al., 2018). The safety of MBNL1 upregulation was addressed by the generation of mouse (Chamberlain and Ranum, 2012) and fly models (Bargiela et al., 2015) that overexpress *Mbnl1*. Both animals displayed surprisingly good tolerability to the premature switching to adult splicing isoforms, demonstrating the therapeutic potential of MBNL1-based strategies.

An alternative strategy to increase MBNL levels relies on the suppression of *Mbnl* regulatory miRNAs (discussed in section I.C(g)) (Cerro-Herreros et al., 2016, Cerro-Herreros et al., 2018).

Given the evidence that PKC phosphorylates and stabilizes CELF1 (reference), it was hypothesized that the therapeutic modulation of CELF1 could be achieved through the manipulation of PKC activity. Indeed, modulation of PKC-dependent CELF upregulation mitigated some of the cardiac symptoms in the inducible EpA960 mice (see Appendix I Table1-2). The blockade of PKC activity by injection of the small inhibitor Ro-31-8220, prevented CELF1 hyperphosphorylation and ameliorated CELF-dependent missplicing as well as cardiac pathogenesis, reducing mortality in these mice (Wang et al., 2009). However, knocking out *Pkc* in DM5 mice was insufficient to yield benefits in skeletal muscle (Kim et al., 2016). The conflicting results suggest that either the

mechanisms of CELF1 upregulation differ between tissues, or that RO-31-8220 acts through a mechanism, other than PKC inactivation and CELF1 downregulation.

CELF1 activity is also modulated by GSK3 β : increased GSK3 β levels are detected in HSA^{LR} mice, before the onset of muscular pathology. Inhibiting GSK3 β restored CELF1 protein function and reduced muscle weakness, myotonia and histopathological alterations in these animals (Jones et al., 2012, Wei et al., 2018) and improved the survival, growth and neuromotor phenotypes in DMSXL mice (Mei et al., 2019). Notably, in some cases the benefits were maintained several months after treatment withdrawal. Based on these results, AMO Pharma held a phase 2 clinical trial (NCT02858908) to test the safety and efficacy of the GSK3 inhibitor *Tideglusid* in CDM and juvenile DM1 patients (NCT02858908). Improvements on cognitive and neuromuscular function prompted the pharmaceutical to continue the study to phase 2/3 (NCT03692312).

New therapeutic directions were recently pointed out by the screening of metabolic stress-induced responses. The study reported the dysregulation of AMP-activated protein kinase (AMPK) and mechanistic target of rapamycin kinase (mTOR) in skeletal muscle of starved HSA^{LR} mice. The inhibition of these pathways with AICAR and rapamycin, respectively, reduces foci content and *Clcn1* splicing, while improving the muscle strength and myotonia (Brockhoff et al., 2017).

B. Management of CNS symptoms

While waiting for an ideal treatment that will simultaneously address the complex multisystemic symptomatology of DM1, chemical and non-medical therapies have been used to improve quality of life. Clinical standardized recommendations were recently published to guide clinicians dealing with adult DM1 patients (Ashizawa et al., 2018). In this section I will focus on a few empirical therapies and new strategies to relieve CNS-related symptoms.

In order to address sleep disturbances, patients are advised to take the anti-narcoleptic *modafinil* that reduces the excessive daytime sleepiness (Turner and Hilton-Jones, 2010). Although the degree of improvement provided by this medicine is variable among patients, the majority of reports agree on the positive effects of the modafinil in the DM1 patients (Damian et al., 2001, MacDonald et al., 2002, West et al., 2016). Sleep-related breathing difficulties are also a concern for patients. Therapeutic measures, such

as continuous positive airway pressure and non-invasive ventilation, help relieve obstructive sleep apnea and prevent respiratory failure, with positive consequences on patient's mood (West et al., 2016).

In the past few years, members from the European DM1 community have assembled the multi-center OPTIMISTIC (Observational Prolonged Trial In Myotonic dystrophy type 1 to Improve QoL-Standards, a Target Identification Collaboration) clinical trial. This project explored the benefits of adapted cognitive behavioral therapy and exercise on DM1 patients suffering from extreme fatigue (van Engelen and The, 2015). The OPTIMISTIC program applied for 10 months increased physical activity capacity and improved fatigue (Okkersen et al., 2018). Even though the contribution of cardiac and muscular deficits or the cognitive dysfunction to the activity outcomes remains to be elucidated, overall this trial provided evidence of the applicability of cognitive behavioral therapy as a new tool to reduce disease burden in DM1 families.

C. Remaining challenges

All the prospect therapies described above produce benefits to the DM1 phenotype to some extent. However most of those strategies were directed to overcome RNA toxicity in muscle, usually taking advantage of the HSA^{LR} model to set up animal pre-clinical experiments. This strategy is obviously limited, since it does not take into account the multi-organ dysfunction of DM1. Despite the critical contribution of these studies towards translational research, validation of the therapeutic strategies tested in tissues, other than skeletal muscle, such as heart and brain, is of utmost importance. Indeed the biodistribution and efficacy of therapeutic compounds remain a critical issue to be addressed, especially for the ASOs-based therapies, and particularly in the CNS. Moreover, there is shared concern about the fate and the consequences of free toxic CUG transcripts released by molecular strategies that do not degrade the expanded RNAs.

More general challenges parallel to all therapeutic strategies under development are still unanswered: (1) the estimation of the long-term risks and benefits from gene therapy strategies targeting toxic RNA or the expansion itself; (2) the definition of standardized, sensitive and, if possible, non-invasive outcome measurements, particularly in the CNS; (3) the standardized classification of clinical disease types, that

will help define homogenous cohorts for therapeutic intervention; and (4) the definition of clear criteria for the initiation and discontinuation of a treatment.

In spite of the progresses made over the last years, a cure for DM1 is still awaited. Searching for the “perfect” gene-therapy treatment that will ideally cure the disease does not invalidate parallel and complementary research on alternatives therapeutic means to improve DM1 tissue function and quality of life. Even the combination of single treatments to address distinct symptoms would be a great improvement for the patients.

VI. Neurobiology of oligodendroglia

In higher vertebrates, the CNS has as primordial role in the coordination of basic vital functions in order to maintain the organism alive. However, its role goes far beyond that - it enables fast, precise and coordinated movements; response to pain stimuli and performance of complex cognitive tasks such as learning, memorization or social interaction and behavior. The proper function of the CNS is only possible when all the counterparts, *i.e.* all the brain cells, are under a fine-tuned, synchronized and connected network. The lost of this balance leads to severe neurodevelopmental and neurodegenerative disorders, as well as abnormal behavior.

A. Different brain cell types – historical and functional distinction

The basal principles of today’s neuroscience are grounded on notions first advanced in the 19th century: functional localization and neuron doctrine. The first postulated that different brain regions must have different functions, while the second proposed that brain and spinal cord consisted in discrete elements with functional continuity, the neurons. In other words, neurons can touch but cannot fuse with each other. However, the term “neuron” was not coined until later because the consensual idea at the time was that the brain was a network of fused tubular elements, even if the largest neurons in the brain, the Purkinje cells, had already been described in 1837 by Purkinje. The work by Golgi, around 1873, in which he impregnated brain tissue blocks with silver nitrate (Golgi staining), described in detail pyramidal and cerebellar neurons.

However, his analysis was biased by the current reticular view of the nervous system. Instead, Cajal's publications from 1896 onwards, described different type of neurons from distinct brain regions and specified their structure and spines, allowed the conception of neuron as individual units. These publications served as the structural foundations of current neuroscience (Glickstein, 2006).

The second great debate of the neuroscience community at the time was about the composition and function of the interstitial nerve tissue, the "neuroglia" introduced by Virchow in 1856. Although initially described as "nerve-glue" that served to hold nerve tissue together, the idea that neuroglia contains structural cellular elements was already implicit. Those cellular elements had been observed before. Remak, in 1838, reported in his PhD thesis a sheath around nerve fibers (named "myelin" in 1853, and reported as part of Schwann cells more than 100 years later). Then Müller, in 1851 and Bergmann, in 1858 described non-neuronal cells in retina (Müller cells) and cerebellar cortex (Bergmann glia), respectively. Subsequent publications reported different star-shaped cells, including studies from Golgi and Cajal, until the settlement of the "second element" as astrocytes, in 1893 by von Lenhossek (Kettenmann and Verkhratsky, 2008). The "third element" of the nervous system was introduced into the neuroglia concept in the beginning of the 20th century by Rio-Hortega, who distinguished oligodendrocytes and microglia from astrocytes by their fewer and smaller processes when stained with silver carbonate (Ndubaku and de Bellard, 2008, Pérez-Cerdá et al., 2015). A younger type of macroglia, the nerve/glial antigen 2 – expressing glia (NG2-glia) (French-Constant and Raff, 1986), owns the delay of its identification to the morphological similarity and capacity to generate oligodendrocytes, remaining mistaken with oligodendrocyte progenitors for long. NG2 cells are an abundant heterogeneous population, spread throughout the developing and adult brain, with astrogliogenic potential (Dimou and Gallo, 2015).

Besides the morphological distinction, brain cell types are functionally very different and yet complementary: while neurons are the functional element of the nervous system in charge of integrating synaptic transmission, glial cells provide them with structural and functional support at different levels. In a simplistic view, astrocytes control the local cell-cell interaction and maintain the neurochemical homeostasis (Santello et al., 2019); microglia are the resident immune cells, responsible for

surveillance and clearance of brain insults (Colonna and Butovsky, 2017); NG2-glia act as active sensors, modulating different aspects of the brain function in case of alterations, in particular remyelination (Parolisi and Boda, 2018); and oligodendrocytes are the myelinating cells of the CNS, insulating the axons for a faster and efficient propagation of the electrical impulse, and actively regulating the circuit function (Pepper et al., 2018). The physical interplay of all these cell types, initially conceptualized in the drawings by Rio-Hortega, in 1928 (Figure VI.1A) remains impressively accurate until today (Figure VI.1B), in spite of the later progress in deciphering their functions and communication routes. Although the glia:neuron ratio was initially estimated to be much higher, with a value of 3 glia cells to 1 neuron (or even 10:1), today we know that this is only true for certain brain regions, such as cerebral cortex. Globally the glia:neuron ratio is estimated at around 1:1 in the human brain, with about 80 billion neuronal cells and a similar amount of glia (von Bartheld et al., 2016).

Given that oligodendrocytes were the cell type studied in this project, their physiology and function will be the focus of the following sections.

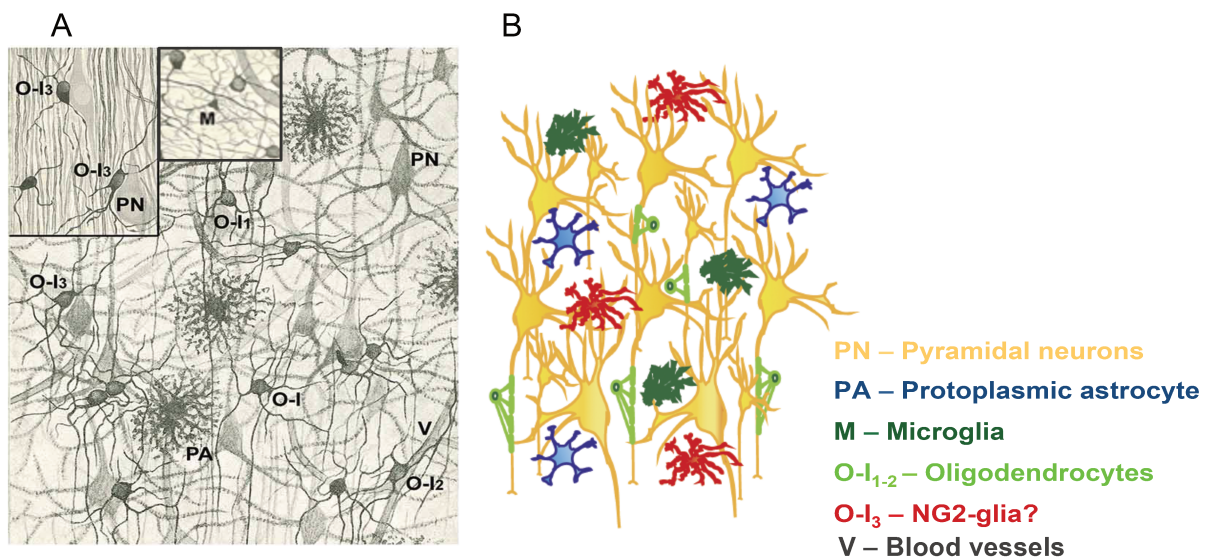


Figure VI.1 - Representation of different brain cell types interplay.

(A) Drawings by Rio-Hortega from his publication in 1928, showing the cerebral cortex cellular content after Golgi-Hortega/silver carbonate staining. (B) Current schematic representation of the communication between different brain cells, from a recent publication (2018) (Adapted from (Pérez-Cerdá et al., 2015) and (Kato et al., 2018)).

B. Oligodendrocyte physiology – Overview on migration, proliferation and differentiation

Oligodendrocytes derive from bipolar motile and proliferative oligodendrocyte progenitor cells (OPC) that give rise to ramified, immature pre-myelinating oligodendrocytes (preOL), which then differentiate into mature, myelinating oligodendrocytes (mOL), containing long radial processes and branching, which when in contact with axons, initiate myelination (Figure VI.2) (Emery, 2010). This discrete classical classification is useful to ease communication, but it is not entirely natural though. Recently, single cell RNA sequencing allowed the analysis of the transcriptome of cells from the oligodendrocyte lineage, isolated from different brain regions, distinguishing 12 subpopulations dispersed in a continuum from platelet - derived

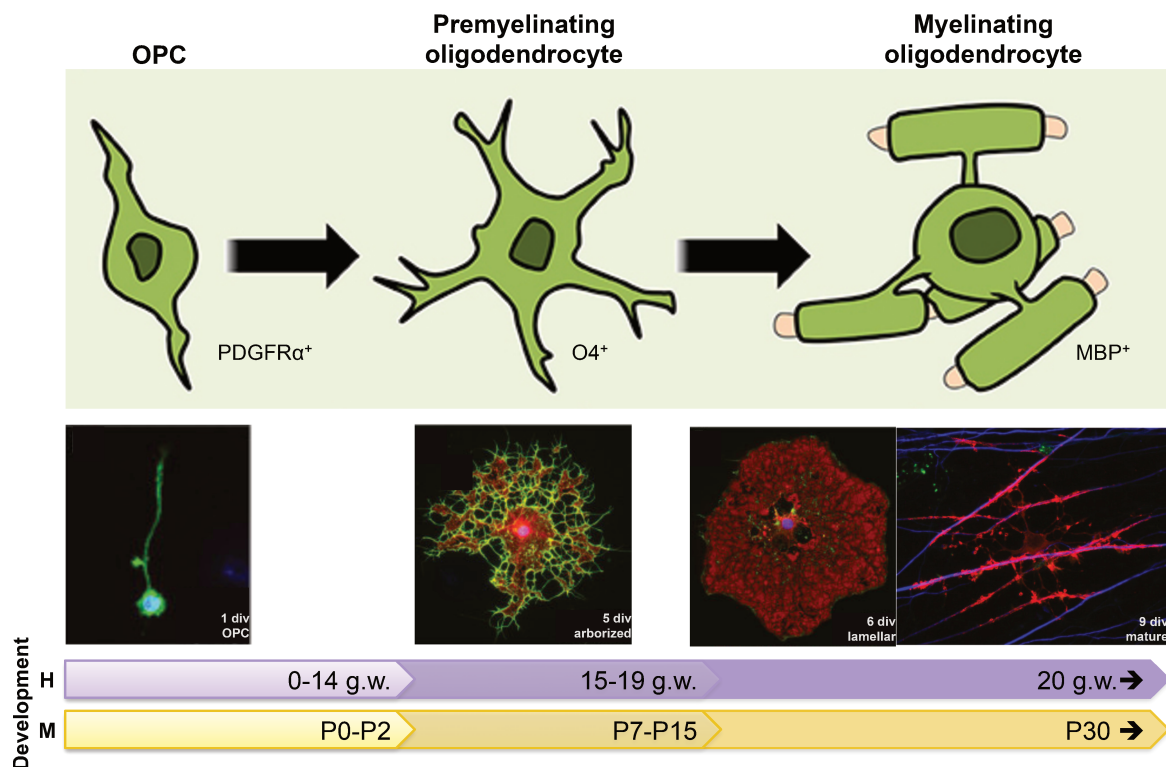


Figure VI.2 – Time-course of oligodendrocyte lineage differentiation.

Schematic representation (top panel) of morphological changes experienced by OPCs and oligodendrocytes throughout differentiation, with indication of representative markers for each cell stage: PDGFR α for OPC, oligodendrocyte antigen 4 (O4) for preOL and myelin basic protein (MBP) for mOL. Illustrative images of oligodendrocytes at different days of *in vitro* (div) differentiation, cultured alone or with neurons (middle panel). The time-line indicates human (H) and mouse (M) developmental periods (bottom panel) corresponding to each cell stage. (Adapted from {Galloway, 2016 #5988;Jakovcevski, 2009 #5989;Zuchero, 2015 #5990})

growth factor (PDGF) receptor alpha (PDGFRA)-positive OPC, to fully mature mOL (Marques et al., 2016).

At the initial stages, OPC regional heterogeneity is due, at least in part, to their spatiotemporal origin. During mouse development, OPCs are generated from oligodendrocyte-committed NSC, in three successive waves derived from subventricular zones (SVZ). From these initial sets, in postnatal forebrain, only OPCs from the last two waves (at embryonic day (E) 15.5 and postnatal day (P) 0) remain, while cerebellum and spinal cord retain cells originated from the two first waves (E11.5-12.5 and E15.5-16.5) (Goldman and Kuypers, 2015, van Tilborg et al., 2018b). Then, OPCs migrate to colonize both WM and GM, being guided by attracting extracellular cues to achieve a relatively uniform distribution. Among the extracellular signals that promote OPCs migration are morphogenic proteins, growth factors (GF) (such as PDGF-A and fibroblast growth factor (FGF)) and extracellular matrix (ECM) components (like laminin (LN), fibronectin or vitronectin). Additionally, axon guidance molecules (e.g. neuronal cell adhesion molecule (NCAM) or semaphorins), as well as glutamatergic neuronal activity can also modulate OPC migration (van Tilborg et al., 2018b). Once reaching the final territory, OPCs follow two alternative fates: keep proliferating to reach an homeostatic cell number, above which OPC exit the cell cycle and start to differentiate into mOLs or stay as parenchymal OPCs, serving as a reservoir of mOLs to maintain the normal myelin turnover and remodeling in adult brain (Goldman and Kuypers, 2015, Franklin and ffrench-Constant, 2008, Young et al., 2013).

OPC proliferation has to be actively sustained by means of extra- and intracellular signals that counterbalance their intrinsic propensity to differentiate. Most of these signals therefore, act simultaneously to promote OPC proliferation (*e.g.* PDGF) and inhibit OL maturation (*e.g.* Notch). Another regulatory layer, which promotes OPC expansion while preventing premature differentiation, is carried out at the nucleus by temporal-regulated transcription factors (van Tilborg et al., 2018b). This “pacemaker” regulation enables proper timing for axonal network establishment (Cui et al., 2010) and proper dispersion of WM myelination (Wang et al., 2007). Interestingly, the responses to proliferation/differentiation cues seem to be also regulated by α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated OPC-axon synapses in mouse corpus callosum (CC): changes in AMPA receptors permeability or function lead

to decreased OPC proliferation (Chen et al., 2018). The disinhibiting of the repressing processes, alone or in combination with maturation promoters, are promising therapeutic targets for myelin repairing in conditions characterized by demyelination (van Tilborg et al., 2018b).

Notably, most of the proliferation cues are survival promoters as well. Indeed, PDGF-A signals through PDGFRA that subsequently interacts with $\alpha 6\beta 1$ integrin receptors within lipid rafts, which can activate (a) tyrosine-protein kinase FYN to promote cell survival, or (b) the PI3K/PKC pathway to promote cell proliferation (Baron et al., 2005). Similarly, other ligands such as the neuronal-derived neuregulin-1 (NRG1), LN or different GF are able to regulate OPC numbers through integrin-dependent and independent mechanisms (O'Meara et al., 2011, Mitew et al., 2014).

Developmental transition from OPC to fully mOL involves a complete and fine tune reprogramming of the cellular status. Changes occurring during this process comprise remarkable morphological remodeling and gene expression reprogramming, precisely integrating anti- and pro-maturation signals (Swiss et al., 2011, Mitew et al., 2014). The switch requires a multitude of intra- and extracellular cellular cues that provide spatial and temporal control of differentiation and myelination. These signals comprehend extrinsic GF and ECM ligands, which activate kinase cascades that influence intracellular event, such as chromatin remodeling, transcriptional and translational reprogramming and actin cytoskeleton reorganization (described in more detail in the next section)(Galloway and Moore, 2016, Elbaz and Popko, 2019, Zuchero and Barres, 2013). A summary of the most relevant pathways and their effect on oligodendrocyte differentiation and myelination is presented Table VI.1.

C. Oligodendrocyte cell shaping and CNS myelination

Parallel to the myelin gene expression, oligodendrocytes experience massive morphological alterations: as they evolve into mOL, they extend cytoplasmic protrusions or processes (as defined by Rio-Hortega) and ramifications, forming membrane structures that culminate in myelin sheath formation. This mechanical remodeling is step-wise regulated in time and space by dynamic rearrangements of microtubule- and actin filaments-based cytoskeleton, as well as cell membrane adhesion complexes, and it ultimately leads to myelination (reviewed in (Seixas et al., 2019)).

Table VI.1 - Summary of extrinsic and intrinsic signals controlling oligodendrocyte differentiation and CNS myelination.

| Type of signal | Ligand/molecule tested | Effect on OL differentiation | Effect on myelination | Reference |
|-----------------------------------------|---------------------------------------------------------------------------------------------------------------------------|------------------------------|---------------------------------------------|-----------------------------------------------------|
| Extrinsic signaling | | | | |
| Axonal expressed molecules | Jagged/Notch | Inhibition | ND | (Wang et al., 1998) |
| | PSA-NCAM downregulation | ND | Increased internodes | (Charles et al., 2000) |
| | LINGO-1 downregulation | Promoting | Increase | (Mi et al., 2005) |
| | Gal-4 | Inhibition | Inhibition | (Stancic et al., 2012, Díez-Revuelta et al., 2017) |
| | Nrg1 KO/Nrg1 OE | No effect /ND | Normal or hypomyelination /Hypermyelination | (Brinkmann et al., 2008)8 (Makinodan et al., 2012a) |
| Secreted molecules and receptors | Wnt/ β -catenin activation | Inhibition | Hypomyelination | (Fancy et al., 2009) |
| | Tcf4 KO (Wnt effector) | Inhibition | ND | (He et al., 2007) |
| | Gpr17OE/KO | Delay/Promote | Demyelination/early onset | (Chen et al., 2009) |
| | γ -secretase inhibition | Promoting | Increased internodes | (Watkins et al., 2008) |
| | Igf1KO/IGFR KO | Reduction | Delayed | (Ye et al., 2002)2 (Zeger et al., 2007) |
| | T3 | Promoting | Promoting | Reviewed in (Mitew et al., 2014) |
| Neuronal activity | LN2 (via integrins) | Promoting | Promoting | Reviewd in (O'Meara et al., 2011) |
| | Glutamate | Inhibition | ND | (Gallo et al., 1996) |
| | Glutamate/ATP | Promoting | ND | (Wake et al., 2011) |
| | Activity-dependent NRG1 reduction/ErbB3 KD | Inhibition | Hypomyelination | (Makinodan et al., 2012a) |
| Intrinsic control | | | | |
| Transcriptional regulation | OLIG1, OLIG2, ASCL, NKX2.2, SOX10, YY1, KLF9, NFAT proteins, SOX2, SOX3, SOX4, SOX17, SREBP proteins, TCF7L2, ZEB2, ZFP24 | Promoting | Mostly increase | Reviewed in (Elbaz and Popko, 2019) |
| | ID2, ID4, HES1, HES5, SOX5, SOX6, NFIA, OLIG2, | Inhibition | Mostly repression | |
| | Mrf KO/Mrf OE in OPC | Block at preOL/Early onset | Inhibition/Early onset | (Emery et al., 2009) |
| | ZBTB7A, ZFP488 | No effect | Remyelination | Reviewed in (Elbaz and Popko, 2019) |
| Chromatin remodelling | HDACs inhibition or KO | Delay | Delay | (Ye et al., 2009) |
| MicroRNAs | Dicer KO in mOLs | Inhibition | Hypomyelination | |
| | miR-219 | Promoting | Promoting | (Dugas et al., 2010) |
| | miR-138 | Promoting | Promoting | |
| | miR-338 inhibition | Inhibition | ND | (Zhao et al., 2010) |
| | miR-23a KO in mOLs | Promoting | Pathological | (Lin et al., 2013) |

| | | | | |
|-----------------------------|-------------------|-----------|------------------|------------------------|
| | miR-17-92 cluster | Promoting | Hypremyelination | (Budde et al., 2010) |
| | Sfmbt2 cluster | Promoting | Remyelination | (Kuypers et al., 2016) |
| Long non-coding RNAs | lncOL1 KO | Delay | Hypomyelination | (He et al., 2017) |
| | lnc158 | Promoting | ND | (Li et al., 2019) |

ND – no data shown

(a) *Oligodendrocyte process outgrowth*

Oligodendrocyte processes tips consist in growth cone-like motile structures with actin-rich lamellipodia and filopodia that sense the surrounding environment, driving rapid changes in cell shape during differentiation (Fox et al., 2006). Initial protrusion extension and wrapping depend on actin polymerization at the leading lamellipodia and rear depolarization via actin-related protein 2/3 (Arp2/3) complex (Zuchero et al., 2015), while ensheathment and compaction is accounted for by actin depolymerizing factor (ADF)/cofilin1-dependent actin depolarization (Figure VI.3) (Nawaz et al., 2015). Interestingly, compaction seems to be temporally regulated by myelin basic protein (MBP), which competes with cofilin1 for the binding of phospholipid PI(4,5)P2, releasing it from the membrane and thus activating depolarization (Zuchero et al., 2015). Furthermore, expression of a subset of genes coding for promoters of actin-nucleation is developmentally regulated during OPCs/mOLs transition and their repression leads to ramification defects and hypomyelination (Kim et al., 2006, Bacon et al., 2007, Zuchero et al., 2015).

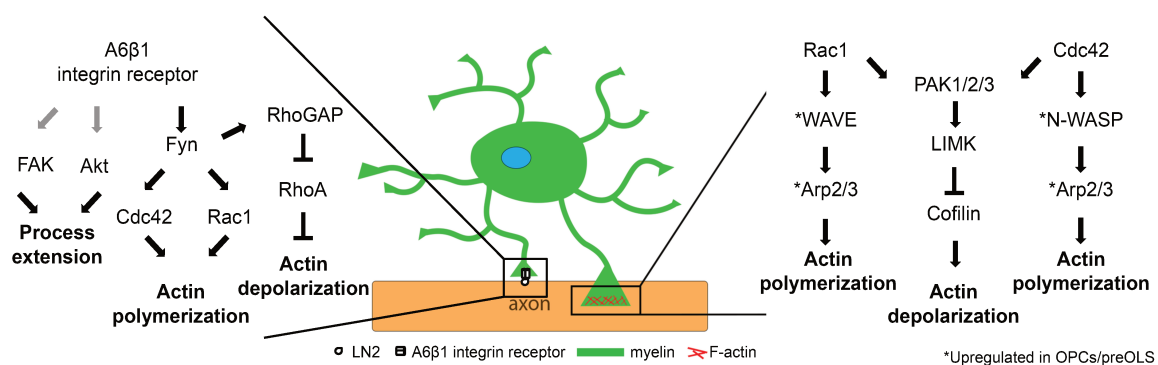


Figure VI.3 - Signaling pathways involved in oligodendrocyte cytoskeleton rearrangement during development.

Initial contact with LN2 activates the integrin $\alpha 6 \beta 1$ signaling cascade, which activates several kinases and its downstream effector (mainly Rho GTPases) leading to actin reorganization and process remodeling. (Adapted from (O'Meara et al., 2013, Brown and Macklin, 2019))

Similar to survival or proliferation, protrusion outgrowth and myelination are also regulated by extracellular signals, such as ECM-derived molecules that signal through well-established pathways to activate actin cytoskeleton reorganization (Figure VI.3). Differences in temporal and regional expression of factors, signals and effectors during CNS development are associated with the diversity of OPC subpopulations, the developmental timing of differentiation and specific groups of neurons to be myelinated (Bercury and Macklin, 2015). Different temporal expression of these factors dictates the sequential expression of a panel of stage-specific markers, among which myelin proteins, enabling the characterization of different phenotypic stages (Table VI.2) (Goldman and Kuypers, 2015). As the oligodendrocyte lineage progresses, preOLS acquire a more ramified morphology, lose migratory capacity and start to present O4-positive membranes, followed by the expression of myelin proteins (Table VI.2) during the immature phase until reaching the mOLs stage, characterized by the expression of the major myelin components, 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNP), MBP and proteolipid protein (PLP) (Bercury and Macklin, 2015).

(b) Axon selection

Myelin features vary among regions, and most importantly between CNS and PNS. Oligodendrocytes from cortex or corpus callosum (CC) regions can build up to 80 internodes (segment of myelinated axon between two Ranvier nodes) ranging between 20 to 200 microns in length and up to 60 lamellae (concentric cellular protrusions around axons) thick. Interestingly, these features are regulated in response to the axon biophysical and activity characteristics (Simons and Lyons, 2013).

Experimental evidence in zebrafish favors the importance of axonal caliber for myelination initiation. In this animal, the first myelinated axon is the larger Mauthner axon (Almeida et al., 2011). Interestingly, the decision of which axon is to be myelinated seems to depend on physical features, since OPCs co-cultured with inert nanofibers selectively myelinate fibers above 400 nm (Lee et al., 2012b). These results suggested that for oligodendrocytes, big caliber is sufficient to initiate axon myelination, while for smaller axons mechanical constraints prevent their wrapping and other factors, such as the density of OPCs, influence their myelination (Rosenberg et al., 2008, Lee et al., 2012b). Supporting the uncoupling of the myelination mechanisms in different types of axons, an earlier study showed the selective hypomyelination of smaller axons in *Fyn*-

deficient mice (Umemori et al., 1994), possible through its function on cytoskeleton regulation (Figure VI.3)(Krämer-Albers and White, 2011).

A role of neuronal activity on myelination has been suggested. The expression of axonal-derived factors and ECM molecules (Table IV.1) may be involved in axon selection, impacting mainly the small-caliber axons (Simons and Lyons, 2013). The fact that social isolated young mice display hypomyelination further supports a role for neuronal activity in axonal myelination. In line with this view, zebrafish showed a biased myelination towards electrically active axons: although vesicular release was not needed to initiate contact of the oligodendrocyte process with the inactive axon, it promoted myelin stabilization (Hines et al., 2015).

Table VI.2 - Characteristic oligodendroglia lineage stage-specific markers, used in cell identification and isolation.

| Stage-specific marker | OPC | preOL | mOL | |
|-------------------------------------------------|---------------------------|----------------------------------------------------------------------------------|------------|-------------|
| Selective surface antigens | A2B5 | O4 | O1 (GALC) | |
| | PDGFR α (CD140a) | | | |
| | NG2 | | | |
| Developmentally-regulated proteins | PTPRZ1 | TSPAN27, CLDN11, PLP1 , TRF, CNP , MAG , SMARCA4, APC (CC1) | | |
| | | | MAL | MOBP |
| | | | MBP | MOG |
| Developmentally-regulated transcription factors | MYT1, OLIG1, OLIG2, SOX10 | | | |
| | NKX2.2 | | | |
| | ID2 | ASCL1 | | |
| | ID4 | SOX9 | | |
| | HES1 | NKX6.2, SOX17, ZFP191, ZFP488, ZFP536 | | |

The myelin proteins are in bold.

(c) Myelin biogenesis

Myelination starts relatively late in the development time-scale: in mice it initiates at birth and it is nearly completed within 2 months in the majority of brain regions (Baumann and Pham-Dinh, 2001), while in humans it starts at embryonic stage, reaching the peak during the first year of life, and lasting until early adulthood (Fields, 2008). Models for myelination had been proposed in earlier studies; however it was only in the last decade that the combination of powerful state-of-art techniques enabled to

propose the “liquid croissant” model to describe the process of myelin formation *in vivo* (Sobottka et al., 2011). According to this model, myelin evolves from the center of a nascent myelin sheath in two coordinated motions: (1) wrapping the axon by the inner tongue (leading edge) and (2) coordinated lateral spreading of the layer, at the end of which cytoplasmic subdomains anchor the myelin sheath to the axon, forming the “paranodal loops” (Figure VI.4A-C) (Snaidero et al., 2014). Myelin compaction follows a precise timing and directional extension driven by MBP local translation at the inner layers and its transport backwards to the outer layer, where MBP binds to the cytoplasmic side of the myelin bilayer to begin compaction. Conversely, CNP act as a spacer, keeping the membranes apart long enough so MBP can be properly deposited, avoiding uncompact pockets within the myelin sheath (Snaidero et al., 2014). Incoordination of myelin compaction and growth might underlie myelin phenotypes that can be broadly divided into two groups: (1) unfolding and hypomyelination (*e.g.* by RhoGTPases signaling dysregulation) and (2) overgrowth and increased myelin thickness, with or without hypermyelination (*e.g.* AKT/mTOR over activation).

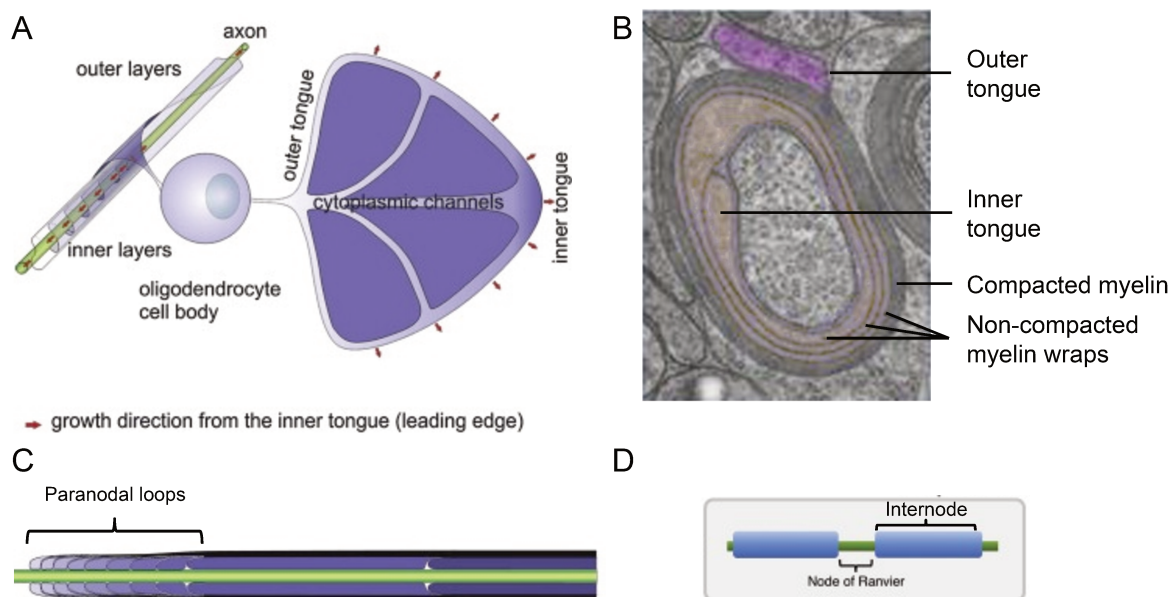


Figure VI.4 - Current model for myelin biogenesis.

(A) Proposed model for myelin sheath formation with wrapped and unwrapped membranes and representation of the direction of myelin growth and the position of cytoplasmic channels. (B) Transversal cross-section of a developing nerve fibre with compacted and non-compacted myelin showing the relative position of inner and outer tongues. (C) Representation of the longitudinal cross-section of the paranodal formation by lateral extension of adjacent layers. (D) Representation of Ranvier node and internode structures and their relative position in the axon. (Adapted from (Snaidero and Simons, 2014, Snaidero et al., 2014)).

(d) Adaptative myelination

Interestingly, mOL are long-lived cells, both in human and mouse brains: once formed they persist throughout adulthood (Yeung et al., 2014, Tripathi et al., 2017). Yet, the myelin turnover resulting from the replacement of damaged OL is just 0.3%, even if there is constant remodeling of WM, by new mOL (Yeung et al., 2014). Adult-born oligodendrocytes continue to be generated in mouse brain and have high survival rates as well (Hill et al., 2018, Hughes et al., 2018), adding myelin to partially unmyelinated axons (Hill et al., 2018). Changes in developed WM volume can occur in response to prolonged practice of new complex skills (Bengtsson et al., 2005) or upon acquisition of cognitive functions (Schlegel et al., 2012). These findings point to an active role for new mOL that act on unmyelinated substrate, modifying pre-existent circuits and providing an additional form of neural plasticity, rather than simple cell replacement or myelin turnover.

In the mouse brain axons are discontinuously myelinated (Tomassy et al., 2014). Through myelin remodeling, internodes can be added or modified in those axons, thus providing means of plasticity at the internode level. Adult myelin internodes are similar to those formed during development within the same regions, thus changes observed between internodes from different origins are due to posterior remodeling (Hughes et al., 2018). The remodeling rates decrease slightly in older mouse brains (Hughes et al., 2018), and differ among brain regions (Young et al., 2013). One expected consequence of the remodeling process is the modulation of the axonal conduction speed. Indeed, optogenetic-induced neuronal activity in adult mice premotor cortex promoted oligodendrogenesis and adaptive increase of myelin sheath, in association with motor improvements up to 4 weeks after stimulation (Gibson et al., 2014).

D. Novel functions for oligodendrocytes

Apart from myelination and myelin remodeling, several studies have implicated oligodendrocytes, especially mOL, in diverse CNS functions beyond internode synthesis.

(a) Maintenance of Ranvier nodes

Perhaps not surprisingly, mOL participate on the formation of the Ranvier nodes (1 μm long excitable, channel-rich axon domains where the action potentials are

regenerated) (Figure VI.4D), in a concomitant process with myelination (Freeman et al., 2016). Particularly, oligodendrocytes coordinate the clustering of voltage gated sodium channels (NaV) at the nodal region by releasing signaling molecules; myelination is required for nodal maturation, as well as to maintain saltatory conduction (Kaplan et al., 2001, Freeman et al., 2015). Its pathological disassembly leads to the dispersion voltage gated channels (KV) towards the paranodal region, leading to short-circuiting of the node and defective neuronal conduction (Suzuki et al., 2004, Hamada and Kole, 2015). Additional studies have suggested a role for oligodendrocytes and myelination in mediation of neuronal transmission. The deletion of the KV Kir4.1 in mOL results in defects in potassium clearance and consequent hyperexcitability in CC and optic nerve, without myelin impairment (Larson et al., 2018).

(b) Regulation of neuronal activity and synchronicity

Similarly, defects in oligodendrocyte population, myelination or myelin ultrastructure are associated with increased synthesis or release of neurotransmitters or metabolites, such as dopamine, glutamine or GABA, in both WM and GM (Roy et al., 2007, Xu et al., 2010, Maheras et al., 2018). These results indicate that oligodendrocytes and myelination are not only important to potentiate conduction speed but also to limit neuronal activity.

Promoting high conduction velocity might be one of the major functions of myelin in big caliber axons, however, in small caliber axons (with substantial lower conduction), myelin might be more important to control the synchrony of action potential firing (Freeman et al., 2016). Interestingly, maturation of the neuronal synchronicity occurs through adolescence in association with cognitive and executive function acquisition (James et al., 2008). It is thus conceivable that mOLs and myelin participate in the establishment of synchrony between different brain regions, ensuring precision and proper frequency of neuronal oscillations (Uhlhaas et al., 2009). Evidence for this comes from cuprizone-induced demyelination in mouse brains where the loss of mOL and myelin integrity in neocortical areas affects synaptic connectivity (Hamada et al., 2017).

(c) Metabolic support

The role of mOL as a metabolic supplier for axons was only elucidated in the last decade. The initial studies indicated that mice with unstable myelin or myelinic channels

(PLP- and CNP-null mice, respectively) showed axonal degeneration, while thin and uncompact myelin (MBP-null mice) was sufficient to preserve axon integrity (Philips and Rothstein, 2017). The different abilities of these mutant mice to propagate action potential and protect axons from degeneration, together with the transfer of lactate from mOL into axons through the monocarboxylate transporter 1 (MCT1) (Figure VI.5A) (Lee et al., 2012c, Funfschilling et al., 2012, Philips and Rothstein, 2017) illustrate well the role of oligodendrocytes as essential metabolic providers. Oligodendrocytes can either import glucose to produce lactate (Figure VI.5B), or exchange metabolic nutrients with neighboring cells, notably astrocytes, through connexin-based gap junctions (Figure VI.5C) (Hirrlinger and Nave, 2014). Importantly, metabolic dysregulation in oligodendrocytes is involved in severe neurodegenerative diseases, such as multiple sclerosis (MS) and ALS (Philips and Rothstein, 2017).

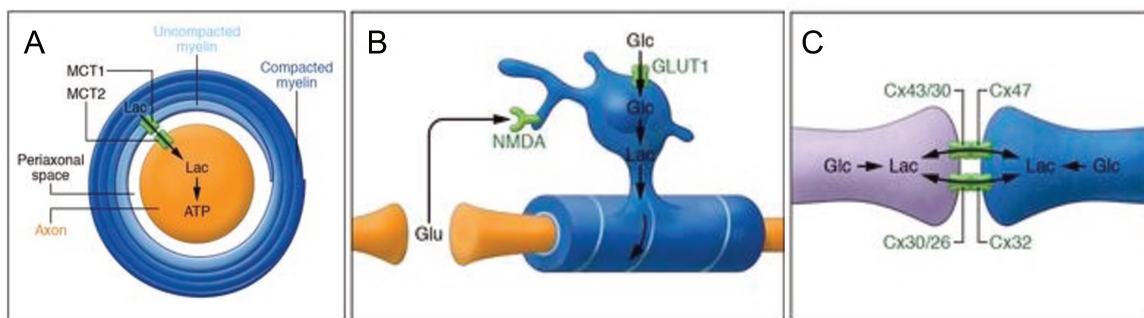


Figure VI.5 – Myelin-mediated metabolic support to neurons.

(A) MCT1 at the oligodendrocyte membrane transports lactate (Lac) to the periaxonal zone where it is uptaken by MCT2 at the axonal surface and then translocated to the lumen and processed for ATP synthesis. (B) Glutamate (Glu) resultant from neuronal activity binds to NMDAR enhancing glucose (Glc) uptake through glucose transporter 1 (GLUT1) and following conversion in lactate, which is then transported to the neurons. (C) Alternative source of intracellular lactate in oligodendrocytes is its uptake through connected gap junctions with astrocytes (violet). (modified from (Philips and Rothstein, 2017))

(d) Motor function

The disruption of motor and coordination capacities in mouse models of demyelination, such as experimental autoimmune encephalomyelitis, associates myelination with motor circuitry function (Moore et al., 2013). In the non-diseased context, the requirement of myelin for motor function is illustrated by the incoordination phenotype of *Plp1* null mice (Gould et al., 2018). Even small changes in

myelin ultrastructure (Klugmann et al., 1997) slow down action potential conduction in *Plp1*-deleted mice (Petit et al., 2014), which is associated with progressive fine motor incoordination without overt gross motor deficits (Gould et al., 2018). Similar effects are found when formation of new mOLs is blocked by genetic deletion of *Myrf* (McKenzie et al., 2014). However the question whether motor deficits are primarily due to mOL cell loss and myelin deterioration, or are a secondary effect of neuronal loss and/or neuroinflammation remains unresolved. The possibility of a contributing effect of microglia in the control of motor function has also been suggested (Schneider et al., 2016).

(e) *Behavior and cognition*

In humans, diseases causing WM alterations with changes or loss of myelin are frequently associated with psychiatric conditions, such as anxiety, depression and social withdrawal (Parnanzone et al., 2017, Arnett et al., 2008). Both focal demyelination and generalized WM lesions can lead to anxiety- and depressive-like behavior in humans and mice (Yang et al., 2017, van Tilborg et al., 2018a), raising the hypothesis that myelin alterations and/or altered oligodendrocyte function triggers these behaviour phenotypes.

Transient loss of NG2 OPCs in adult mice leads to depressive phenotypes, associated with anxiety- and anhedonic-like behavior, within 7 days of cell ablation (Birey et al., 2015). Two altered molecular pathways provide possible explanations for these phenotypes: (1) reduced glutamate uptake by the astrocytes and impaired glutamatergic systems in prefrontal cortex, decreasing excitatory neurotransmission, and/or (2) loss of secreted FGF2 by NG2 glia, since the blocking of this GF in other mice resulted in similar depressive phenotypes (Birey et al., 2015). Moreover, paradigms of induced stress in mice can cause changes in NG2 glia population as well as depressive phenotypes (Birey et al., 2015, Yang et al., 2016). The influence of impaired myelination on behavior is also demonstrated in mice deleted for *ErbB* (Roy et al., 2007) or *Olig2* (Chen et al., 2015), which show decreased movement and increased anxiety, in association with neurotransmitters dysregulation. Altogether these data indicate a role for the oligodendrocyte lineage and myelination in the development of mood disorders.

Cognitive decline is a prevalent feature in MS, with more than half of the patients showing WM alteration in imaging studies, supportive of a disconnection syndrome,

which triggers disease symptoms (Rocca et al., 2015). Importantly, cognitive impairment correlate positively with GM demyelination (Geurts and Barkhof, 2008), and even patients in early stages or milder forms perform poorly in neuropsychological tests to assess attention, executive function, learning and memory (Baysal et al., 2014). Similarly, diverse mouse models of oligodendrocyte loss, altered myelin or demyelination show working memory deficits (Xu et al., 2010, Xu et al., 2017, Yang et al., 2017, Makinodan et al., 2012b, Cao et al., 2017), while increasing myelin thickness of developed internodes improves fear memory acquisition (Jeffries et al., 2016). Interestingly, the formation of mOL and myelination are boosted in mice by the learning of a new motor task or enhanced exploratory activity (Xiao et al., 2016, Sampaio-Baptista et al., 2013, Keiner et al., 2017, Hughes et al., 2018). In summary, increasing human and mouse data support the role of myelin and oligodendrocyte plasticity in cognition and motor function.

E. Oligodendrocyte and myelin defects in trinucleotide diseases

Unstable microsatellite diseases, in particular those caused by trinucleotide repeat expansions, are frequently neuromuscular, neurodevelopmental and/or neurodegenerative Table I.1 and they are associated with some degree of cognitive deficit and behavioral dysfunction. Although oligodendrocytes and myelin are the primary targets of some neurological diseases, such as ALS or MS, their implication in repeat expansion diseases has been overlooked. Oligodendroglia cells and their functions have gained more attention in the last decade though, owing to the development of new imaging techniques and availability of relevant animal models.

In FXS for instance, WM microstructure are altered showing increased fractional anisotropy in specific tracts starting from early age (Haas et al., 2009, Hall et al., 2016), which correlates with behavior alterations in patients, when compared with IQ-matched controls (Green et al., 2015). Early but transient myelin defects have been also reported in different brain regions of *Fmr1* KO model (Pacey et al., 2013, Giampetruzzi et al., 2013), suggesting a contribution of myelination to the characteristic behavior and cognitive symptoms in FXS.

Myelin breakdown is a well-described event in HD patients, thanks to imaging studies in patients and the development of transgenic mouse models. Early imaging

studies in pre-symptomatic, symptomatic HD patients and post-mortem tissues have consistently reported myelin deterioration that was more severe in early- than in late-myelinated brain areas, such as striatum or medial temporal lobe, respectively (Bartzokis et al., 2006, Reading et al., 2005), thus indicating a regional pattern for myelin breakdown, in association with iron toxicity (Bartzokis et al., 2007). Additionally, morphometric analysis showed reduced WM and GM volumes, in correlation with caudate atrophy (the most affected brain structure) (Fennema-Notestine et al., 2004). In particular, the definition of a “temporal-specific and regionally selective” WM degeneration, which occurs as early as in pre-symptomatic stages, prior to the loss of axonal integrity and correlates with cognitive scores, but not with atrophy, was proposed as a biomarker to distinguish pre-symptomatic patients from early HD stages. In this context, myelin changes have been proposed as a causative factor of HD cognitive decline (Paulsen et al., 2010, Di Paola et al., 2014, Rosas et al., 2018, Bourbon-Teles et al., 2019).

Studies in several HD mouse models have further corroborated WM alterations in association with oligodendrocytes and myelin dysfunction in early stages of the disease (Teo et al., 2016, Huang et al., 2015, Jin et al., 2015). Worth of note are the HD-like motor deficits in mice, in which the expression of mutant *HTT* is restricted to the oligodendrocyte lineage (Jin et al., 2015, Huang et al., 2015, Ferrari Bardile et al., 2019). The clinical relevance of these observations has supported the recent clinical trial for the use of Laquinimod in HD (NCT02215616, phase 2), a drug that acts primarily in restoring oligodendrocyte physiology and myelin loss (Garcia-Miralles et al., 2016, Garcia-Miralles et al., 2019).

As described on the section I.D(b), imaging studies in DM1 patients show overt alterations in the major WM tracts, hyperintensities throughout the brain and some degree of atrophy and GM volume loss (Okkersen et al., 2017b). Moreover, the connectivity defects found correlated with the personality traits reported in patients, while inter-individual variability was associated with the diversity of the symptoms. All of the above are shared characteristics with HD or MS, which allows for speculation whether myelin is also affected in DM brains. On the other hand, low glucose uptake in DM1 brains (Okkersen et al., 2017b) might impair oligodendrocyte function and

metabolic support provided to neurons, contributing to neuronal dysfunction through cell non-autonomous mechanisms.

Thus, there is a need for in-depth studies on the potential impact of oligodendrocyte and myelin abnormalities and dysfunction in DM1 neuropsychological symptoms. The work presented in this thesis, aimed to fill the gap in the understanding of the potential role of myelinating oligodendroglia in DM1 brain disease, taking advantage of relevant animal and cellular models.

VII. References

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Appendix I

Of Mice and Men: Advances in the Understanding of Neuromuscular Aspects of Myotonic Dystrophy

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Intensive effort has been directed toward the modeling of myotonic dystrophy (DM) in mice, in order to reproduce human disease and to provide useful tools to investigate molecular and cellular pathogenesis and test efficient therapies. Mouse models have contributed to dissect the multifaceted impact of the DM mutation in various tissues, cell types and in a pleiotropy of pathways, through the expression of toxic RNA transcripts. Changes in alternative splicing, transcription, translation, intracellular RNA localization, polyadenylation, miRNA metabolism and phosphorylation of disease intermediates have been described in different tissues. Some of these events have been directly associated with specific disease symptoms in the skeletal muscle and heart of mice, offering the molecular explanation for individual disease phenotypes. In the central nervous system (CNS), however, the situation is more complex. We still do not know how the molecular abnormalities described translate into CNS dysfunction, nor do we know if the correction of individual molecular events will provide significant therapeutic benefits. The variability in model design and phenotypes described so far requires a thorough and critical analysis. In this review we discuss the recent contributions of mouse models to the understanding of neuromuscular aspects of disease, therapy development, and we provide a reflective assessment of our current limitations and pressing questions that remain unanswered.

Keywords: myotonic dystrophy, mouse, trinucleotide DNA repeat, skeletal muscle, cardiac muscle, central nervous system, brain, RNA toxicity

INTRODUCTION

Animal models offer experimental tools to investigate the causes and mechanisms of disease, when the access to human samples is limited. The remarkable progresses in genetic engineering allowed the introduction of human mutations in the mouse genome, to reproduce molecular, cellular and physiological disease manifestations. The resulting phenotypes provide insight to confirm starting hypotheses, reveal novel pathogenic mechanisms and evaluate new therapies. Myotonic dystrophy (DM) illustrates the cardinal contribution of mouse models to the systematic dissection of a complex disease mechanism, from genetic mutation to the design of clinical trials.

DM is the most common form of adult muscular dystrophy, characterized by pleiotropic symptoms, which are highly variable in their nature and severity (1). Major muscular features include myotonia, muscle weakness, atrophy and smooth muscle dysfunction. Cardiac conduction

defects and arrhythmias are associated with cardiomyopathy and may lead to sudden death (1). Brain involvement is illustrated by predominant structural abnormalities of the white matter, cognitive impairment (such as executive dysfunction, visuospatial deficits and abnormal social cognition), behavioral changes (such as apathy and social avoidance) and excessive daytime sleepiness (2). Other peripheral disease manifestations include insulin resistance, iridescent posterior subcapsular cataracts, and gastrointestinal complications (such as constipation/diarrhea) (1).

Two different autosomal dominant mutations in two unrelated genes cause DM and define two genetically distinct forms of the condition. DM type 1 (DM1) is caused by the expansion of a CTG trinucleotide repeat in the 3'-untranslated region (UTR) of the DM protein kinase (*DMPK*) gene (3). The DM type 2 (DM2) mutation consists in the expansion of an intronic CCTG tetranucleotide in the CCHC-type zinc finger nucleic acid binding protein (*CNBP*) gene (4). Although genetically distinct, DM1 and DM2 share a toxic RNA gain of function mechanism. In both conditions, expanded CUG/CCUG transcripts accumulate in the cell nucleus to form RNA aggregates or RNA foci (4–6), which perturb the function of RNA-binding proteins and a number of downstream events (7). Although clinically similar, disease symptoms are usually milder in DM2 than in DM1 (1, 8).

In a scenario where the expansion of simple non-coding DNA repeats has a broad deleterious impact on multiple tissues and physiological processes the generation of mouse models that faithfully reproduce the disease presents unique challenges. In order to be clinically relevant mouse models must have *construct*, *face* and *predictive* value (9). In other words, relevant mouse models must recapitulate the genetics and molecular pathogenesis (construct value); they must mimic clinical human features, both molecularly and physiologically (face value); and they must provide a platform to determine the effectiveness of new therapeutic interventions on a clinical population (predictive value). However, mouse models rarely, if ever, completely recapitulate all aspects of human disease. This is particularly applicable to DM, given the clinical variability of the disease, the involvement of multiple tissues and the complexity of the underlying molecular pathways. Even with this caveat, mouse models, alone or in combination, have been instrumental to understand fundamental molecular pathomechanisms (10). Importantly, they have allowed molecular and cellular analyses at various developmental stages, as well as in cell types and tissues that are not easily accessible in humans. We have previously reviewed the contribution of mouse models to decipher the grounds of RNA toxicity and to evaluate promising preclinical assays (10), but there is little doubt that mouse models have continued to provide in-depth understanding of DM disease mechanisms over the last years.

Here we discuss how recent mouse data refined our understanding of RNA toxicity and unfolded numerous roles and pathogenic implications of the RNA-binding proteins dysregulated in DM. We review other emerging disease intermediates and dysregulated signaling pathways recently uncovered. Pre-clinical therapeutic developments are

discussed in light of their contribution to reinforce fundamental aspects of disease pathogenesis. We focus primarily on the neuromuscular aspects of the disease to establish correlations between mouse data and human pathology. We point out some contradictory findings between mouse models to illustrate the challenges, complexity and variability of DM disease pathogenesis.

FROM DNA REPEATS TO TOXIC RNA TRANSCRIPTS

The toxicity of RNA repeats was unequivocally demonstrated in HSA^{LR} transgenic mice, through the insertion of an expanded CTG sequence in the 3'UTR of an unrelated gene: the human actin, alpha 1 (*ACTA1*) gene. The expression of CUG-containing *ACTA1* transcripts in mouse skeletal muscle generated genuine myotonia and histological signs of myopathy (11). The elimination of the expanded transcripts by antisense oligonucleotides reduced myotonia in these mice (12), confirming the toxicity of CUG RNA repeats.

The absence of muscle weakness in the HSA^{LR} mouse line that expressed the highest transgene levels and showed pronounced muscle histopathology was intriguing and suggested the dissociation between the toxicity of RNA foci and the etiology of muscle weakness (11), an hypothesis that persisted for some years. However, the later analysis of a second HSA^{LR} line, which also expressed high levels of the transgene and showed myotonia, revealed reduced grip strength (13). Contrary to the initial reports, these findings corroborate the view that the expression of toxic RNA repeats is sufficient to trigger muscle weakness. CUG RNA toxicity was further demonstrated and confirmed in other mouse lines, listed in **Table 1**.

The ubiquitous expression of expanded *DMPK* transcripts from the human DM1 locus resulted in multisystemic phenotypes in DMSXL mice carrying more than 1000 CTG repeats. These phenotypes include reduced muscle strength, lower motor performances, peripheral neuropathy, respiratory impairment, abnormal cognition and behavior, and cardiac conduction defects (26–29). Similarly, the inducible expression of a large, interrupted CTG repeat flanked by the 3'UTR of the *DMPK* gene produced cardiac, muscular and neurological phenotypes in EpA960 mice (16, 30, 31). Surprisingly, high expression of short (CTG)₅ repeats within the *DMPK* 3'UTR was pathogenic in DM5 mice, causing DM1-like myotonia and cardiac conduction defects (17). Hence, the expression of many copies of a short CUG repeat may have functional outcomes that are comparable to the expression of a few copies of large CUG RNA repeats. In other words, the toxicity of repetitive RNA is two-fold: it is determined not only by the sequence length but also by the abundance of the repeat transcripts in the cell. While HSA^{LR}, DMSXL and EpA960 animals accumulate foci, nuclear RNA aggregates were not detected in DM5 mice, raising the possibility that submicroscopic RNA foci can cause disease, or that soluble CUG RNA is also pathogenic (32). The DM1 molecular hallmarks reported in the main poly-CUG mouse

TABLE 1 | Summary of transgene design and expression in the DM mouse models most extensively studied.

| Models of toxic RNA expression (poly-CUG models) | | | | | |
|---------------------------------------------------------|----------------------------------------------------------------------------------------------------|--------------------------------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------|------------|
| Mouse model | (CTG)n | Flanking sequence | Promoter | Tissue expression | References |
| HSA ^{LR} | ~250 | Human skeletal actin 3'UTR | Human <i>ACTA1</i> | Skeletal muscle | (11) |
| DMSXL | > 1,000 | Human DMPK locus | Human <i>DMPK</i> | Ubiquitous | (14, 15) |
| EpA960 | 960 | Human DMPK 3'UTR | CMV | Inducible (ubiquitous or tissue-specific) | (16) |
| DM5/DM200 | 5/200 | Tet-responsive, human <i>DMPK</i> promoter | Human <i>DMPK</i> | Inducible (ubiquitous or tissue specific) | (17) |
| Models of altered RNA-binding proteins | | | | | |
| Mouse model | Mutation/construct | | Tissue expression | | References |
| <i>Mbn1</i> KO | Constitutive deletion of <i>Mbn1</i> exon 3 | | Ubiquitous | | (18) |
| <i>Mbn2</i> KO | Constitutive deletion of <i>Mbn2</i> exon 3 | | Ubiquitous | | (19) |
| <i>Mbn3</i> KO | Constitutive deletion of <i>Mbn3</i> exon 3 | | Ubiquitous | | (20) |
| <i>Mbn1/Mbn2</i> DKO | Constitutive deletion of <i>Mbn1</i> exon 3 Constitutive or conditional deletion of <i>Mbn2</i> | | Ubiquitous deletion of <i>Mbn1</i> . Ubiquitous or tissue-specific deletion of <i>Mbn2</i> | | (21) |
| <i>Mbn1/Mbn3</i> DKO | Constitutive deletion of <i>Mbn1</i> exon 3 Constitutive deletion of <i>Mbn1</i> exon 2 | | | | (22) |
| <i>Mbn1/Mbn2/Mbn3</i> TKO | Constitutive deletion of <i>Mbn1</i> exon 3 Conditional deletion of <i>Mbn2</i> and <i>Mbn3</i> | | Ubiquitous deletion of <i>Mbn1</i> . Tissue-specific deletion of <i>Mbn2</i> and <i>Mbn3</i> | | (23) |
| TRECUGBP1 | Human CELF1 sequence downstream of Tet-responsive CMV promoter | | Inducible (ubiquitous or tissue-specific) | | (24) |
| TRECUGBP2 | Human CELF2 sequence downstream of Tet-responsive CMV promoter | | Inducible (ubiquitous or tissue-specific) | | (25) |

TABLE 2 | Molecular hallmarks of RNA toxicity in the mouse models expressing CUG RNA repeats.

| Models of toxic RNA expression (poly-CUG models) | | | | | |
|---------------------------------------------------------|--------------------------------|----------------------|--------------------------------------------|----------------------------------------------------|--------------|
| Mouse model | RNA foci | MBNL co-localization | CELF1 upregulation | Missplicing | References |
| HSA ^{LR} | Skeletal muscle | MBNL1 | Skeletal muscle | Severe in skeletal muscle | (11, 13, 33) |
| DMSXL | Multiple tissues | MBNL1 MBNL2 | Brain (and CELF2) Trend in heart | Mild, age-dependent in multiple tissues | (26, 27) |
| EpA960 | Skeletal muscle; Heart; CNS | MBNL1 MBNL2 | Skeletal muscle Heart Brain | Severe in skeletal muscle and heart. Mild in brain | (16, 30, 31) |
| DM5/DM200 | Absent | Not detected | Skeletal muscle. Normal levels in heart | Mild in skeletal muscle. Absent in heart | (17) |

models are summarized in **Table 2**. No poly-CCUG DM2 mouse model has been fully characterized yet.

RNA foci are dynamic ribonucleoprotein structures that disrupt important RNA-binding proteins (**Figure 1**). Members of the MBNL (muscleblind-like) family of splicing factors are sequestered and partially inactivated by the RNA foci in DM1 and DM2 (34, 35), while CELF (CUGBP Elav-like family) proteins

are abnormally upregulated, at least in DM1 (16, 36, 37). MBNL sequestration, CELF upregulation and missplicing have been detected to different extents in mouse models expressing poly-CUG RNA transcripts (**Table 2**). MBNL and CELF proteins bind independently to RNA targets and functionally compete to regulate their downstream processing (25). The two protein families comprise key regulators of developmental splicing

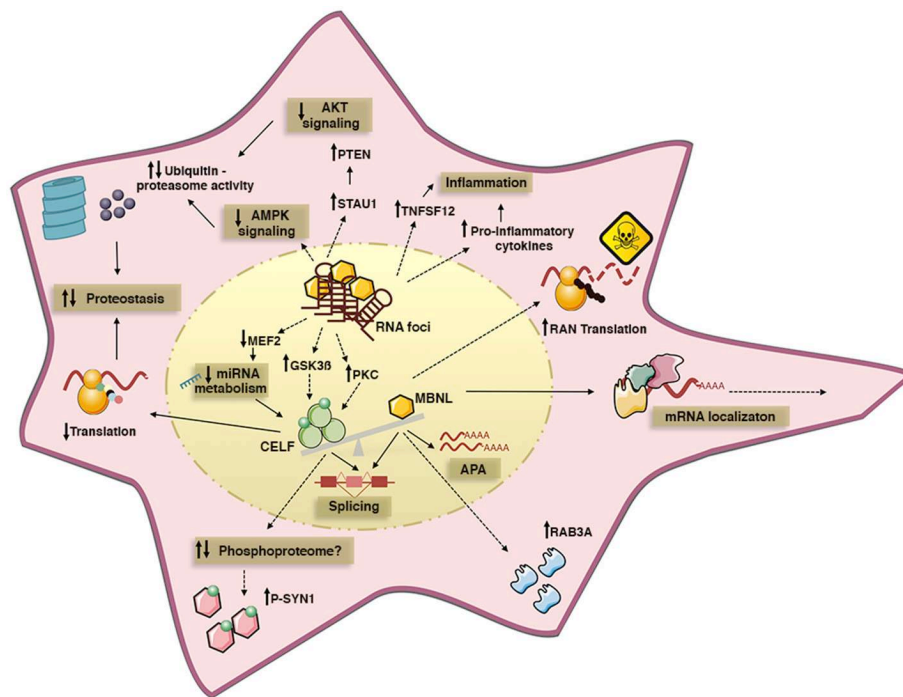


FIGURE 1 | Summary of some of the cell pathways and signaling cascades dysregulated by toxic RNA repeats in DM cells. The expression of toxic RNA transcripts sequesters MBNL proteins into nuclear RNA foci, and upregulates CELF proteins. Different mechanisms may account for CELF upregulation, such as altered PKC and GSK3 β kinase activity, or changes in miRNA levels due to altered MEF2 transcription program. MBNL inactivation and CELF gain-of-function cause pathogenic missplicing. Functional MBNL inactivation alone disrupts alternative polyadenylation and intracellular localization of mRNA targets; it is also believed to dysregulate protein expression, independently of splicing, and to promote RAN translation of toxic peptides. In turn, CELF1 upregulation affects translation efficacy and it may affect the phosphorylation of a subset of proteins through unidentified mechanisms. Protein homeostasis is also perturbed by the downregulation of AKT and AMPK signaling pathways, which likely promotes protein catabolism by increased ubiquitin-proteasome activity, hence contributing to muscle atrophy and weakness. Finally, the increased expression of pro-inflammatory cytokines suggests ongoing inflammation in DM. Solid lines represent well defined disease mechanisms, while dashed lines represent circumstantial data with poorly defined mechanistic links.

transitions. The combined MBNL sequestration and CELF upregulation results in the pathogenic expression of fetal isoforms in adult DM tissues (7). In other words, DM spliceopathy does not produce “unusual” splicing isoforms; instead, it is associated with the expression of normal splicing products that are not well-suited to adult tissue function, leading to the onset of typical disease manifestations. In this context myotonia is the consequence of the abnormal splicing of the CLCN1 chloride channel (38, 39), while insulin resistance is most likely associated with the missplicing of the insulin receptor (38, 40). It is important to note the significant overlap between the splicing abnormalities in DM and other muscular dystrophies (41). The similarities depict a scenario in which splicing dysregulation in DM is not only a primary disease process, but also a secondary event caused by general tissue degeneration.

In addition to the canonical sense transcripts, both DM1 and DM2 loci produce antisense transcripts, a feature shared with many microsatellite repeat loci and that has been suggested to regulate local gene expression (42). The CTG expansion interferes with the relative levels of sense and antisense RNA in DM1 patients (43) and in transgenic mice carrying the human DM1 locus (44). The pathogenic

impact of these changes on local gene expression and disease mechanisms requires further studies in experimental models.

THE MULTIFACETED ROLE OF MBNL PROTEINS IN DM PATHOGENESIS

Humans and mice (as well as most vertebrates) express three *MBNL* genes (*MBNL1*, *MBNL2*, and *MBNL3*) (45). Endogenous *MBNL1* and *MBNL2* co-localize with CUG and CCUG RNA foci in DM1 and DM2 cells, respectively (4, 46–48), whereas *MBNL3* protein was not detected in adult tissues (48).

The three *MBNL* paralogs show differences in spatial distribution in adult mouse tissues. *Mbnl1* and *Mbnl2* transcripts are ubiquitously expressed, but *Mbnl1* RNA levels are higher in heart, whereas *Mbnl2* is more homogeneously distributed (49). The steady-state levels of *MBNL2* protein, however, are low in adult skeletal muscle (19). *Mbnl3* transcript levels are very low in adult mice (49). Differences in protein distribution extend to cell types: the analysis of primary mouse cultures revealed higher relative levels of *MBNL1* in astrocytes, while *MBNL2* was more abundant in primary neurons (50).

The involvement of MBNL proteins in DM was tested in knockout lines generated either through the deletion of *Mbnl* genes alone, or the combined inactivation of multiple *Mbnl* genes (Table 1). These mice revealed some degree of functional specialization between individual members of the MBNL family and clarified their roles in disease molecular pathogenesis.

Functional Specialization of MBNL Proteins: Insight From Single Knockout Lines

Direct evidence of detrimental MBNL sequestration was provided by the generation of *Mbnl1* KO mice. *Mbnl1* inactivation impacted primarily the skeletal muscle and caused pronounced myotonia, but it also resulted in DM1-like subcapsular cataracts, lack of motivation and apathy in knockout mice (18, 51). The impact on cardiac function was less obvious and dependent on the genetic background of *Mbnl1* KO mice: cardiac conduction defects were more pronounced on a homogenous 129/Sv background (52), relative to a mixed 129/Sv x C57BL6 background (21). The reasons behind strain-specific cardiac differences between the homogenous and the mixed background have not yet been resolved, but the comparison between these two lines may provide unique insight into the modifiers of disease severity. It is important to note that DM is a highly variable condition, and that variability in disease manifestations may be explained by a complex interplay between genetic modifiers and environmental factors. The backcrossing of different mouse models onto different genetic backgrounds may facilitate the identification of relevant genetic modifiers of disease.

Although reproducing critical muscular and cardiac phenotypes, *Mbnl1* KO mice did not develop prominent muscle weakness/wasting or marked cognitive deficits, aside from decreased motivation (51). Additional MBNL members may therefore serve as key disease intermediates. Indeed, the inactivation of *Mbnl2* yielded mild muscle pathology, but marked CNS phenotypes, suggesting a tissue-specific impact of *Mbnl* gene inactivation. Neurological phenotypes of *Mbnl2* KO include sleep disturbance, defective spatial memory, abnormal synaptic plasticity and seizure susceptibility (19).

The deleterious effect of *Mbnl1* inactivation on muscle physiology was accompanied by splicing defects that are more severe in skeletal muscle and in heart than in the CNS (18, 52, 53), and it related to the role of MBNL1 in the control of fetal-to-adult splicing transitions in muscle (18, 33). Similarly, MBNL2 appears to serve a similar function in the CNS (19). As a result of this regional specialization, *Mbnl1* KO mice express embryonic splicing isoforms predominantly in the muscle (18, 33), while *Mbnl2* KO mice exhibit embryonic splicing profiles mainly in the CNS (19). Still, we cannot exclude other significant roles of MBNL1 in the CNS independent of splicing: MBNL1 controls the steady-state levels of RAB3A, and possibly other synaptic proteins (27, 54); and it also determines the length of neuronal dendrites and axons (31) (Figure 2).

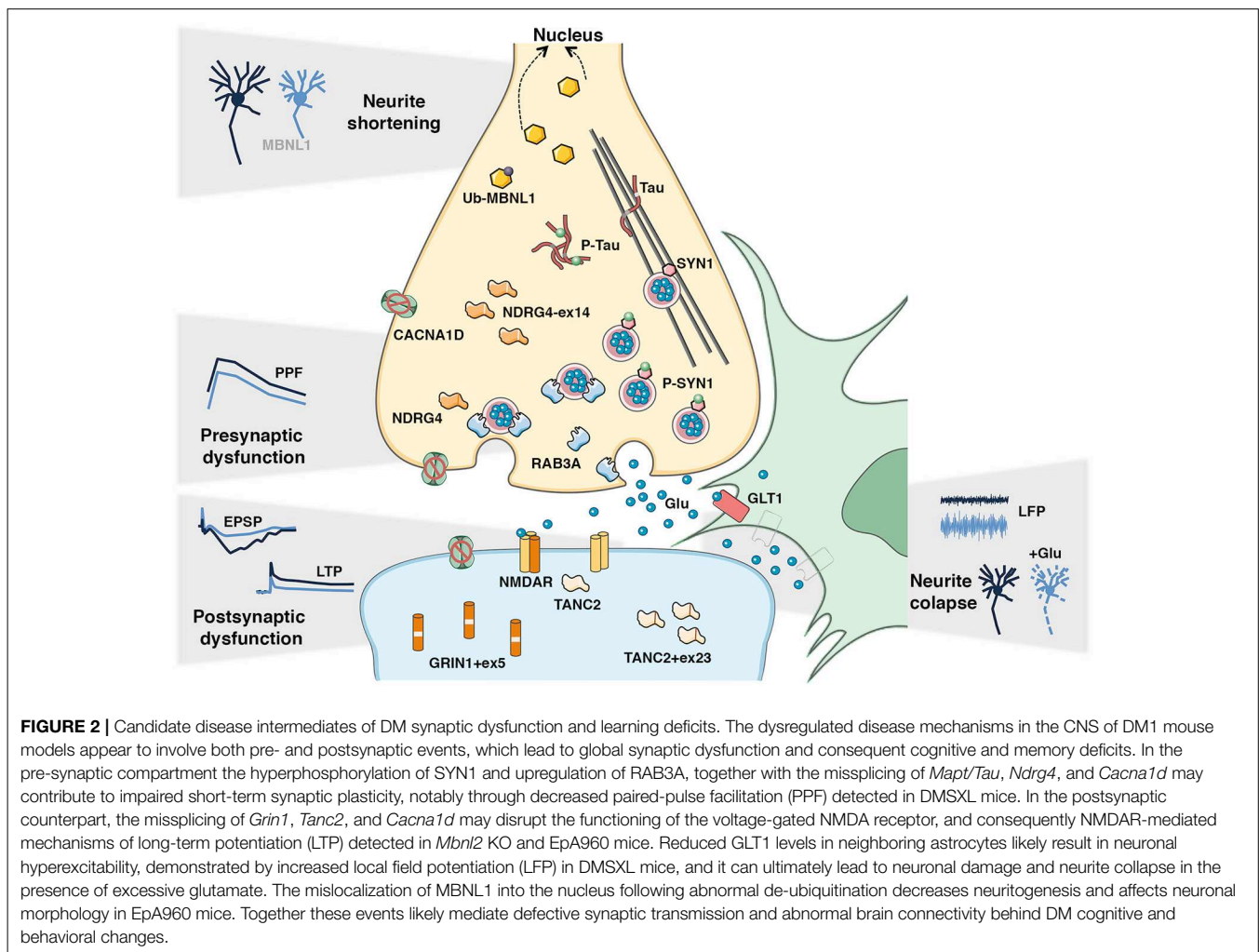
The inactivation of *Mbnl3* yielded intriguing results: despite low *Mbnl3* expression in adult muscle, *Mbnl3* KO mice exhibited reduced grip strength and age-dependent decline in skeletal muscle regeneration (20). Other age-associated phenotypes were described in an independent *Mbnl3* KO line, such as glucose intolerance, cardiac deficits and subcapsular cataracts (55). MBNL3 loss of function may therefore contribute to the accelerated aging suggested in DM (56). Interestingly, the phenotypes of *Mbnl3* KO mice are not accompanied by significant changes in alternative splicing (20, 55). Together with the primary localization of MBNL3 in the cytoplasm (23), these findings predict roles of MBNL proteins other than splicing regulation.

Combined Inactivation of MBNL Proteins

Despite the significant phenotypes of *Mbnl1* and *Mbnl2* single gene knockout lines, they do not model the full disease spectrum, possibly due to compensatory mechanisms of the remaining *Mbnl* genes (21). In *Mbnl1* KO mice, *Mbnl2* expression is upregulated and MBNL2 protein binds to target transcripts that are normally regulated by MBNL1 (21). In order to recreate a situation that resembles more closely the human disease, in which the three MBNL paralogs are sequestered by toxic RNA foci (35), compound knockout mice were generated (Table 1). While *Mbnl1/Mbnl2* double knockout (DKO) mice were embryonic lethal, the inactivation of one *Mbnl2* copy in a *Mbnl1* KO background was sufficient to exacerbate myotonia and trigger muscle weakness, loss of mature neuromuscular junctions and cardiac conduction defects, which were absent in single *Mbnl1* KO mice (21, 57). The aggravated phenotypes were accompanied by an increasing severity in spliceopathy and significant changes in alternative polyadenylation (APA) (58). The molecular analysis of *Mbnl1/Mbnl2* DKO mice was instrumental to reveal the role of MBNL proteins in the regulation of APA: MBNL proteins and the APA machinery compete to bind to APA sites of a subset of transcripts, in a mechanism that regulates the processing and length of the 3' end of target transcripts, with subsequent implications for their stability and localization (Figure 1). Similar to DM splicing abnormalities, the sequestration and functional inactivation of MBNL proteins by toxic RNA results in the persistence of fetal APA profiles in adult muscle and brain of DM1 and DM2 patients (58, 59). The direct contribution of individual APA defects to specific symptoms is unclear (60), but the mouse models available offer unique tools to address this question, not only in DM but also in other conditions in which MBNL proteins are sequestered by toxic RNA repeats.

Dual depletion of *Mbnl1* and *Mbnl3* also enhanced myotonia, muscle weakness and myopathy in skeletal muscle (22). However, in contrast to *Mbnl1/Mbnl2* DKO, increased myotonia was not associated with a greater extent of splicing dysregulation in *Mbnl1/Mbnl3* DKO mice. Instead, enhanced myotonia was the result of the synergy between *Clcn1* missplicing caused by *Mbnl1* deletion alone, and defective CLCN1 translation, caused by combined inactivation of MBNL1 and MBNL3 proteins (22).

More recently, conditional triple knockout (TKO) mice were generated by muscle-specific deletion *Mbnl2* and *Mbnl3* on an



Mbnl1 knockout background (Table 1). *Mbnl1/Mbnl2/Mbnl3* TKOs present high neonatal mortality, growth defects, respiratory distress, muscle weakness and wasting in association with pronounced splicing and gene expression defects (23). Interestingly, the total spliceopathy in muscle was only modestly increased in *Mbnl1/Mbnl2/Mbnl3* TKO mice, relative to *Mbnl1/Mbnl2* DKOs, supporting the view that congenital spliceopathy is primarily due to compound loss of MBNL1 and MBNL2, and further pointing to MBNL3 functions, other than splicing regulation. Hence, the congenital form of the disease seems to require combined inactivation of the three *Mbnl* paralogs from an early developmental stage. DMSXL mice, which also show growth retardation from birth, and DM1 individuals express both sense and anti-sense *DMPK* transcripts from embryonic and fetal stages (44), confirming that the toxic RNA mechanisms behind congenital cases could operate early on during development.

Overall the generation and characterization of single and compound *Mbnl* KO mouse models, demonstrated that the simultaneous sequestration of various MBNL proteins is instrumental for the development of clinical manifestations of

DM. Given the sparse availability and technical difficulties of working with human DM tissue, constitutive and conditional *Mbnl* KO mice grant the possibility to investigate abnormal RNA processing in different tissues, cell types and developmental stages.

Insight From MBNL Replacement Strategies

MBNL1 loss of function accounts more than 80% of missplicing events and nearly 70% of expression defects in the skeletal muscle of HSA^{LR} mice (61, 62), strongly anticipating the benefits of therapeutic gene replacement. Overexpression of MBNL1 through viral infection or genetic manipulation ameliorated myotonia and splicing abnormalities in the tibialis anterior of HSA^{LR} mice, however it was insufficient to fully correct muscle histopathology (63, 64). While further confirming the role of *Mbnl1* loss of function in the onset of myotonia, these findings also hint at the involvement of additional disease intermediates in muscle pathology. It is conceivable that other MBNL proteins might be necessary to fully reverse muscle phenotypes. Prior to the further development of MBNL

replacement strategies, it is important to evaluate to which extent MBNL proteins are interchangeable and capable to functionally replace each other in muscle and in other tissues of DM1 mouse models expressing expanded CUG transcripts. Alternatively, the incomplete rescuing of muscle physiology in HSA^{LR} mice by MBNL1 overexpression points to the involvement of other families of disease intermediates alongside MBNL proteins.

Cytoplasmic Roles of MBNL: RNA Trafficking and Proteotoxicity

MBNL proteins are also present in the cytoplasm (23, 48), where they likely regulate mRNA stability (61, 62, 65), as well as the intracellular localization of mRNA transcripts through binding to the 3'UTR of their targets (66, 67). The role of MBNL proteins in mRNA trafficking might be particularly relevant in highly polarized brain cells, such as neurons. Altered MBNL activity or intracellular localization in DM1 could be detrimental for correct transport of mRNAs toward specialized cell compartments (such as axons, dendrites and synapses), which would subsequently affect local translation and ultimately cell function.

In further support of a cytoplasmic function of MBNL proteins, the expression of CUG RNA in the forebrain of EpA960 mice (Table 1) affects MBNL1 ubiquitination and distribution between the nucleus and the cytoplasm (Figure 2), prior to the shortening of neuronal dendrites and axons (31, 68). Morphological impairments occurred in the absence of missplicing, suggesting a contribution of cytoplasmic MBNL1 to disease process.

MBNL proteins have been recently proposed to act as guardians against proteotoxicity. CUG and CCUG RNA generate toxic peptides through non-conventional repeat-associated non-ATG (RAN) translation (69, 70). The combination of bidirectional transcription with RAN translation of expanded repeats produces multiple toxic species, which co-localize with markers of apoptosis, supporting a role in disease pathology (71). Interestingly, RNA accumulation seems to co-exist and exacerbate RAN translation in the same cell, in a mechanism mediated by MBNL proteins: MBNL sequestration and inactivation by nuclear RNA foci promotes RAN translation in DM1 and DM2 cell models (70, 72). RAN products have been reported in DMSXL mice (69). Future mouse studies are required to elucidate the relationship between RNA foci, MBNL protein and RAN translation, and the pathogenicity of RAN peptides in multiple tissues and cell types.

CELFB PROTEINS: SPLICING, TRANSLATION AND DM PATHOGENESIS

CELF1 upregulation correlates with muscle histopathology in DM1 patients and DM5 transgenic mice (Table 1) (73), pointing to a direct role of CELF1 in disease pathogenesis (Figure 1). The upregulation of CELF1 in DM2 skeletal muscle is contentious, with conflicting reports of normal and increased protein levels (33, 74–76).

To directly address the role of CELF1 gain of function, overexpressing mice were generated. Ubiquitous CELF1

upregulation resulted in severe developmental phenotypes and muscle histopathology (77), which correlated with transgene expression levels (77, 78). The high mortality of these mice limited their face and predictive value. Conditional mouse lines were more informative, since they offered the opportunity to focus on individual tissues and assess the pathogenic contribution of CELF protein overexpression alone (Table 1). Induction of CELF1 transgene expression in mouse skeletal muscle was sufficient to reproduce muscle wasting, defective motor performance and myopathy (79); while CELF1 upregulation in heart caused cardiac conduction defects, cardiomyopathy with hypertrophy and early mortality (24). As expected, muscular and cardiac phenotypes were accompanied by missplicing events in muscle and heart, respectively (24, 79). A splicing-mediated effect was further supported by the expression of a dominant-negative *Celf1* variant in HSA^{LR} mice, which partially corrected missplicing in skeletal muscle (80).

CELF proteins can also regulate the alternative splicing of transcripts involved in neuronal function (81). Therefore, the upregulation of CELF1 and CELF2 reported in human DM1 brains (27, 82) may have a substantial contribution to the etiology of neurological dysfunction. Conditional overexpressing models could help investigate the cognitive and behavior consequences of CELF1 or CELF2 upregulation, and identify subsets of transcripts that specifically respond to these two RNA-binding proteins. In support of target discrimination between CELF proteins, it was shown that the splicing of *MAPT* exon 10 responds specifically to CELF2, but not to CELF1 upregulation (82).

Although already generated, CELF2-overexpressing mice have only been used in molecular approaches to study the antagonistic role of MBNL and CELF proteins in splicing regulation (25). The phenotypic consequences of CELF2 overexpression have not been reported yet.

Cytoplasmic Functions of CELFB Proteins

In addition to regulating alternative splicing in the nucleus, CELFB proteins have cytoplasmic roles in the regulation of mRNA stability, translation and deadenylation (83). The distribution of CELF1 between the nucleus and the cytoplasm is regulated by AKT phosphorylation (84). As a result, CELFB gain of function in DM1 has intricate consequences that affect multiple cellular pathways in different cell compartments.

CELF1 activity is controlled by multiple phosphorylation events. The role of CELF1 in translation depends on the phosphorylation of Serine-302: phosphorylated CELF1 acts as an activator, while unphosphorylated CELF1 represses translation (84, 85). In DM1, the increase in the total levels of CELF1 is accompanied by the elevation of both phosphorylated and unphosphorylated forms of CELF1 at Serine-302 (40, 86, 87). This results in the reprogramming of protein translation, altered proteostasis and global cell stress, which ultimately affects cell function (75, 78, 84).

DM5 mice have corroborated the cytoplasmic functions of CELF1 in DM1 pathogenesis. The genetic inactivation of *Celf1* in DM5 mice did not mitigate missplicing, but instead corrected the expression of CELF1 translational targets in skeletal muscle (73); a tissue that shows CELF1 upregulation

in DM5 mice (17). The molecular changes were sufficient to improve motor performance, grip strength and histopathology, but left myotonia unchanged (73). These results demonstrate the pathogenic relevance of CELF1-regulated translation, and point to CELF1-independent myotonia mechanisms in DM. Interestingly, in line with the absence of CELF1 upregulation in DM5 hearts (17), *Celf1* deletion did not ameliorate the cardiac function (73).

Finally, it is worth noting that CELF1 overexpression alone is associated with the hyperphosphorylation of Synapsin-1 in cell culture (27) (Figure 2), suggesting a contributing role of this RNA-binding protein in the regulation of the phosphoproteome.

Mechanisms of CELF Upregulation and Therapeutic Strategies

CELF1 upregulation in DM1 operates at protein level, since transcript load remains unchanged in skeletal muscle (73). In the heart of DM1 patients and induced EpA960 mice, upregulation correlates with CELF1 protein hyperphosphorylation, higher protein stability and increased PKC activity (87) (Figure 1). Consistent with a direct role of PKC in CELF1 metabolism, treatment of EpA960 mice with PKC inhibitors immediately after transgene induction avoided CELF1 upregulation, reduced mouse mortality and improved cardiac function (88). These findings provided pharmacological evidence of the involvement of PKC in CELF1 function and in DM1 cardiac phenotypes. Surprisingly, the genetic inactivation of *Pkc* did not lower CELF1 expression or correct histopathology in the skeletal muscle of DM5 mice (89).

Different reasons may account for the differing outcomes of CELF1 results obtained with EpA960 and DM5 mice. First, the inherent differences between the two mouse models: while in EpA960 interrupted large repeats are expressed under the control of a non-*DMPK* promoter (16), DM5 mice express short (CUG)₅ RNA repeat in multiple tissues and cell types under the control of the human *DMPK* promoter (17). Second, it is conceivable that the molecular mechanisms of CELF1 upregulation differ between heart and skeletal muscle: CELF1 upregulation in the skeletal muscle might be independent of PKC. Third, off-target effects of kinase inhibition might have introduced confounding factors in the analysis. Indeed, the PKC inhibitor Ro 31-8220 used in EpA960 mice has since then been found to reduce RNA foci, release MBNL1 and correct MBNL1-dependent splicing events in a cell model of DM1 (90). Furthermore, Ro 31-8220 can also inhibit other kinases, including GSK3 β (91).

Experimental evidence of the role of GSK3 β in DM1 muscle pathology was obtained in HSA^{LR} mice: GSK3 β inhibition restored CELF1 protein levels and translational activity, improved muscle strength and corrected histopathological changes (13, 92). The possibility remains that different aspects of CELF1 metabolism are controlled by different phosphorylation events: phosphorylation by PKC increases protein stability (87, 92), GSK3 β controls the translational activity of CELF1 (13) and AKT regulates the nucleus-cytoplasm distribution of CELF1 (84) (Figure 1). We currently do not know the molecular link between the repeat expansion and altered kinase activity.

Kinase-independent mechanisms of CELF1 upregulation have been proposed. Under physiological conditions, CELF1 protein decreases in adult mouse tissues in response to a developmental increase in a subset of microRNA (miRNA) species. Transgene induction in EpA960 revealed that CUG RNA toxicity disrupts the MEF2 transcription network, lowers miRNA expression reversing the physiological miRNA developmental program and causing CELF1 upregulation (93). Therefore altered levels of miRNA in DM1 tissues could explain CELF1 upregulation.

To discard the possibility of a direct or indirect regulation of CELF1 by MBNL proteins, CELF1 expression was measured in *Mbnl1* KO mice, and revealed no changes (18). In contrast, induction of CELF1 over-expression in transgenic mice yielded MBNL1 upregulation, possibly mediated by tissue regeneration (79).

Despite progress in the understanding of the multifaceted metabolism of CELF1 in DM1, the jury is still out on the molecular mechanisms of upregulation in DM1 and the extent of the therapeutic benefits of CELF targeting in tissues, other than the heart. The mechanisms behind CELF2 upregulation in the CNS of DMSXL mice are less clear (27). Useful mouse models are available to address these questions (Table 1 and Table 2), through pharmacological or genetic manipulation of CELF1 and CELF2 levels, as well as the activity of candidate kinases and miRNA metabolism.

UNRAVELING DISEASE INTERMEDIATES AND PATHWAYS BEHIND NEUROMUSCULAR PATHOLOGY

Additional layers of DM1 molecular pathogenesis, beyond the canonical involvement MBNL and CELF RNA-binding proteins, have emerged from recent mouse studies of muscle and heart phenotypes. Hereditary myotonia is usually caused by the malfunction of ion channels (94). In line with this view, compelling evidence has demonstrated the direct role of *CLCN1* chloride channel missplicing in the onset of DM1 and DM2 myotonia (39, 63). The mechanisms behind muscle weakness/wasting and cardiac dysfunction can be more diverse, and mediated by a combination of interacting intermediates. In this section we first discuss some critical splicing events, whose contribution to muscle and heart pathology has been corroborated by mouse studies. Then we review the emerging role of additional pathways, whose mechanistic link with MBNL and CELF canonical disease intermediates has not yet been elucidated and deserves further attention.

The Role of Missplicing in Muscle and Heart Disease: Many Roads Leading to Rome

Progressive muscle weakness and wasting are among the most prominent clinical features of DM1, in association with centralized nuclei and myofiber atrophy, without overt regeneration, fibrosis or necrosis (1). Previous studies have shown associations between muscle weakness and MBNL1-dependent splicing of *BINI* (95), *CACNA1S* (96) and *DMD*

(97). The recreation of the DM1 missplicing of *Bin1*, *Cacna1s* or *Dmd* in wild-type mice, through RNA antisense technology, corroborated the contribution of these events to muscle weakness and myopathy (95–98). However, it is still unclear if the combined inactivation of multiple MBNL proteins is the sole responsible for muscle weakness. Elevation of CELF1 protein may certainly play a determinant role too, as suggested by the muscle phenotype of CELF1-overexpressing mice (30) and by the improved muscle strength following CELF1 downregulation in HSA^{LR} mice (13). Some CELF1-responsive splicing events may provide connecting dots in the mechanisms of muscle pathology: while *RYR1* missplicing alters excitation-contraction coupling in skeletal muscle (99), the shift of *PKM* splicing to an embryonic isoform results in less efficient energy production, likely associated with muscle weakness and wasting (98).

An expected role for splicing dysregulation has also been suggested in DM heart disease. In spite of the confirmed contribution of MBNL1/MBNL2 loss of function (21) and CELF1 upregulation toward cardiac conduction defects (24), the downstream disease intermediates remained elusive. MBNL1-dependent missplicing of *SCN5A* was found in the heart of DM1 patients and *Mbnl1/Mbnl2* DKO mice. When the DM1 splicing isoform is expressed in wild-type mice, it causes DM1-like cardiac conduction defects and arrhythmias (100). The influential role of *SCN5A* does not rule out the contribution of other yet unidentified splicing events that may reinforce heart spliceopathy and aggravate cardiac disease in DM.

Cellular Energy Sensors, Proteasome Activity and Muscle Weakness

The RNA binding protein Staufen1 is significantly upregulated in DM1 muscle biopsies, in the absence of missplicing of the corresponding transcript, and it correlates with disease severity (101). Sustained expression of Staufen1 in the skeletal muscle of overexpressing transgenic mice causes muscle weakness and myopathy, characterized by an increase in the frequency of small fibers and central nuclei. Staufen1 impairs muscle differentiation through enhanced translation of c-myc (102), which in turn upregulates the transcription of the *PTEN* tumor suppressor gene and ultimately inhibits downstream AKT signaling (103). The AKT pathway promotes cell survival, proliferation and growth and mediates cell metabolism, transcription and translation in response to extracellular stimuli and changes in energy balance (104). The increased expression of atrogenes in Staufen1-overexpressing mice was linked to AKT signaling inactivation and *PTEN* upregulation, which interfere with the activity of the ubiquitin-proteasome system to promote catabolic protein degradation, which likely contributes to the muscular phenotypes (103). In further support of elevated protein degradation in DM1 muscle weakness and myopathy, DMSXL mice show enhanced proteasome activity in association with muscle weakness and myopathy (26, 105).

The dysregulation of the adaptive switch between catabolic and anabolic states in DM may extend beyond AKT missignaling, and encompass other intermediates. Maintaining an adequate supply of energy is an essential requirement for cell function,

notably in muscle and CNS, which depends on the cross talk between AKT and AMPK signaling pathways (104). Interestingly, the activation of AMPK signaling is also impaired in the skeletal muscle of HSA^{LR} mice following fasting (106), corroborating the idea that DM perturbs cell master sensors of energy balance. Importantly, pharmacological treatments to normalize this pathway improved muscle strength and corrected myotonia in these mice (106). Although these data suggest a role of the AMPK cascade in DM1 muscle pathology, it was also noted that the pharmacological activation of AMPK reduced RNA foci in HSA^{LR} mice. Hence, it is possible that rather than a direct role on the etiology of muscle pathology, AMPK dysregulation perturbs the dynamics of CUG RNA, stabilizes foci and accentuates spliceopathy, thereby aggravating muscle manifestations. Conversely, the AMPK activator alone may simply destabilize RNA foci and lead to an amelioration of mouse phenotypes through a restoration of splicing.

Given the role of Staufen1 in neuronal dendrite arborization and synaptic development (107), it will be of interest to study the implication of Staufen1 in the neurological deficits of DM1. Both AKT and AMPK signaling pathways are implicated in multiple aspects of brain development and function, and their dysregulation has been associated with neurological disease (104, 108). Their role in DM may, however, be restricted to muscle, since no altered AKT/AMPK signaling activity was detected in DM1 neural stem cells (109). Nonetheless, these results must be confirmed in relevant DM mouse models of brain dysfunction.

A Role for Inflammation in Muscle Pathology

Tumor necrosis factor superfamily member 12 (TNFSF12) was found upregulated in the skeletal muscle of DM5 and DM200 mice (Table 1), shortly after transgene induction and prior to the onset of muscle pathology (110). Genetic deletion of *Tnfsf12* or the inhibition of the downstream signaling cascade by anti-TWEAK antibodies improved the muscle strength of DM5 mice, demonstrating the physiological relevance of TWEAK signaling in DM1. The binding of TWEAK to its receptor, TNFSF12, regulates cell proliferation, differentiation, inflammation and apoptosis (111). In muscle, the TWEAK-TNFSF12 complex becomes particularly engaged in response to disease, triggering the activation of pro-inflammatory responses that can contribute to DM1 myopathy (110). Further support of ongoing inflammation in muscle was provided by global analysis of gene expression in congenital DM1, which revealed significant upregulation of pro-inflammatory genes (112).

It is conceivable that muscle weakness and atrophy in DM1 is multifactorial process, resulting not only from simultaneous dysregulation of splicing, unbalanced protein synthesis/degradation, but also inflammation.

Changes in miRNA Levels: Defective Transcription or Maturation?

miRNA profiling revealed significant changes in the heart (113), skeletal muscle (114–118) and serum (119) of DM1 and/or DM2 patients. Despite the divergence of some of the results reported,

miRNA dysregulation emerged as a disease feature, which could either be a direct consequence of RNA toxicity, or a lateral event secondary to altered cell physiology. The investigation of miR-1 dysregulation favored the former. Mature miR-1 appears to be downregulated in DM1 and DM2 hearts, in association with an expected increase in miR-1 downstream targets: the upregulation of GJA1 (connexin 43) gap junction protein and CACNA1C calcium channel might subsequently contribute to heart phenotypes (113). In an effort to shed light onto the mechanisms of miR-1 misregulation, MBNL1 knocking down in cell culture blocked the maturation of pre-miR-1, which suggested a role of MBNL1 in miRNA processing and biogenesis, in agreement with the normal or elevated levels of pre-miR-1 found in DM1 and DM2 patients, respectively (113). However, this hypothesis is at odds with subsequent findings. First, miR-1 remained unaltered in *Mbnl1* KO mice (93). It is possible that MBNL2 upregulation in these mice (21), which compensates for the lack of MBNL1, could avoid miR-1 downregulation. To answer this question it would be important to study miR-1 levels in *Mbnl1/Mbnl2* DKO. Second, global analysis of miRNA species revealed that CUG-associated changes occurred already at the precursor stage in the induced EpA960 mouse model, arguing against a primary defect in subsequent miRNA processing and maturation. Instead, these results were consistent with defects in miRNA transcription and were attributed to the dysregulation of the MEF2 transcriptional program (93). It is possible that the high expression levels of the expanded (and interrupted) transgene in EpA960 mice trigger severe molecular defects and more pronounced dysregulation of miR-1 transcription, upstream from processing and maturation, relative to DM1 and DM2 patients. Finally, recent findings on CELF1-overexpressing mice did not fully match previous results in human tissue either. In contrast with the upregulation of miR-1 targets reported in DM1 and DM2 hearts (113), GJA1 protein levels decrease in the heart of CELF1-overexpressing mice (120). The discrepancy between patients and these mice might be explained by a combined effect of the heterogeneous regional distribution of GJA1 in disease hearts, and the study of different disease stages: GJA1 levels may show an initial compensatory increase during the early adaptation disease stages studied in human samples (113), followed by a late decrease during maladaptation disease stages, like in CELF1-overexpressing mice (120). Further studies are required to clarify these questions and to extend the implications of miRNA metabolism to other affected tissues, notably the CNS.

DM1 Cardiac Function: Revisiting DMPK Loss of Function

The sequestration of expanded *DMPK* RNA in the nucleus of DM1 cells causes a 50% reduction in protein levels (121). Initial reports suggested a role of *DMPK* haploinsufficiency in disease etiology, a hypothesis corroborated by a dose-dependent effect in mouse heart: the deletion of one copy of the murine *Dmpk* gene was sufficient to disrupt cardiac conduction (122, 123). In contrast, late and mild myopathy in skeletal muscle required full deletion of both *Dmpk* copies in knockout mice (124). These early findings suggest that therapeutic hopes aiming to eliminate

DMPK transcripts may aggravate some aspects of the disease pathology, particularly in heart. In this context, it is worth reviewing our actual knowledge on the contribution of *DMPK* protein to disease.

The recent re-evaluation of the impact of *Dmpk* deletion in knockout mice, bred onto homogeneous genetic backgrounds, showed no functional impact on cardiac or skeletal muscle, thereby excluding a role of *DMPK* loss of function in muscle phenotypes (125). The reasons behind the diverging results relative to early findings may relate to the strain background and the role of unidentified modifiers. Alternatively, the differences may relate to the replacement strategy used to inactivate the *Dmpk* gene, which might have interfered with the expression of flanking genes in the knockout lines previously generated (125). In summary, these data provide evidence of the limited functional impact of *DMPK* inactivation on heart and skeletal muscle, and validate the anti-sense therapies being developed, which are discussed below. Nonetheless, the role of *DMPK* protein in the CNS, as well as in other tissues, needs to be further explored.

DM MOUSE MODELS OF NERVOUS SYSTEM DYSFUNCTION

The burden of CNS dysfunction has shifted DM research from an initial focus on muscle pathology, to the investigation of brain disease mechanisms. Sophisticated imaging techniques have characterized structural and metabolic abnormalities in human brains (2, 126). Molecular studies have also been performed in the nervous system, but they rely on samples collected at the end-stage of the disease. Animal models overcome this critical limitation, as they provide tissue samples throughout disease progression, offering the possibility to characterize molecular, cellular and electrophysiological changes in the nervous system prior to the onset of disease symptoms. In this section we critically review relevant neurological phenotypes of various DM mouse models, and the insight they provide to the understanding of disease mechanisms in the central and peripheral nervous system.

The Expression of Toxic RNA in the CNS

Two DM1 mouse models express large CUG RNA transcripts in the CNS: the ubiquitous DMSXL line and the inducible EpA960 mice (Table 1). Both DMSXL and forebrain-induced EpA960 mice show impaired spatial learning and memory in the Morris Water Maze, resembling the visuoconstructive defects in DM1 patients (27, 31). DMSXL mice have also shown signs of anhedonia and novelty inhibition of exploratory activity (27). The electrophysiological profiling of the hippocampus revealed synaptic dysfunction behind these phenotypes: while DMSXL mice show impaired short-term paired-pulse facilitation (27), suggestive of pre-synaptic dysfunction; EpA960 exhibit reduced long-term potentiation (LTP) (31), which is more often associated with post-synaptic abnormalities (Figure 2). The diverging effects on pre- and post-synaptic neuronal plasticity between the mouse lines may be accounted for, at least partly, by their intrinsic differences: DMSXL mice express pure CUG repeats in multiple brain cell types from an early embryonic

stage; while induced EpA960 mice express higher levels of interrupted CUG repeats post-natally, in the neurons of the forebrain (Table 1).

Typical RNA foci accumulation and co-localization with MBNL1 and MBNL2 were detected in various cell types of DMSXL brains (27) and in EpA960 neurons (31). Still, both lines showed only limited spliceopathy (27, 31). In contrast, *Mbnl2* KO and *Mbnl* DKO displayed more pronounced splicing dysregulation, which may contribute to impaired LTP and spatial learning of *Mbnl2* knockout mice (19, 59); the missplicing of *Grin1* may reduce dendritic localization of the glutamate receptor, which may be further aggravated by *Tanc2* abnormalities (127, 128); while *Cacna1d* and *Ndr4* misregulation might impair neuronal activity and learning (129, 130) (Figure 2). Other MBNL-dependent pathways may, however, contribute to brain disease, such as defects in APA (59) and changes in the expression and phosphorylation of synaptic proteins (27, 50, 54).

MAPT/Tau protein has long been associated with DM1 brain disease. Abnormal MAPT isoform distribution was first described at the protein level (131), in association with the intranuclear accumulation of hyperphosphorylated protein fibers, or tangles in patients (Figure 2). Abnormal missplicing was later described in patients (46, 132) and in the brain of DMSXL mice (27). The pronounced *Mapt* RNA missplicing in *Mbnl1/Mbnl2* DKO indicates the critical role of the spliceopathy resulting from the dual loss of these two RNA-binding proteins (59). The DM1 tauopathy has been suggested to interfere with axonal transport and neurosecretion (133), but further animal studies are required to decipher the mechanisms.

REGIONAL DISTRIBUTION OF DM PATHOLOGY IN THE BRAIN

Imaging and neuropsychological assessment have uncovered candidate brain regions primarily affected by DM. The identification of critical brain areas will be important to direct future therapies toward the most relevant brain territories, and it will likely depend on an intricate interplay of factors, such as somatic repeat length, levels of toxic RNA, foci abundance and the activity of RNA-binding proteins. A small number of studies has investigated repeat instability (132) and *DMPK* gene expression in different brain areas in a limited number of human patients (134). DM1 mice offer the possibility to surmount the limited availability of human tissue and perform more detailed analyses. Transgenic DM1 mice expressing ~500 CTG repeats under the control of the human *DMPK* promoter and the regulatory regions of the DM1 locus (14) showed age-dependent accumulation of larger repeat sizes in most brain regions (Figure 3). The semi-quantitative results did not reveal brain regions with exceptionally high somatic mosaicism, in which we could anticipate the accumulation of very long CUG repeats. The cerebellum, however, exhibited lower levels of somatic instability, as reported in humans (135) and in another model of CTG repeat instability (136). The average repeat size in the cerebellum was nonetheless within the disease-associated

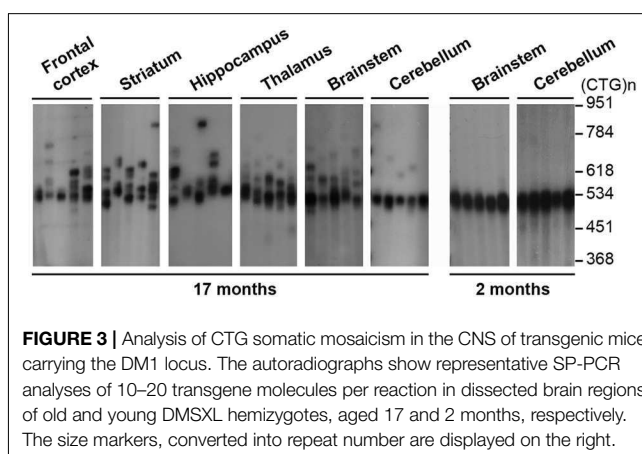


FIGURE 3 | Analysis of CTG somatic mosaicism in the CNS of transgenic mice carrying the DM1 locus. The autoradiographs show representative SP-PCR analyses of 10–20 transgene molecules per reaction in dissected brain regions of old and young DMSXL hemizygotes, aged 17 and 2 months, respectively. The size markers, converted into repeat number are displayed on the right.

range. It is possible that future analyses of somatic repeat instability in smaller brain areas or individual cell types of these mice will reveal susceptible cell populations that accumulate significantly longer repeat expansions.

Similarly, the expression levels of the *DMPK* transgene showed modest variation between CNS regions (54). In contrast, RNA foci were not homogeneously distributed and accumulated preferentially in the frontal cortex and certain areas of the brainstem of DMSXL mice (26, 54), and they appeared to be more abundant in cortical astrocytes relative to neurons (27). The analysis of well-defined histological layers of the mouse cerebellum has also shown greater foci accumulation and more severe spliceopathy in Bergmann astrocytes, relative to the neighboring Purkinje cells (50). Together these findings demonstrate more pronounced pathologic events in defined brain cell populations and cell types, a view further supported by the preferential accumulation of anti-sense RAN-translated products in the oligodendrocytes of DM2 brains (70).

The factors governing the distribution of DM pathology in the brain remain elusive and must be addressed in future mouse studies, but variations in the expression of MBNL and other RNA-binding proteins between brain regions (54) and cell types should be considered (50).

THE ROLE OF GABA, GLUTAMATE AND GLIA IN DM1 NEURONAL HYPEREXCITABILITY

While waiting for efficient gene therapy to correct the causing genetic defect (the DNA repeat expansion) or neutralize the pathogenic molecule (the toxic RNA), one can imagine pharmacological means to ameliorate or prevent progression of neurological symptoms. Such strategies require comprehensive characterization of neuronal activity and integrative brain dysfunction.

Perturbed balance between excitatory and inhibitory neurons disrupts cognition in neurological diseases. Several mouse studies favor a scenario of neuronal excitability in DM1. Both DMSXL and *Mbnl2* KO mice present elevated

susceptibility to PTZ-induced seizures, suggesting GABA-mediated hyperexcitability (19). In line with elevated neuronal excitability, *Mbnl2* KO mice show augmented responsiveness to intracortical train stimulation, in a mechanism partially mediated by abnormal glutamate neurotransmission (137). Reduced expression of the glial GLT1 glutamate transporter in DMSXL brains is associated with elevated neuronal firing *in vivo* (50) (Figure 2). This finding supports a role of defective glutamatergic transmission and neuronal excitability in DM1, mediated by abnormal neuroglial interactions. Neuronal hyperexcitability is a frequent cause of epilepsy. Although epileptic episodes are rare in DM, patients present high sensitivity to GABA agonists (138) as well as abnormalities in glutamatergic transmission in the frontal lobe (139).

In addition to GABA and glutamate, circumstantial evidence points to the involvement of other signaling molecules. HPLC quantification revealed region-specific defects in dopamine and serotonin neurochemicals in DMSXL brains, in association with high foci content in dopaminergic and serotonergic brain centers (27). Importantly, DM1 brains have shown loss of neurons signaling through these two types of neurotransmitters (140, 141).

Today optogenetics allows the neuronal manipulation of neuronal circuits *in vivo*. In combination with electrophysiology, imaging and behavior assays, these techniques can provide insight into the contribution of neuronal activity to the cognitive performance of DM mice, and elucidate the neuronal circuits most profoundly affected by the disease.

Structural, Developmental and Functional Features of DM Brains: Insight From Mouse Models

The brain structural changes found in DM1 and DM2 are mainly characterized by white matter hyperintensities, some general atrophy and dispersed gray matter reduction across the four cortical lobes, the basal ganglia, and cerebellum. Importantly, white matter abnormalities correlate with disease duration and cognitive deficits (2, 126). Functional imaging revealed low glucose uptake and cerebral hypoperfusion, as well as abnormal connectivity patterns that correlate with atypical personality traits and executive dysfunction (142, 143). The correlation between imaging data and neuropsychological profiles hints to the involvement of complex neuronal networks, through defective neurodevelopment, neurodegeneration or neurodysfunction. Today we still do not know the contributing weight of each of these components to DM brain disease. The molecular and histological mouse studies have shed some light on this question.

Higher expression of embryonic splicing isoforms in the brains of DMSXL (27), *Mbnl2* KO and *Mbnl1/Mbnl2* DKO mice (19, 59) points to a disrupted developmental program. In contrast, the dysregulation of synaptic proteins does not recreate embryonic events (54), supporting functional deficits in DM brains, rather than a developmental delay.

Inducible EpA960 mice have recently given further insight. Transgene induction in adult forebrain (after the completion

of CNS development) yielded progressive loss of axonal and dendritic integrity, together with brain atrophy (31)—a sign of ongoing neurodegeneration in adults, possibly in line with the reported premature and accelerated cognitive decline in DM1 patients (144). However, the EpA960 mouse data do not exclude developmental disruption, should toxic RNA be expressed during early embryonic stages.

Understanding the contribution of defective development, neurodysfunction and neurodegeneration is critical to design therapeutic schemes: we must intervene prior to the establishment of irreversible developmental defects, irreparable cell damage or permanent network dysfunction. Mouse models, and in particular the inducible lines (Table 1), will help assess the reversibility of neurological phenotypes and whether neurological disease progression can be halted and even reversed.

The Peripheral Nervous System and the Neuromuscular Junction

The involvement of the peripheral nervous system (PNS) and the presence of peripheral neuropathy in DM1 has been open to debate (145). The scarce availability of human samples has slowed down research on this topic, but mice expressing toxic CUG repeats in the PNS and in the neuromuscular junction (NMJ) have surpassed this limitation.

Axonopathy was detected in the DMSXL sciatic nerves, characterized by smaller nerve sections, loss and reduced size of myelinated fibers, in association with thinner myelin sheaths, which may highlight ongoing pathogenicity in myelinating cells. The neuronopathy extends to the spinal cord of DMSXL, where a reduction in the number of motor neurons was reported (146).

The analysis of the neuromuscular junction (NMJ) in DM1 muscle biopsies revealed abundant accumulation of RNA foci both in pre-synaptic motoneurons and in post-synaptic nuclei, with pronounced MBNL1 sequestration (147). As a result the NMJ is at risk of developing DM1-associated spliceopathy, but we currently do not know which MBNL1-dependent targets and pathways are dysregulated. In addition, the expression of two members of the SLITRK family of membrane proteins is dysregulated in DM1, in a MBNL1-independent manner, affecting neuromuscular connections (148). Together these findings suggest that both MBNL-dependent and MBNL-independent mechanisms may disturb the organization, stability and function of the NMJ, thereby contributing to PNS pathology and, importantly, to muscle pathology. In support of this view, the expression of expanded CUG RNA in the diaphragmatic NMJ of DMSXL mice is associated with disorganized endplates, lower density of postsynaptic acetylcholine receptors and reduced number of myelinated neurons, possibly mediating the respiratory impairment of these mice (28). In contrast, HSA^{LR} transgenic mice exhibit poor foci accumulation in subsynaptic nuclei (147), indicating that the muscle phenotypes of this line (such as myotonia, central nuclei and ring fibers) do not require the expression of toxic RNA in the NMJ. Subs synaptic RNA toxicity in the NMJ would preferably contribute to DM1 muscle features that are not detected in HSA^{LR} mice, such as angular fiber atrophy and pyknotic nuclear clumps (147). In conclusion,

defective communication between nerve endings and skeletal muscle might be a common feature in DM1, likely contributing to muscle pathology.

THERAPY DEVELOPMENT: PRE-CLINICAL MOUSE STUDIES

Following the identification of CUG repeats as the pathogenic element in DM1, expanded RNA transcripts became an attractive therapeutic target, endorsed by the reversion of disease phenotypes in an inducible mouse model of DM1 (17). Hence, the neutralization of CUG repeats has been tested in relevant DM1 mouse models, taking advantage of antisense oligonucleotides (ASO) or small molecules (Table 3).

Antisense Oligonucleotides to Neutralize Toxic RNA

ASO have been designed to disperse nuclear RNA foci and redistribute MBNL proteins, or to induce the degradation of expanded transcripts. Early approaches aimed to destabilize CUG RNA foci by direct injection of morpholino-type ASO into the skeletal muscle of HSA^{LR} mice. The reduction in nuclear foci, redistribution of MBNL1 protein and splicing correction was sufficient to improve muscle histology and myotonia (150). Similarly, 2'-O-methyl phosphorothioate (PS) modified ASO reduced foci number and corrected missplicing in two independent mouse models (149); unfortunately the molecular benefits were insufficient to improve muscle phenotypes (Table 3). Both strategies reduced the levels of toxic transcripts without RNase H activation, likely through the degradation of expanded transcripts released from nuclear foci. Alternative approaches used RNase H-active ASO to enhance nuclear RNA degradation of CUG repeats. Intramuscular injection and electroporation of 2'-O-methoxyethyl (MOE) gapmers knocked-down expanded CUG transcripts in EpA960 mice and reduced RNA foci (151). Further reduction in toxic RNA was achieved by the combination of RNase H-active MOE gapmers and morpholinos (151). However, local injection caused some degree of muscle damage, which aggravated histopathology and splicing dysregulation in these mice. The systemic delivery of ASO overcomes this problem and is particularly attractive given the vast number of tissues and organs affected in DM1: systemic administration of MOE gapmers reduced expanded CUG RNA, corrected global transcriptome, ameliorated histopathology and resulted in long-term suppression of myotonia in HSA^{LR} mice (12). Similarly, 2'-4'-constrained-ethyl (cEt) ASO administered systemically yielded robust reduction of expanded *DMPK* transcripts, improved body weight, muscle strength and histology of DMSXL mice (154). The demonstration that expanded CUG RNA is a potential target for the RNA interference (RNAi) pathway (163) suggested the therapeutic use of siRNA. Both intramuscular injection and viral delivery of siRNA molecules activated toxic CUG degradation, reduced molecular signs of RNA toxicity and improved the phenotypes of HSA^{LR} mice (152, 155).

ASO offer today a promising pipeline for therapeutic development, but their efficient delivery and biodistribution are still critical hurdles to overcome.

Ligands and Small Molecules to Disperse RNA Foci

Small soluble chemicals with high biodistribution and low toxicity may provide an alternative to ASO. Some of these compounds were tested in DM1 mouse models (Table 3). Derivatives of pentamidine (and other diamidines), hoescht and aminoglycoside, as well as synthetic peptides yielded limited correction of missplicing in HSA^{LR} mice (156, 159, 160). While diamidines inhibit the transcription of toxic CUG RNA, the others likely disrupt RNA-protein complexes, releasing MBNL proteins from nuclear CUG foci. Although the benefits of some of these molecules were modest in mice, the results established a scaffold for chemical redesign to optimize biodistribution, reduce toxicity and increase efficacy.

Approaches limited to restoring MBNL function are unlikely to fully address the consequences of RNA toxicity and additional intermediates should also be targeted. The dissection of the molecular pathways implicated in DM1 pathogenesis revealed some of these targets and hinted at novel routes of pharmacological intervention (Table 3). In the future, therapeutic combination of multiple approaches to eliminate the primary offending RNA with approaches to correct downstream pathogenic events might be required.

DNA as Therapeutic Target

Strategies targeting the DNA repeat expansion mutation were previously tested in DM1 mouse cell culture systems (164) or directly in HSA^{LR} skeletal muscle (165), and proved capable of stabilizing the trinucleotide CTG repeat tract. Although substantial effort has concentrated on the deleterious accumulation of toxic RNA, recent gene editing tools provide new means to target the upstream DNA mutation that causes DM1. CRISPR/Cas9 systems were tested in DMSXL mouse cells to induce repeat contractions (43), while modified Cas9 was used in HSA^{LR} mice to block the transcription of toxic RNA (166).

DM1 ANIMAL MODELS BEHIND MICE

By definition, an animal model provides a simplification of the complex human system, or at least, part of it. Mouse models offer a good compromise between easy manipulation, affordable research cost and similarity to the complex physiology of humans. However, mice have limitations too, and today there is no perfect DM1 mouse model that fully recreates all disease aspects. Conversely, reduced body mass has been repeatedly reported in mice (21, 23, 26, 77, 78) but no direct parallel has been established with human clinical symptoms, nor is it known to what extent this phenotype reflects a DM1-associated developmental delay.

Given the nature of the constructs used, transgene expression varies between models and introduces some drawbacks that should not be overlooked. Some constitutive models (such as the DMSXL mice) express low transgene levels, and require

TABLE 3 | Therapeutic strategies tested in DM1 mouse models.

| Compound | Target | Administration | Mechanism | Mouse model | Benefits reported in DM1 mice | References |
|------------------------------------------------|-----------------------------------|-----------------------------------------------------|----------------------------------------------------------------------------|-----------------------------------------|-------------------------------------------------------------------------------------------------------|------------|
| ANTISENSE OLIGONUCLEOTIDES | | | | | | |
| PS | CUG sequence | Intramuscular injection (local) | Steric hindrance and foci dispersion | DMSXL ^a HSA ^{LR} | Dispersion of RNA foci Reduction of CUG RNA Splicing correction | (149) |
| Morpholino | CUG sequence | Intramuscular injection and electroporation (local) | Steric hindrance and foci dispersion | HSA ^{LR} DMSXL ^a | Reduction of CUG RNA Splicing correction Mitigation of myotonia | (150) |
| MOE-gapmer | Flanking region | Subcutaneous injection (systemic) | RNase H-mediated degradation | HSA ^{LR} | Reduction of CUG RNA Splicing correction Improved histology Sustained mitigation of myotonia | (12) |
| MOE-gapmer | CUG sequence | Intramuscular injection and electroporation (local) | RNase H-mediated degradation | Induced EpA960 | Dispersion of RNA foci Splicing correction | (151) |
| MOE-gapmer and morpholino | CUG sequence | Intramuscular injection and electroporation (local) | Combined RNase H and foci release | Induced EpA960 | Enhanced reduction of CUG RNA | (151) |
| siRNA | CUG sequence | Intramuscular injection and electroporation (local) | RNAi-mediated RNA degradation | HSA ^{LR} | Reduction of CUG RNA Dispersion of RNA foci Splicing correction Mitigation of myotonia | (152) |
| cEt gapmer | <i>DMPK</i> 3'UTR | Subcutaneous injection (systemic) | RNase H-mediated degradation | DMSXL | Reduction of CUG RNA Body weight gain Improved muscle strength Improved histology | (153, 154) |
| siRNA | hACTA1 3'UTR | Intravenous injection of rAAV vectors (systemic) | RNAi-mediated RNA degradation | HSA ^{LR} | Reduction of CUG RNA Splicing correction Improved histology Mitigation of myotonia | (155) |
| NUCLEIC ACID BINDING CHEMICALS | | | | | | |
| Pentamidine, heptamidine and diamidine analogs | CUG transcription | Intraperitoneal injection (systemic) | Inhibition of CUG transcription Foci dispersion and CUG RNA degradation | HSA ^{LR} | Reduction of CUG RNA Splicing correction Mitigation of myotonia | (156–158) |
| Hoescht derivatives | CUG-MBNL complex | Intraperitoneal injection (systemic) | Disruption of RNA foci | HSA ^{LR} | Splicing correction | (159) |
| Kanamycin derivatives | CUG-MBNL complex | Intraperitoneal injection (systemic) | Disruption of RNA foci | HSA ^{LR} | Splicing correction | (160) |
| Synthetic peptide | CUG-MBNL complex | Intramuscular injection (local) | Disruption of RNA foci | HSA ^{LR} | Improved histology Splicing correction | (161) |
| Actinomycin D | CUG transcription | Intraperitoneal injection (systemic) | Inhibition of CUG transcription | HSA ^{LR} | Reduction of CUG RNA Splicing correction | (162) |
| PHARMACOLOGICAL APPROACHES | | | | | | |
| Ceftriaxone | GLT1, glial glutamate transporter | Intraperitoneal injection (systemic) | Upregulation of GLT1 | DMSXL | Correction of Purkinje cell firing Improved motor coordination | (50) |
| Bio, Lithium, TDZD-8 | GSK3 β | Intraperitoneal injection (systemic) | GSK3 β inhibition | HSA ^{LR} | Improved histology Improved muscle strength Mitigation of myotonia | (13) |
| Ro-31-8220 | PKC | Intraperitoneal injection (systemic) | PKC inhibition | EpA960 | CELF1 downregulation Splicing correction Amelioration of cardiac function | (88) |
| AICAR | AMPA signaling | Intraperitoneal injection (systemic) | AMPK activation | HSA ^{LR} | Dispersion of RNA foci Splicing correction Mitigation of myotonia | (106) |
| Rapamycin and AZD8055 | mTOR signaling | Intraperitoneal injection (systemic) | mTORC1 inhibition | HSA ^{LR} | Improved muscle function and strength | (106) |
| Anti-TWEAK antibody | TWEAK/Fn14 signaling | Intraperitoneal injection (systemic) | TWEAK | DM5 | Improved muscle histology Improved muscle strength Greater survival | (110) |

rAAV, recombinant adeno-associated viral; ASO, antisense oligonucleotide; BIO, 6-bromindirubin-39-oxime; cEt, 2',4'-constrained ethyl-modified; LNA, locked nucleic acids; MOE, 2'-O-methoxyethyl; PS, 2'-O-methyl phosphorothioate. ^aThe DMSXL mice used in these studies were hemizygous and carried ~500–800 CTG.

breeding to homozygosity to develop disease phenotypes. In contrast, the high expression levels in tissue-specific models (such as the EpA960 and DM5 mice) may trigger some non-specific disease features. Finally, tissue and cell type-specific expression in HSA^{LR}, EpA960 and DM5/DM200 mice can mask non-cell-autonomous mechanisms, critical for some features of disease pathogenesis. In summary, the collection of mice available today covers different aspects of DM1 pathology to a certain extent, partially fulfilling the absence of a perfect mouse model, and providing means for data validation by independent laboratories.

Simple organisms can also provide complementary models for basic, translational and pre-clinical research. Although phylogenetically distant from humans, *Drosophila melanogaster*, *Caenorhabditis elegans* or zebrafish (*Danio rerio*), have multiple advantages over mice, including their easy manipulation, low maintenance cost and fast generation of large offspring. The expression of toxic RNA in *D. melanogaster* recreated molecular features of DM1, such as RNA foci accumulation, muscleblind protein sequestration and missplicing (167–169). Some lines showed eye degeneration (167, 168), a general readout of neurotoxicity, but which does not necessarily relate to human pathology. The development of muscle phenotypes, such as muscle wasting (167) and hypercontraction (169) seems more relevant. The expression of expanded CUG repeats also resulted in RNA foci and muscle phenotypes in zebrafish (170, 171) and *C. elegans* (172, 173). Together, these data suggest the conservation of the core mechanisms of RNA toxicity across species, and corroborate the use of simple organisms in large screenings for disease modifiers. Such studies have already resulted in the identification of genetic modifiers (167, 174, 175), chemicals that correct DM1 splicing abnormalities (176) and miRNA sponges that regulate MBNL protein levels and rescue fly phenotypes (177). The physiology of small organisms and humans are nonetheless substantially different, and therefore parallels must be established with care.

CONCLUSIONS

Transgenic mouse models, alone or in combination, have been key to understanding fundamental molecular pathomechanisms of DM. Over the last decade, the progress in mouse studies and the advances in high throughput approaches (e.g., transcriptomics and proteomics) have led to the identification of hundreds of misregulated genes and proteins, through changes in alternative splicing, polyadenylation, protein translation and phosphorylation. Understanding the contribution of these molecular events to the etiology of DM will help depict the course between repeat expansion and the onset of disease manifestations. Future studies should continue to address “which” disease intermediates and cell populations, “where” in the tissue and “when” during disease course experience the most pronounced abnormalities. Linking these variables will identify critical events and developmental windows during which specific cell pathways are particularly sensitive to pathological insults and targetable by corrective therapies. A better understanding

of pathophysiological trajectories will guide the development of efficient therapeutic approaches.

Some models have deliberately focused on specific disease features and recapitulated a small number of disease phenotypes (e.g. muscle pathology in HSA^{LR} models, cardiac function in inducible CELF1-overexpressing mice). Although oversimplifying the situation, this reductionist approach has offered the opportunity to break the complexity of disease down to tractable “building blocks” and to unravel the mechanisms behind individual aspects of the disease. The future combination of these different models, by intercrossing different transgenic lines, might be considered to “rebuild” the convoluted human disease and to explore the interdependence of individual factors. The complexity of DM pathobiology and variation in mouse models design require, however, a critical approach in the interpretation and comparison of the results obtained with different lines.

There is little doubt that mouse models will continue to provide in-depth understanding of disease. One of their major advantages is the opportunity to monitor early pathological changes, prior to the onset of disease symptoms, which is difficult to achieve in humans with the current diagnostic standards. We anticipate that future studies will uncover additional cellular pathways impacted during the disease course, while revealing targetable events to reverse disease.

AUTHOR CONTRIBUTIONS

JA: experimental work and data acquisition; GG and MG-P: study design, interpretation, data analysis; SB and MG-P: preparation of figures; SB, GG, and MG-P: manuscript preparation.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Objectives and thesis outline

My PhD research project was developed within the framework of the general aim of our laboratory. Among the research lines developed in our lab we are particularly interested in understanding the molecular and cellular mechanisms dysregulated in DM1 brains. Specifically, I aimed to gain insight into the contribution of myelin abnormalities and oligodendrocyte dysfunction towards DM1 brain pathology, and characterize the molecular events specifically dysregulated in response to the CTG expansion. To do so, I combined *in vivo* studies in DMSXL mice with *in vitro* approaches using primary oligodendrocyte cultures derived thereof and validated the findings in human samples and relevant iPS-derived cell models.

The three main objectives of my PhD work are covered in the following three chapters:

Chapter II – Development of faster and improved videomicroscopy-based semi-automated strategies to analyze phenotypic alterations in brain cells.

The laboratory is very much interested in the study of the contribution and degree of dysfunction of different brain cell types to DM1 brain manifestations. Upon my arrival, I started to set up DMSXL primary oligodendrocyte cultures and, in order to study their phenotypes and molecular abnormalities I develop and optimize videomicroscopy-based semi-automated strategies to analyze phenotypic alterations in different brain cell models. The results of this part of my project are at the moment in press in *Methods in Molecular Biology*, in an issue dedicated to Trinucleotide Repeat Protocols.

Sandra O. Braz, Diana M. Dinca, Geneviève Gourdon, Mário Gomes-Pereira

Real time videomicroscopy and semi-automated analysis of brain cell culture models of trinucleotide repeat expansion diseases.

Chapter III - Investigate myelination defects in the DM1 brain disease

I initiated the analysis of oligodendrocytes dysfunction in DM1, through the analysis of the molecular hallmarks of RNA disease in this cell type. In chapter III, I describe the molecular evidence of RNA toxicity in the oligodendrocytes of a transgenic mouse model of DM1. Then I investigated the structural and physiological consequences

of these abnormalities onto myelin structure and oligodendrocytes cell biology *in vivo*. The results of this work are in preparation and will be soon submitted for publication.

Sandra O. Braz, Alain Schmitt, Dominique Langui, Geneviève Gourdon and Mário Gomes-Pereira

Developmental delay in myelination and oligodendroglia lineage progression in DMSXL mice

Chapter IV - Study the contribution of DM1 oligodendrocytes dysfunction to the myelin phenotypes

Following the description of RNA foci in the DM1 oligodendrocytes and the evidence of transient hypomyelination in DM1 mice, the final aim of my PhD was to study the oligodendroglia cell abnormalities behind the myelin defects, and identify candidate pathways dysregulated by the repeat expansion. To this end I took advantage of homogeneous primary cultures of OPC cultures derived from DMSXL, that I phenotyped during their differentiation into oligodendrocytes. The results of this work are in preparation and will be soon submitted for publication.

Sandra O. Braz, Raphaël Blain, Aurélien Cordier, Olivier Alibeu, Cyril F. Bourgeois, Cristine Bole, Didier Auboeuf, Geneviève Gourdon and Mário Gomes-Pereira

GUG RNA toxicity is associated with differentiation deficits in oligodendrocytes in a model of myotonic dystrophy type I.

In parallel to my main project, I have contributed to other projects ongoing in the laboratory, focused on the deregulation of GLT1 glutamate transporter, that we have found in DMSXL and DM1 brains. Given the essential role of GLT1 in the fine regulation of glutamate levels in the CNS, my aim was to determine whether the GLT1 downregulation in DMSXL was already preset at transcriptional level and whether it was due to the lack of Bergman glia cells in the mouse cerebellum. This work was published recently in *Cell Reports*.

Géraldine Sicot *, Laurent Servais *, Diana M. Dincă *, Axelle Leroy, Cynthia Prigogine, Fadia Medja, **Sandra O. Braz**, Aline Huguet-Lachon, Cerina Chhuon, Annie Nicole, Noëmy Gueriba, Ruan Oliveira, Bernard Dan, Denis Furling, Maurice S. Swanson, Ida Chiara Guerrera, Guy Cheron, Geneviève Gourdon and Mário Gomes-Pereira **Downregulation of the Glial GLT1 Glutamate Transporter and Purkinje Cell Dysfunction in a Mouse Model of Myotonic Dystrophy** – Cell Reports (2017) 19, 2718- 2729

Chapter II

Real-time monitoring of brain cell models of trinucleotide expansion diseases

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I. Abstract

Proper brain function requires the coordinated and intricate interaction between neuronal and glial cells. Like many other neurological conditions, trinucleotide repeat expansion disorders are likely initiated by the synergistic combination of abnormalities hitting different brain cell types, which ultimately disrupt brain function and lead to the onset of neurological symptoms. Understanding how trinucleotide repeat expansions affect the phenotypes and physiology of neurons and glia is fundamental to improve our understanding of disease mechanisms in the brain and shape the design of future therapeutic interventions.

Here we describe a protocol for semi-automated videomicroscopy analysis of cultured brain cells, maintained under suitable and controlled conditions. Through real-time monitoring of basic cell phenotypes (such as proliferation, cell morphology, differentiation and migration) this method provides an accurate primary assessment of the impact of the repeat expansion on the physiology of neurons and glia. The versatility of the system, the automated image acquisition and the semi-automated processing of the data collected allow rapid phenotypic analysis of individual cell types, as well as the investigation of cell-cell interactions. The stability of the acquisition system provides reproducible and robust results. The raw data can be easily exported to other software to perform more sophisticated imaging analysis and statistical tests. In summary, the methods described offer versatile, reproducible and time-effective means to dissect the impact of the repeat expansion on different brain cell types and on intercellular interactions.

Key words:

Real-time videomicroscopy; primary cell cultures; neuron; astrocyte; oligodendrocyte; trinucleotide repeat expansion; cell phenotypes; semi-automated analysis.

II. Introduction

Trinucleotide repeats expansion diseases comprise neuromuscular, neurodevelopmental and neurodegenerative disorders [1, 2], which are usually associated with cognitive deficits and behavioural changes. Today we know that repeat expansions mediate disease etiology through a variety of mechanisms, including loss of function (*e.g.* fragile X syndrome, FXS; and Friedreich ataxia, FRDA) [3, 4], toxic gain of function of polyglutamine tracts (*e.g.* Huntington disease, HD) [5] and RNA toxicity (*e.g.* myotonic dystrophy type 1, DM1) [6]. On top of these mechanisms, the accumulation of toxic homopolypeptides by repeat-associated non-AUG (RAN) translation may also play a determinant pathogenic role [7].

Mouse models have been crucial to decipher the molecular pathogenesis of trinucleotide repeat expansion diseases, notably in the brain, where they recreate the intricate and multidirectional interactions between different cell types [8]. However, the broad view revealed by the analysis of tissue samples, does not grant sufficient resolution to focus on pathological events occurring in specific cell populations. This is particularly relevant in conditions in which it is conceivable that disease susceptibility and the cascade of pathogenic events vary between different cell types and/or subpopulations. Cell type selective pathogenesis in trinucleotide repeat expansion disorders is illustrated by the preferential degeneration of medium spiny neurons in the striatum of HD patients [9] and by the pervasive loss of Purkinje cells in many spirocerebellar ataxias (SCAs) [10], while other neuronal types remain relatively spared. Rat primary cultures corroborated the increased vulnerability of striatal neurons to expanded huntingtin, relative to cortical neurons [11]. More recently, the generation of human iPSC-derived Purkinje cells confirmed the burden of SCAs to this neuronal cell type [12].

In spite of the neuronal damage that precedes the neurological manifestations of trinucleotide repeat expansion diseases, a less “neurocentric” view of brain pathology is emerging, pointing to the key role of glial cells. It has been reported that astrocyte dysfunction prevents normal neuronal development and proper synaptogenesis in FXS [13, 14] and FRDA [15, 16]. Astrocyte deficits may also cause Purkinje cell hyperexcitability in SCA7 [17] and DM1 [18]. Signs of astrocyte pathology were also

reported in HD [19], fragile X-associated tremor/ataxia syndrome (FXTAS) [20] and SCA1 [21]. In addition to astrocytes, oligodendrocytes are also affected by trinucleotide repeat expansions. The dysfunction of this glial cell type was documented in mouse models of HD and FXS, and it was characterized by delayed and reduced myelination in association with defective maturation of oligodendrocytes [22, 23]. The involvement of microglia in trinucleotide repeat expansion diseases has been less explored. Nonetheless, microglia activation was reported in HD patients, as well as in mouse models of HD [24, 25] and SCA1 [21]. In HD models, microglia activation is accompanied by abnormal cell morphology and defective migration response to chemotaxis [26].

Together these findings demonstrate the diverse response of different brain cells to disease, and the critical insight that can be gained from the analysis of individual cell types. Understanding the disease mechanisms that operate in different cell populations and underlie the complex neurological symptoms is crucial for future therapeutic developments. Cell culture techniques provide a powerful setting to address this question. While homogenous cell cultures can reveal the impact of the disease on the phenotype and physiology of different cell types, mixed cultures (established through the combination of different cells) can help untangle the mechanisms of intercellular miscommunication in the brain and their contribution to disease. These studies were traditionally performed on cultured cells fixed at different time points. Although informative, this strategy presents two main limitations: (a) the readouts represent isolated snapshots of physiological processes that are continuously ongoing and they may therefore miss relevant events; (b) the methodology is time-consuming, involving cell manipulation, cell labeling, signal detection, data collection and laborious data analysis.

The alternative is to perform prolonged recordings of live cell phenotypes, by real-time semi-automated videomicroscopy. The success of this methodology depends on the maintenance of well-defined and suitable culture conditions, which must guarantee cell function and stability over long periods of time. This requirement is particularly important for brain cells, given their high sensitivity to environmental changes: subtle uncontrolled fluctuations may have deleterious consequences to cell homeostasis and affect the quality and reproducibility of the results. The advanced Incucyte® live-cell analysis system (Essen Bioscience) reduces these technical

challenges and maximizes the quality and reproducibility of the data collected. The videosystem is incorporated in a regular CO₂ incubator, allowing the cells to be kept in their optimal temperature and humidity throughout the experiment. Image acquisition is automatic: once the experimenter has defined the acquisition settings he/she can walk away, leaving the cells unperturbed. The semi-automatic analysis of the IncuCyte® system is another major strength: the system offers pre-defined analysis settings that can be adapted by the scientist to better fit the type of cells and the biological process being studied. Once defined, the same settings and analysis can be immediately applied to the images as they are acquired by the video system - the computerized workflow translates into real-time results and traceability of eventual problems, therefore saving precious time.

The methods described here provide a videomicroscopy semi-automated approach to assess the primary impact of the disease on different cell lineages of the brain (neurons, astrocytes and oligodendrocytes), using proliferation, differentiation, cell adhesion, morphological complexity and migration (among others) as basic readouts of cell dysfunction. The results generated offer meaningful clues to design future experiments and extend the analysis of the disease mechanisms operating in individual cell populations, through more refined approaches. In spite of our focus on brain cells, the protocols can be easily adapted and applied to a myriad of other cell types.

II. Materials

A. Specific equipment and reagents

1. IncuCyte Zoom videomicroscope equipped with IncuCyte® Scratch Wound Cell Migration Software Module (9600-0012, Essen Bioscience) and IncuCyte® NeuroTrack Software Module (9600-0010, Essen Bioscience).
2. Computer equipped with IncuCyte Zoom software, FIJI-ImageJ software [27] and DiPer program [28].
3. IncuCyte® Cell Migration Kit (4493, Essen Bioscience).
4. IncuCyte® ImageLock Plates (4379, Essen Bioscience).
5. IncuCyte® NeuroLight Red Lentivirus - Synapsin Promoter (4584, Essen Bioscience).

6. GentleMACS Dissociator (130-093-235 or superior, Miltenyi).
7. Neural tissue dissociation kit (130-092-628, Miltenyi) and magnetic anti-CD140a microbeads (130-092-628, Miltenyi).
8. Equipment maintenance: 0.5% Alconox, 1% Virkon, double distilled H₂O and 70% Ethanol.

B. Mouse experiments

For experiments involving animal models you must follow the institutional, national and/or international guidelines for ethical conduct in the care and use of animals.

C. Cell culture media and coating reagents

1. Dissection medium for neurons and astrocytes: Leibovitz's L-15 Medium, 30 μ M glucose.
2. Dissection medium for OPC: Hank's balanced salt solution without (HBSS) Ca²⁺ and Mg²⁺.
3. Neuronal medium: Neurobasal-A, 1X B27 supplement, 0.5 mM L-Glutamine, 0.25 μ g/mL Amphotericin B, 100 U/mL penicillin and 100 μ g/mL streptomycin.
4. Astrocytes medium: DMEM low glucose, 10% fetal bovine serum (FBS) and 0.05 mg/mL Gentamycin.
5. OPC medium: DMEM F-12, 1% N2 supplement, 2% B27 supplement, 50 units/mL penicillin and 50 μ g/mL streptomycin, 0.01% bovine serum albumin, 40 ng/mL FGF2 and 20 ng/mL PDGF-AA.
6. Oligodendrocytes medium: DMEM F-12, 1% N2 supplement, 2% B27 supplement, 50 units/mL penicillin and 50 μ g/mL streptomycin, 50 μ g/mL insulin, 40 ng/mL triiodo-thyronin and 1 ng/mL CNTF.
7. Coating: 0.1 mg/mL of poly-D-lysine (PDL) and 10 μ g/mL laminin (See **Note 1**).

III. Methods

A. Primary mouse cell cultures

Different protocols are available in the literature and can be used to establish primary mouse neurons, astrocytes and oligodendrocytes cultures. We briefly describe below the protocols used in our laboratory to ensure healthy and reproducible cell cultures prior to cell seeding (see **Note 2**).

(a) For primary neuron cultures:

1. Prepare primary dissociated cell cultures of cortical neurons from embryonic day (E) 16.5 mouse embryos (see **Note 3**). Dissect the brain and place it on a dish with dissociation medium for neurons, under a stereoscope.
2. Separate the cortices from the rest of the brain and carefully remove the meninges with the help of micro tweezers.
3. Maintain the cortices in dissection medium on ice until end of the dissection.
4. Wash the cortices twice with neuronal medium, previously warmed at 37°C.
5. Incubate the cortices in neuronal medium containing 0.05% of trypsin and 10 U/mL of DNaseI 15 min at 37°C, shaking gently the tube every 5 minutes (min).
6. Wash the cell suspension twice with neuronal medium supplemented with 5% FBS to inhibit further tissue digestion by trypsin.
7. Dissociate the cells passing them through a 21 G needle, wash twice in neuronal medium, collecting the cells at 200 g for 5 min at room temperature (RT) in between washes.
8. Count cells (see **Note 4**) before plating at appropriated cell density in PDL and laminin-coated 96 well plates (see **Note 5**) in pre-warmed neuronal medium containing 5% FBS.
9. Replace the medium 4 hours (h) after plating with neuronal medium supplemented with 100 μ M AraC and withdraw the FBS to avoid astrocytes proliferation (see **Note 6**).
10. Change half of the medium every 3-4 days (see **Note 7**).

(b) For primary astrocyte cultures:

1. Prepare primary dissociated cell cultures of cortical astrocytes from postnatal day (P) 1 pups (see **Note 3**). Dissect the cortices as described above.
2. Wash the tissue twice with 1 mL of astrocytes medium and mechanically dissociate, passing through 21 G needle twice and then three times through a 26 G needle, waiting 2-3 min for the sedimentation of the fragments in between each dissociation.
3. Centrifuge the cell suspension 200 g for 5 min at RT, remove the supernatant and resuspend the cells in 10 mL of pre-warmed astrocytes medium.
4. Plate the cells in a T75 dish at 300,000 cells/mL.
5. On the following day, change the medium (see **Note 7**) to remove the dead cells.
6. Primary astrocytes adhere, grow and proliferate on both coated and uncoated dishes (see **Note 1** and **6**).
7. Incubate the cells for 2 weeks in astrocyte medium, changing the medium every 3 days (see **Note 8**).

(c) For primary oligodendrocyte progenitors (OPC) cell cultures:

1. Prepare dissociated cell cultures of cortical OPC from P0-P2 mice (see **Note 3**). Perform the dissection as described above using dissection medium for OPC.
2. Dissociate the tissue using the neural tissue dissociation kit and following the manufacture's protocol. Dissociate the tissue by cycles of mechanic and enzymatic dissociation to produce a single cell suspension, then filter the suspension through a 70 μ m strainer and wash with HBSS with Ca²⁺ and Mg²⁺.
3. Centrifuge the cell suspension at 300 g for 10 min at RT, remove the supernatant and resuspend the cells in a 1X PBS with Ca²⁺ and Mg²⁺, supplemented with 5% BSA (BSA buffer).
4. Isolate the OPC using magnetic anti-CD140a (PDGFR α) labelled microbeads (protocol adapted from Dincman et al. 2012 [29]).
5. After blocking, incubate the cells with the magnetic microbeads to allow the binding of the PDGFR α ⁺ cells, then wash the cells in BSA buffer and collect at 300 g for 10 min at RT, to eliminate cell-free microbeads.
6. To separate the OPC, pass the cell suspension through a column attached to a magnetic field, where the PDGFR α ⁺ cells are retained.
7. Remove the column from the magnetic field and flush the cells in OPC medium.

8. Count OPC and plate them at a density of 9,000-15,000 cells/cm² in PDL and laminin-coated 96 well plates (see **Note 5**) and keep in OPC medium to proliferate (See **Note 9**).
9. Two days later, replace the medium with oligodendrocyte medium, in order to induce the differentiation of OPC into oligodendrocytes (see **Note 6**).

B. Cell proliferation (Astrocytes and OPC)

In this protocol, cell proliferation is estimated by the percentage of well surface area occupied by cells (i.e. confluence) over time.

1. Seed the astrocytes and OPC at 20,000 or 5,000 cells/well, respectively (see **Note 10**), on 96-well plates (see **Note 5**) and keep the cells in their corresponding culture media.
2. Set the system to acquire a phase-contrast picture (see **Note 11**) every 45 min over 50 h for astrocytes, or every 2 h during 7-8 days for oligodendrocytes, selecting the “Standard” type of scan and using the 20X objective (see **Note 12**). Change the cell medium every 2-3 days (see **Notes 6 and 13**).
3. Set the image analysis by first creating a collection of images (see **Note 14**) representative of the phenotypes to be monitored throughout the experiment. Select the first picture and click on “Create or Add to Image Collection” (Figure 1 option **A**), select the “Basic Analyzer” job type mode and add the image to the new image collection. Select another 4-6 new images (see **Note 15**) and add them to the same

image collection.

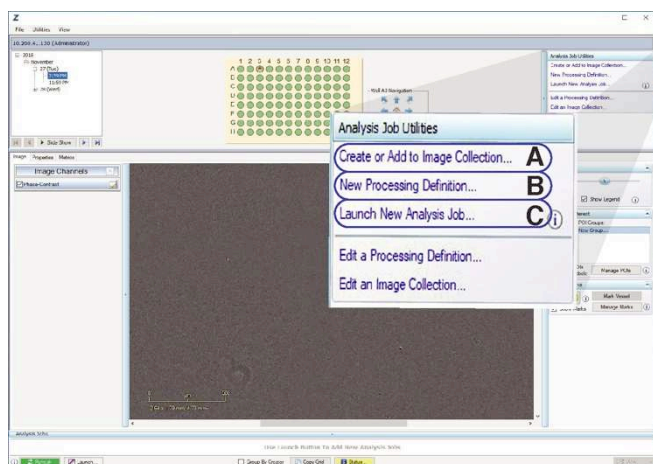


Figure 1 - Incucyte® Zoom software interface.

Software interface screenshot showing the panel for the definition of the semi-automated analysis. The process starts with the creation of an image collection (option A), followed by the definition of the analysis settings that will be applied to all the images subsequently acquired (option B). Finally the analysis is launched (option C).

4. Using this collection of images, define the settings to be applied to the analyses. Under the option “New Processing Definition” (Figure 1 option **B**) run the “Preview Current” command to automatically generate the “Confluence Mask” that will contour and cover the surface of what the software considers to be cells in the first picture selected.
5. Since the automatic mask is unlikely to fit all the cell shapes/densities through time and/or all cell conditions (Figure 2A), adjust this mask in order to fully outline the shape of the cells. To do so use the filters named “Cell/Background”, “Size” and “Eccentricity”, which are available on the “Segmentation Adjustment” panel (See **Note 16**).
6. Apply the defined settings to all the image collection by selecting the option “Preview All”, and check if the mask fits perfectly the majority of the cells in the different images of the image collection previously chosen (Figure 2B).

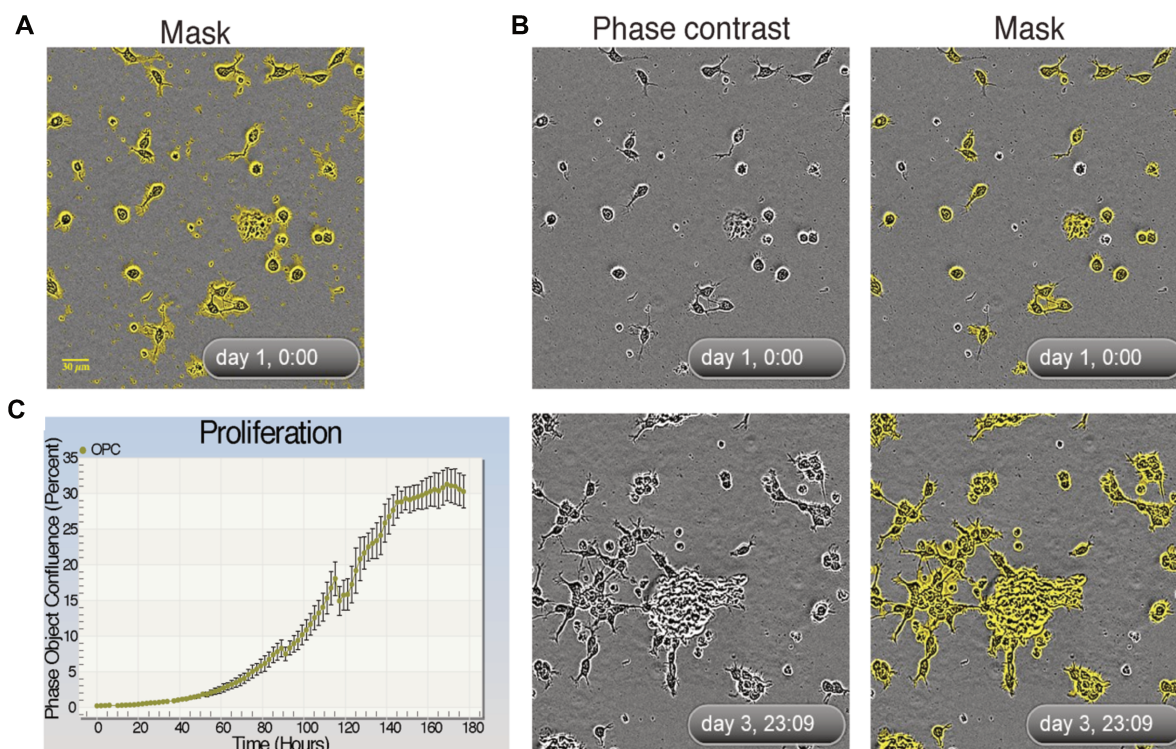


Figure 2. Live-cell monitoring of OPC proliferation.

(A) Phase contrast image showing a merged a poor “Confluence Mask” (yellow) fit on OPC imaged 1 day after plating. The confluence mask is inadequately counting small cell debris and plate/coating imperfections as objects (cells), also including some space between adjacent cells. (B) Phase contrast images of OPC on day 1 and 3 after plating. Adequate confluence masks (yellow) faithfully delineate the shape of living cells, excluding cell debris, imperfections and

space between cells. (C) The quantitative analysis of cell proliferation is plotted as the percentage of confluence (surface of the well occupied by cells) over 8 d.

7. Initiate the analysis by selecting “Launch New Analysis Job” (Figure 1 option C). The software will apply the defined settings to all the pictures acquired from the plate over time (see **Note 17**).
8. The results will estimate cell proliferation, measured as the percentage of cell confluence (see Notes 18 and 19) or as a phase-contrast object (cell) count (see Notes 11 and 20) during the time-course of the experiment. The results are plotted as the percentage of confluence over time for each well, or as the average of technical/biological replicates (Figure 2C) (See **Note 21**).

C. Wound healing migration (Astrocytes and OPC)

This analysis evaluates the capacity on the cells to migrate towards and fill an empty space created by a wound on a cell monolayer. It estimates the percentage of the wound surface occupied by migrating cells over time.

1. Seed 20,000 astrocytes or 10,000 OPC per well (see **Note 10**), on a 96-well plate Incucyte® Image Lock (see **Notes 1, 22 and 23**) and keep the cells in their medium. Incubate the cells for 1-2 days to obtain a confluent monolayer of astrocytes, or a 70-90% confluent layer of OPC.
2. Place the open plate in the base of the Incucyte® Wound Maker (see Note 24) and carefully introduce the wound maker pin block into the wells. Perform the wound on the bottom of the well by pushing and holding the lever (see Note 25). Wash the cell
3. gently with pre-warm astrocyte or OPC medium twice, to remove any cell debris remaining in the wound (while avoiding to disturb the cells) and refill the wells with 100 µL of medium (See **Note 7**) using a multichannel micropipette.
4. Set the system to acquire a phase-contrast picture (see **Note 11**) every 45-60 min over 3 days (See **Note 22**), selecting the “Scratch Wound” on the “Scan Type” dropdown and using the 10x objective.
5. Set the image analysis by creating a representative set of images taking into account the phenotype to be analyzed over time (See **Note 14**). To this end, select the first picture and click on “Create or Add to Image Collection” (Figure 1 option A), select the “Scratch Wound” option on the job type mode and add the image to the new

image collection. Select another 4-6 new images (see **Note 15**), including the first scan (time 0) after wounding, which will be used to define the initial mask for the surface of the wounded (cell-free) area. Add the images to the previous image collection.

6. Using this collection of images, define the settings to be applied to the analyses. Under the option “Process Definition” (Figure 1 option **B**) run the “Preview Current” command on the first picture to automatically generate the “Confluence Mask” (which will cover and contour the surface of what the software supposes to be a cell layer (Figure 3A-1) and the “Scratch Wound Mask” (which will cover the cell-free space created by the scratch (Figure 3A-3).
7. Since the automatic mask may not be able to perfectly detect and outlines the cell layer limits and/or some cell debris may remain on the wounded area (Figure 3A), adjust the mask so it fully covers and delineates cell shapes and the cell front on each side of the wound, using the filters named “Cell/Background”, “Size” and “Eccentricity” available on the “Segmentation Adjustment” panel (See **Notes 16** and **26**).
8. Apply the defined settings to the entire image collection previously created by running “Preview All” and check if the masks provide a perfect fit for the front of migration (Figure 3A-2), cell layer and wounded space for the majority of the different images previously selected (Figure 3B).
9. Initiate the analysis by clicking on the “Launch New Analysis Job” (Figure 1 option **C**). The software will apply the settings previously defined to all the pictures acquired on the plate (see **Note 17**).
10. The results will estimate the rate of occupancy of the space left empty following the scratch. They can be represented by one of three measurements: a) wound width over time (distance between the front of migration of each side of the cell-free space); b) wound confluence over time (the percentage of the wound initial surface occupied by the cells) or c) relative wound density (RWD, the percentage of the cell density in the wound relative to the percentage of cell density in the regions surrounding the wound) (see **Notes 21** and **27**) (Figure 3C).

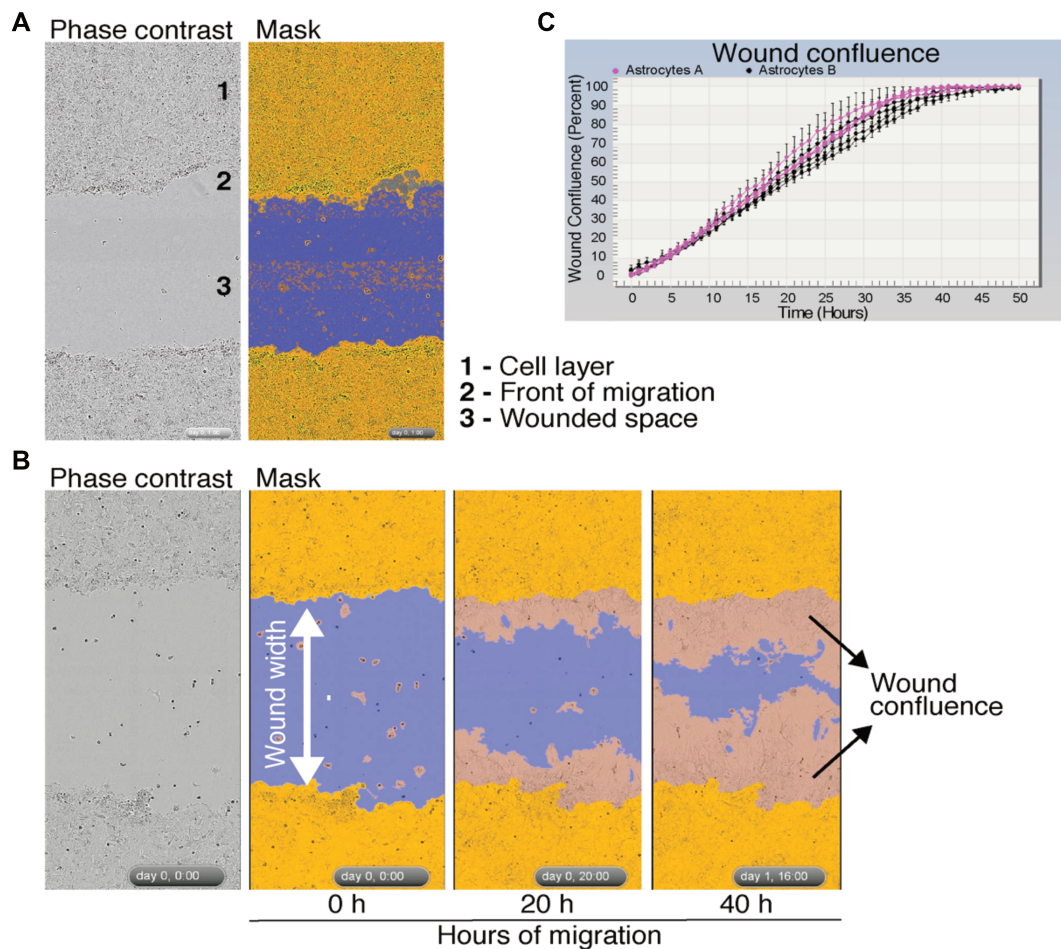


Figure 3 - Astrocyte migration capacity tested by an automated wound healing assay.

(A) Phase contrast image of a cell monolayer after scratching, using the Incucyte® Wound Maker. The picture shows the cell layer (1), front of migration (2) and wounded space (3) on the left. The image on the right shows a merged bad fitting “Confluence mask” (yellow), covering only part of the front of migration and inadequately including empty wounded space without cells. (B) Phase contrast image of a scratched astrocyte monolayer and the fitting “Confluence Masks” (yellow) over time. The merged images show the “Confluence Mask” overlaying the cell layer and outlining some isolated cells left in the wounded space, while excluding cell debris. The “Scratch Wound Mask” (blue) is confined to the wounded space, free of cells. The wound width (white double arrowhead) decreases as the wound confluence increase over 40 hours following the initial scratch. (C) The speed of collective migration of the astrocytes is estimated by the increase in wound confluence over time. Several biological replicates of astrocytes of individual mice (pink and black) are plotted.

D. Single cell tracking (Astrocytes)

This analysis allows the drawing of the migration path, the measurement of speed, directionality and displacement of individual astrocytes by manually tracking the position of the nucleus in each image, using Fiji and the open-source DiPer computer software [28].

1. Following the wound healing assay, export migration videos from the IncuCyte® in uncompressed AVI format and open them using Fiji (see **Note 28**). Make sure to export at least one video (or one image) with the embedded scale, to be able to use it for setting the scale in Fiji (see **Note 29**). The image visualization of the videos can be modified to facilitate tracking (See **Note 30** and **31**).
2. After setting the scale (see **Note 28**) use the “Manual Tracking Fiji” plugin to track the nucleus of at least 40 astrocytes per video. Insert the time interval in the corresponding panel of the Tracking window, and the x/y calibration (corresponding to the pixel width, which can be found when setting the scale in Fiji). Start tracking by clicking “Add track” (Figure 4A) and following the advancement of one selected cell. This will measure the position of the astrocytes on the X and Y coordinates, the distance and the velocity of the astrocytes (Figure 4B).
3. The distance and velocity results can be directly analyzed using the appropriate statistical program, or they can be used to calculate more complex parameters using the DiPer open-source macros for Microsoft Excel [28]. These macros allow the calculation of cells directionality (defined as the straight line between the start and the end point of the migration trajectory divided by the total length of the trajectory), or the mean square displacement (which will assess the area explored by the migrating cells over time), as well as plotting each cell trajectory, individually or collectively, on X/Y axes, emanating from the original starting point of displacement (Figure 4C).

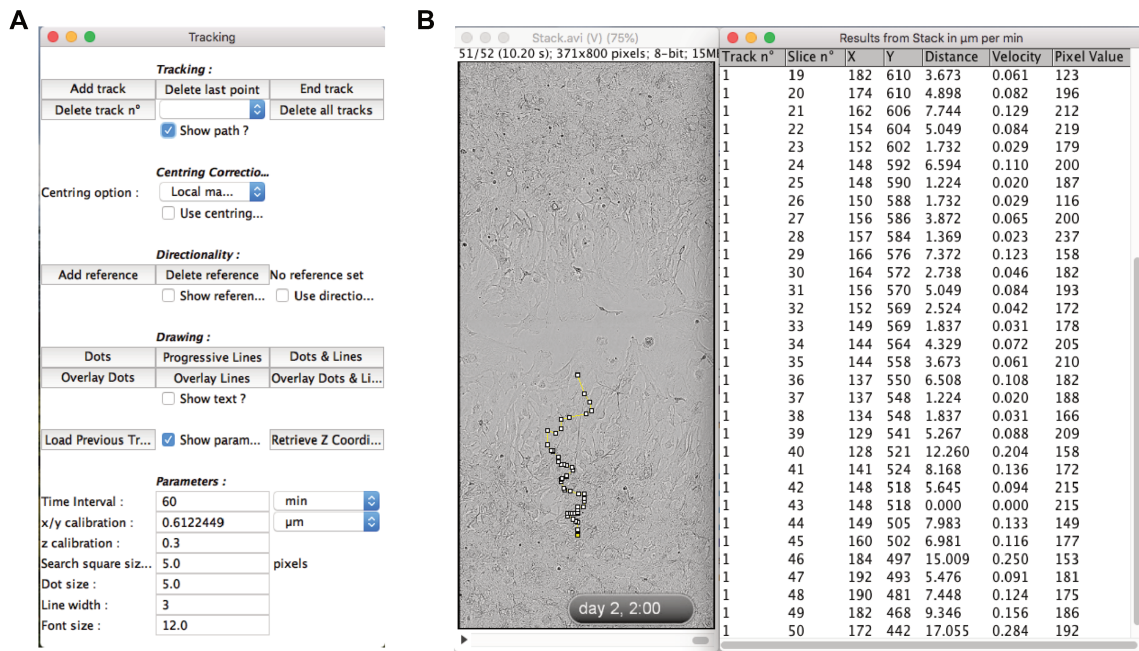


Figure 4 - Individual cell tracking procedure to study the speed, directionality and displacement of individual astrocytes.

(A) Screen shot of the “Manual Tracking” plugin window showing the tracking options and parameters to set up prior to the analysis. (B) Example of one astrocyte path tracked throughout 50 h of migration and the raw data retrieved by the plugin. (C) Example of the projection of the migration trajectory of 20 astrocytes on the X/Y coordinates (in μm) obtained using the DiPer macros in Microsoft Excel.

E. Morphological complexity (Neurons and Oligodendrocytes)

In the following protocols the analyses measure the changes in length and branching of neurites and oligodendrocyte processes over time.

(a) Neurite development:

1. Seed primary neurons at 30,000 cell/well on a coated 96-well plate and keep the cells in neuronal medium (see **Notes 1, 5 and 10**). Incubate the plates at 37°C overnight before placing them in the Incucyte® 16 hours later (in the next morning).

2. Set the system to acquire one phase-contrast picture (see **Note 11**) every 3 h over 7 days, using the “Standard” type of scan and the 20X objective, (see **Notes 12** and **32**).
3. Set the image analysis, starting by creating a representative set of images taking into account the cell morphology to be analyzed during the course of the experiment (see **Note 14**). To this end, select the first picture and click on “Create or Add to Image Collection” (Figure 1 option **A**), select the mode “Neurotrack” as job type and add the image to the new image collection. Select another 4-6 new images (see **Note 15**) and add them to the previous collection.
4. Using this collection of images, define the settings to be applied to the analyses. Under the option “Process Definition” (Figure 1 option **B**) run the “Preview Current” on the first picture to automatically generate the “Phase Cell-Body Cluster Mask” and a the “Phase Neurite Mask” (See **Note 33**). The first will cover and contour only the cell-body surface, while the second will trace what the software assumes to be neurites (cell processes).
5. Due to the complexity of the neuronal cultures, the automatic mask may not be able to perfectly trace all neurites. In that case, select “Phase Neurites (Analyze)” and adjust the masks in order to fully cover and delineate the cell shape. To this end, use the filters “Cell/Background”, “Size” and “Eccentricity” to adjust the “Phase Cell-Body Cluster Mask” (see **Note 16**), and the neurite sensitivity parameters (see **Note 34**) for the “Neurite Mask” refinement. These option and filters are available on the “Cell-body Cluster” and “Neurite” parameter panels.
6. Apply the defined settings to all the image collection by running “Preview All” and check if the masks faithfully reproduce the complexity of the neuronal cell shape (Figure 5A).
7. Initiate the analysis selecting the “Launch New Analysis Job” (Figure 1 option **C**) and select the “NeuroTrack” job type. The software will apply the settings previously defined to all the pictures acquired on the plate throughout the experiments (see **Note 17**).
8. The results will measure the changes in neurite length and number of branching points per well surface over time (Figure 5B) (see **Note 21** and **35**).

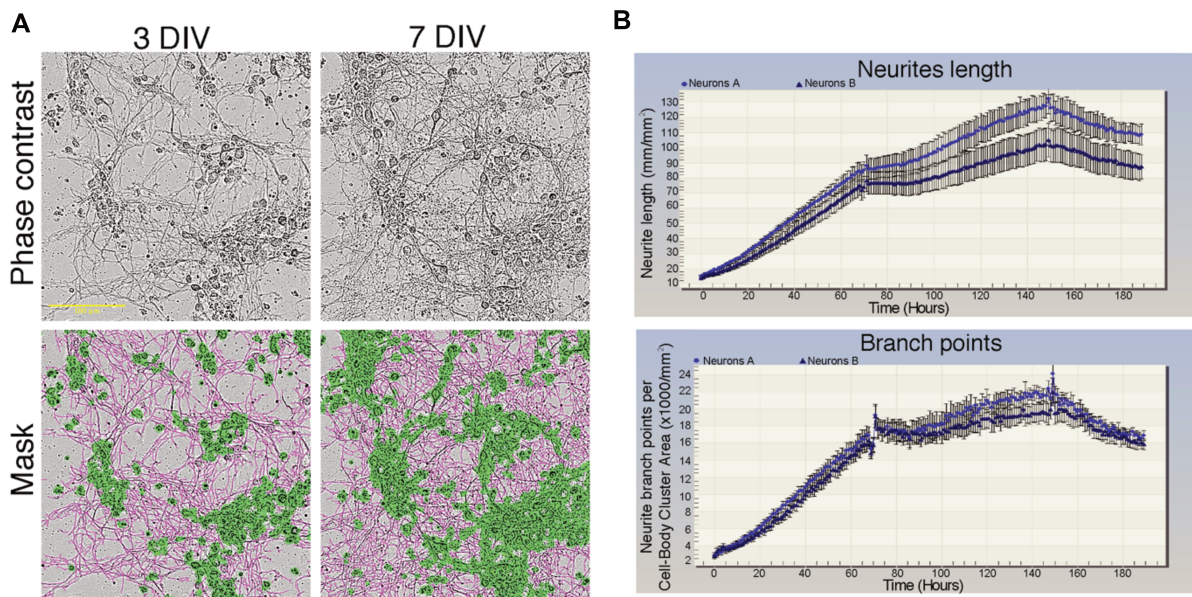


Figure 5 - Semi-automated and label-free measurement of neuritogenesis.

(A) Phase contrast (top) and respective blended mask (bottom) images of neurons at 3 and 7 days of in vitro (DIV) development. The mask images show the perfect fitting of the “Phase Cell-Body Cluster Mask” (green) on neuronal cell bodies. The appropriate “Phase Neurite Mask” (pink) traces neurite development and growth over time. **(B)** The length of neurites (mm per mm² of plate surface) is automatically measured and plotted over time, during the entire course of the experiment. The number of branch points over time is counted and normalized relative to the area occupied by the cell bodies. Neurons derived from two different mice are plotted. Inflection points illustrate the moments when the medium of the cells was changed.

(b) Process extension:

1. Seed OPC at 5,000 cell/well on coated 96-well plates and keep the cells in OPC medium for two days to stabilize the culture (see **Notes 1, 5 and 10**).
2. Set the system to acquire one phase-contrast picture every 2 h over 8-10 days, selecting the “Standard” type of scan and using the 20X objective (see **Notes 12 and 32**).
3. After 2 days, replace the medium with oligodendrocyte medium to induce OPC differentiation into mature, fully ramified oligodendrocytes. Change the medium every 2-3 days (see **Notes 7 and 13**).
4. To set the analysis, start by creating a representative set of images taking into account the changes in cell morphology that occur over time and that you will monitor over the course of the experiment (See **Note 14**). Proceed as described in section II.E(a) (Neurite development, steps 4-5). Please note that the “Neurotrack” algorithm was developed to analyze neurons, so the automatic mask may not be able

to perfectly detect the thin oligodendrocytes processes and secondary branching (See **Note 34**). To adjust the “Neurite Mask” to better fit oligodendrocyte processes and branching, set the “Neurite” parameters to maximum sensitivity (See **Note 36**) (Figure 6A)

5. Launch the analyses as described in section II.E(a) (Neurite development, step 7).
6. The results will show the length of the processes and the number of secondary branching per cell-body cluster, thus reflecting the cell differentiation over time (see **Note 21** and **35**) (Figure 6B and 6C).

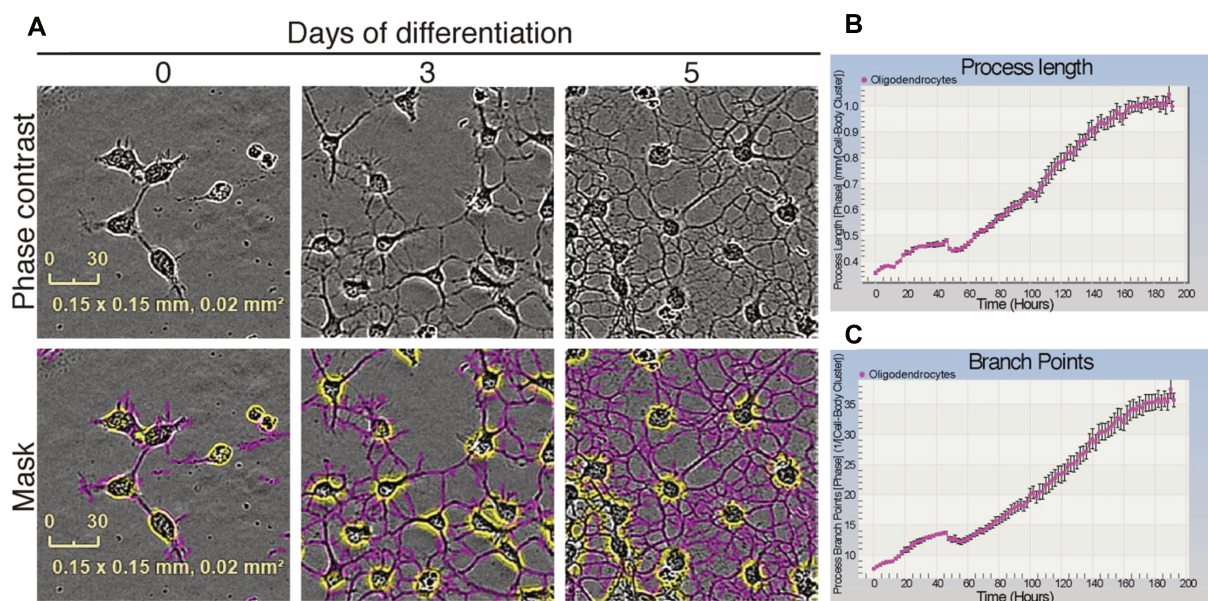


Figure 6 - Analysis of oligodendrocyte differentiation by semi-automated measurements of process extension and branching.

(A) Phase contrast (top) and respective blended mask (bottom) images of oligodendrocytes at different stages of *in vitro* differentiation. The mask images show the perfect contour of the cell bodies by the “Phase Cell-Body Cluster Mask” (yellow) and the tracing of the oligodendrocyte processes and secondary branching by “Phase Neurite Mask” (pink). (B) The imaging system measures and plots the length of cell processes per cell body cluster over 7 days of differentiation. (C) The number of branch points relative to cell body clusters is plotted over the course of the experiment.

(c) *Co-cultures analyses:*

This type of analysis allows the investigation of neuro-glia interaction. In particular, this protocol describes how to assess the effect of astrocytes on neurite development by measuring the neurite length and branching of red fluorescent neurons over time, in the presence of astrocytes.

1. Seed primary neurons at 30,000 cell/well on a coated 96-well plate and keep the cells in neuronal medium supplemented with 5% of FBS (see **Notes 1, 5 and 10**). Incubate cells at 37°C for 4 h.
2. Remove the culture medium. Infect neurons with IncuCyte® NeuroLight Red Lentivirus with the diluted lentivirus at a multiplicity of infection (MOI) of 3 TU/cell (see **Note 37**) in 200 µL of neuronal medium. Incubate cells with the lentivirus overnight at 37°C.
3. The next morning (after 8-10 hours of lentiviral infection), remove 190 µL of lentivirus-containing medium, wash once with neuronal medium, and plate 50,000 previously cultured primary astrocytes in 200 µL of neuronal medium per well, on top of neurons. Perform half-medium changes every 3 days (see **Notes 7 and 13**).
4. Place the plate on the Incucyte® imaging system and set the scan to acquire one image every 3 h with the 20x objective for 10 days, selecting both phase contrast and red fluorescence channels and using the “Standard” type of scan (see **Notes 10 and 12**).
5. Two days after the beginning of the acquisition (when the NeuroLight red signal in the neurites is sufficiently bright) optimize the “z” focus of the Incucyte® objective manually, by moving its scale ring (usually 1.2 it is a good starting point). Test the “z” focus by clicking on “Scan on Demand” and scan some wells of the plate each time you move the ring, until you get a good image resolution of the red neurites across the entire plate.
6. Set the image analysis. Start by creating a representative set of red fluorescent images taking into account the cell morphology and cell structures to be monitored over time (see **Note 14**). To this end, select the first picture and click on “Create an Image Collection” (Figure 1 option **A**), select the mode “Neurotrack” as job type and add the image to the new image collection. Select another 4-6 new images (see **Note 15**) and add them to the previous collection.
7. Using this collection of images, define the settings to be applied to the analyses. Under the option “Process Definition” (Figure 1 option **B**) run the “Preview Current” on the first picture to automatically generate the “Cell-Body Cluster Mask” and a the “Color Neurites Mask”. The first will cover and contour only the cell-body surface, while the second will trace what the software presumes to be neurites.

8. If the automatic mask is not able to perfectly trace all neurites, adjust it in the “Color Neurites (Analyze)” panel in order to fully cover and delineate the cell shape using the filters “Cell-Body Cluster Parameters”, “Cleanup”, “Cell-Body Cluster Filters” and “Neurite Parameters” for the “Color Neurites Mask” (see **Note 34**) (Figure 7A).
9. Launch the analysis as described in section II.E(a)(Neurite development, steps 6-7).
10. The results will measure the changes in the neurite length and number of branching points per well surface of the red fluorescent neurons over time (See **Note 35**) in the presence of unmarked co-cultured astrocytes (See **Note 38**) (Figure 7B and 7C).

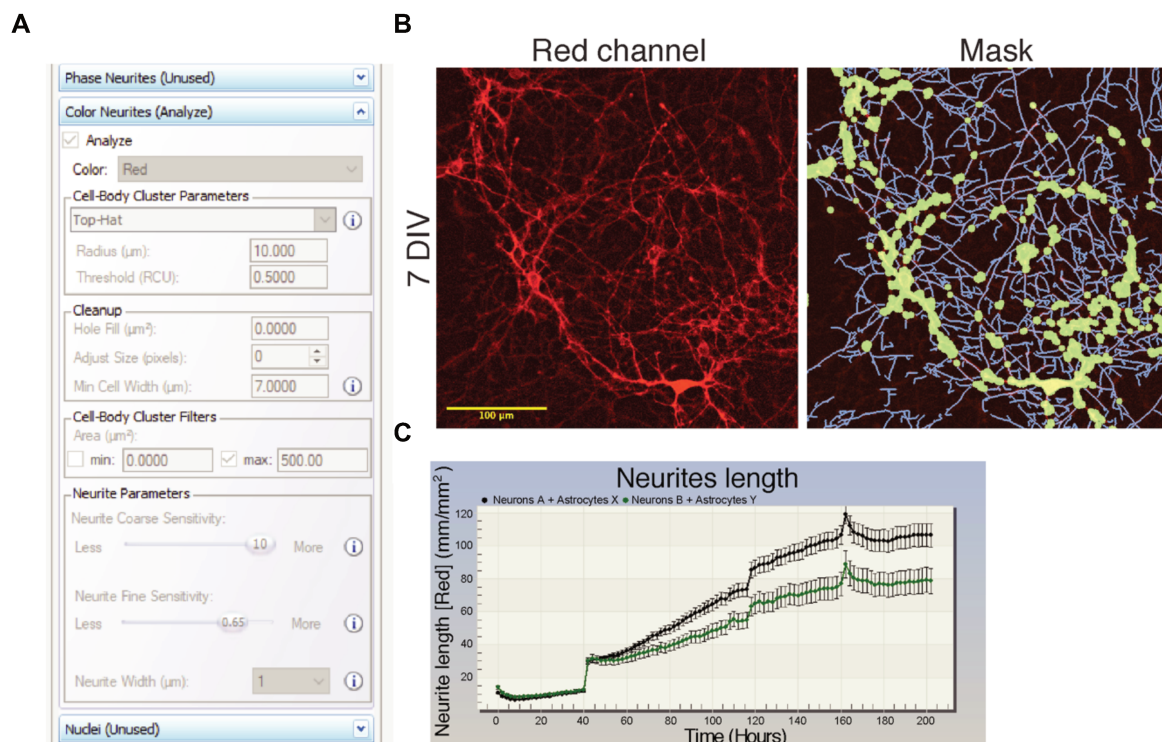


Figure 7 - Monitoring of neuronal neuritogenesis on a monolayer of astrocytes.

(A) Screen shot of the “Color Neurites (Analyze)” panel of filters used to define and fit the settings to identify and measure correctly red-labeled neurites. The settings will be applied to the entire analysis. **(B)** Neurons imaged at 7 days *in vitro* (DIV) transduced with InCuCyte® NeuroLight Red Lentivirus (left) and the blended mask (right). The “Cell-Body Cluster Mask” (green) contours the cell bodies, while the “Color Neurites Mask” (blue) delineates red fluorescent neurites. **(C)** Neurite length is measured during the course of the experiment and plotted in mm per mm² of plate surface over time. Two different combinations of neurons and astrocytes co-cultures are plotted. Inflection points illustrate the moments when the medium of the cells was changed.

IV. Notes

1. Different extracellular matrix coating protocols and reagents can be used according with the experiment (*e.g.* to study cell-to-matrix interactions) and cell type used. First, coat plates with PDL for at least 20 min at room temperature and wash three times with double distilled sterile H₂O. Second, coat plates with laminin at least 3h at 37°C and wash the plates twice with 1X PBS.
2. The described cell culture protocols can be used to culture cells from either one single embryo/pup or pooled animals.
3. To obtain mouse embryos at E16.5 plan the mice breeding in advance. For efficient mouse mating, we recommend to cross in the same cage one male with one/two females in proestrus/estrus phase in the evening, and check for vaginal plugs in the morning of the next day. Since the coupling is not 100% efficient you may want to scale up the number of breeding couples. Also some strains/transgenic mice have lower breeding rates, which decrease the number of embryos. For the P0-P2 mice pups, one male and two females were maintained continuously in the same cage.
4. For the cell counting dilute the cells in trypan blue and count them using a Neubauer chamber. Other counting methods/instruments can also be used.
5. For the protocols described, in our laboratory we use 96 well plates (unless stated otherwise), since this type of microplate allows the use of a lower cell number and volume of reagents and a high number of biological and experimental replicates per plate. However the Incucyte® imaging system supports a high variety of common cell vessels (listed in the manufacturer datasheets) that can be used accordingly with the cell type/experiment need. In addition the system can accommodate and read six microplates simultaneously, allowing high throughput processing of samples and easy reproducibility of the results.
6. The purity of the primary cultures can be verified by immunostaining detection of cell type-specific markers, such as NeuN for neurons, GFAP for astrocytes and Olig2 for oligodendrocytes.

7. Medium should be changed with fresh pre-warmed medium at 37°C. Cell culture medium should be replaced carefully avoiding to touch or disturb the cell layer at the bottom of the plate.
8. In most experiments described we used astrocytes cultured on uncoated plates during 2 weeks *in vitro*, unless stated otherwise.
9. The cell density is a critical issue for the OPC culture: suboptimal densities can lead to cell death (if cell density is too low) or undesired cell differentiation (if cell density is too high). The OPC and oligodendrocytes are loosely attached to the surface of the dishes, for this reason medium changes should be performed with extra care, using pre-warmed medium and gentle handling of the plates.
10. The cell densities indicated are suitable for the cell types mentioned in the protocol. However for other cell types, optimal seeding densities should be established prior to the experiment. After seeding, incubate the plates 15 min at room temperature to allow the cells to distribute evenly across the bottom of the plate, before placing the cells in the Incucyte® incubator. Wait 15 min before starting the scan, to allow the dissipation of eventual condensation accumulated on the plate lid.
11. In addition to the acquisition of images in a stain-free manner, the system is also able to detect fluorescence, which diversifies the array of possible experiments. Notably, the fluorescent labeling of nuclear DNA is an alternative method to precisely count the number of cells and monitor cell proliferation, or the fluorescent labeling of neurites for the length and branching measurements (as described in section 3.4).
12. To avoid variability resulting from scanned images collected from non-representative areas of the well (in case of uneven cell distribution), it is recommended to select multiple pictures per well by editing the “Scan Pattern”.
13. To change the cell medium while the experiment is ongoing, remove the plate from the machine in between two scans, change the medium and reintroduce the plate in the machine before the next scan begins.
14. The aim of creating an image collection is to have a set of representative images to train and test the settings that will be subsequently used for the analysis.

15. Although the number of pictures in the image collection is not limited, adding too many pictures slows down the process of setting definition. The selected images should represent all the possible cell variations in shape, density or other relevant parameters over time and/or across all the conditions tested. Pictures with bizarre cell density or poor image quality should not be included in the image collection and they should be excluded from later data analyses. For example, pictures taken close to the border of the well may show non-homogeneous cell density, due to uneven seeding.
16. The “cells/background” sliding cursor is useful to distinguish more or less cells from the background. If the culture is clean from cell debris and/or the cells are flat/low contrasted, the cursors should be shifted towards the cell side to filter in the entire cell. In contrast, if the culture contains cell debris and the cells have a good phase-contrast, the cursor should be placed towards the background. Additionally, as the cell debris is normally round and of smaller size than cells, it can be filtered out by the “size” and “shape” filters - increasing the minimum “size” and “eccentricity” of the object to be considered as cells.
17. For the same group of acquisitions (“Vessel Scans”) we can generate as many image collections and process definitions as we feel necessary to better adapt the analysis to the biological process being studied. Those can be applied to all the scans at any time of the experiment while they are acquired by the imaging system,, using the option “Open Ended”. The good fitting of the mask can be checked for each individual image once the analysis is launched and finished.
18. Using the “Cell Confluence Mask” as a measurement on cells with high cytoplasm spreading and low contrast can be difficult, because different biological processes, such as cell adhesion/spreading and proliferation, cannot be easily distinguished. To overcome this potential problem cell adhesion/spreading can be discriminated from proliferation by focusing the analysis on the initial 45 min after cell seeding. Additionally it is possible to export single images from the Incucyte® software and manually analyse the cell surface with another software (*e.g.* freehand selection tool available in ImageJ or FIJI).
19. This is a limitation common to other methods to measure cell growth, such as the impedance-based monitoring system xCELLigence® RTCA MP system (ACEA

Biosciences Inc). Although the xCELLigence® is able to detect in real-time very fast changes in cell index (based on the measurement of electrical impedance caused by the cell growth and attachment of the cells to the plate), the results are more difficult to interpret due to the lack of visual readout.

20. The software also counts the number of “objects” (cells) over time, as a measurement of cell proliferation. However, using phase-contrast pictures for this measurement can be misleading. As a result of proliferation, the cells become, naturally more confluent, and the individual cells previously identified as isolated “objects” become adjacent and make physical contact. They are no longer distinguished, as two distinct objects, but as a single one. Consequently the “cell count” provided by the software is underestimated, and it may even decrease over time, while the real cell number on the plate goes up. A proposed solution to overcome this problem is the use of fluorescent live-cell nuclear markers.
21. The results can also be illustrated by an overview of the global population dynamics in the entire plate, and individual plots can be drawn for each well of the microplate. They can also be exported as a numerical data file for subsequent statistical treatment and graphical representation by specialized software.
22. For astrocytes, uncoated plates work well and the wound takes about 50 h to close. However, the coating accelerates cell adhesion and migration, and it can help studying interactions between the cells and the extracellular matrix. For OPC the coating is necessary for cell adhesion. Although single coating with PDL can be used, allowing the wound to close over 72 h, PDL+laminin coating increases the speed of wound healing, which is completed after 48 h. Hence, the duration of the experiment will depend on the type of coating used.
23. The use of “Incucyte ® Image lock” special plate is crucial to assure that all the scans will be performed at exactly the same position throughout the experiment, since each well is marked with a cross that the IncuCyte® will use as a reference point for the positioning of the picture within the well. This will avoid variability in the scan position and therefore in the analysis.
24. Remember to clean the Incucyte® wound maker before using it with successive 5 min washes with 45 mL of double distilled H₂O and 45 mL of 70% Ethanol, using

the reservoirs from the kit. While wounding the plates, all wells of the plate should contain 100 μ L of medium or PBS 1X. After finishing wounding your plates, clean again the wound maker with successive 5 min washes of 45 mL of 0.5% Alconox, 1% Virkon, double distilled H₂O and 70% Ethanol. To maintain the good working conditions of the instrument it's extremely important to never turn the pin block of Incucyte® wound maker upside down, or the solutions/media will crystalize inside the pins and impair the scratching.

25. After the wound is made, lift the pin block straight up to avoid damaging the front of migration. This step will create a precise and reproducible wound throughout the 96-well plate. Using the wound maker instead of the classical pipette tip presents a great advantage since it maximises the homogeneity of the wound between wells, it guarantees the regularity of the fronts of migration and it reduces cell lifting. Overall the process is time saving: the 96 wells of a plate can be scratched in parallel in one single passage of the wound maker.
26. Changes the filter parameters in the “Segmentation Adjustment” panel will change both “Confluence Mask” and “Scratch Wound Mask”. It is not possible to apply different parameters to the different masks.
27. Although this system, like the classical wound healing assays [30], does not avoid the damage-induced cell proliferation, the analysis with RWD algorithm automatically normalizes the confluence in the wound space to the confluence in the cell layer, excluding the contribution of factors other than migration (*e.g.* proliferation or cell death) towards the filling of the wound.
28. To export the videos from the IncuCyte® go to “Utilities>Export Movie or Image set” and then select the right time range (from the beginning of the experiment until the moment when the wound is closed for all conditions) and the right wells. At least one video should have the scale (“Legend”) and Timestamp should be included in all videos, as a reminder for further analysis. Use “Windows AVI video (uncompressed)” format to ensure compatibility with Fiji.
29. Set the scale in Fiji by using the line tool to measure the scale on one Incucyte® image and then select “Set Scale” in the Fiji “Analyze” menu. Remember to check the “Global” box in order to apply the scale to all further images.

30. To better visualize the nucleus of the cells the stack can be flipped on the Z scale (time scale) to start the analysis from the end of the video, when the cell cytoplasm is widely spread. Once delineated, the tracking position data can be flipped back using Microsoft Excel.
31. The color of the migration images can also be processed to facilitate the visual detection of the nucleus. We recommend converting the images to 8-bit and applying the “HiLo LUT” palette.
32. Depending on the experiment the acquisition can be done over different time periods.
33. If you label neurites fluorescently, use the “Color Cell-Body Cluster Mask” and the “Color Neurite Mask” to define the settings for analysis.
34. Use the “Filtering” parameter to filter out small imperfections (*e.g.* due to irregular coating) or cell debris that can be mislabelled as neurites. This parameter can also be used to filter in thinner neurites/cell processes, through the selection of the “Better” or “Best” options in addition to the “Neurite Sensivity” slider. Define the pixel size at which the cell bodies transit into neurites/cell processes using the “Neurite Width” dropdown.
35. The neurite length and branching results can be quantified relative to the area of the well, to the cell-body cluster number or area. For neurons, the quantification of the neurite length per area is normally used, when the physiological neurite dynamics is being tested, since the number of neurons and cell body area will not change over time. However, when compounds are tested, the normalizations to cell body should be considered to exclude confounding effects, such as cell death. For oligodendrocytes, it is more accurate to normalize the measurements of cell processes relative to the cell body clusters, in an attempt to minimise the influence of possible alterations in cell number. The cell-body dynamics is estimated by measuring the percentage of confluence.
36. We adapt the “Neurotrack” algorithm to measure the process extension and branching of oligodendrocytes. However neurites and axons are bigger and thicker than the oligodendrocytes processes. As a result the settings must be adjusted to the highest sensitivity to maximise the detection of truly thin

oligodendrocytes processes and branching, while distinguishing them from cell bodies.

37. A MOI of 3 TU/cell was optimized in our laboratory to infect primary cortical neurons. For other neuronal types the optimal MOI must be determined.

38. The described protocol is useful to test, for instance, the beneficial/deleterious effect of an astrocyte monolayer of a given phenotype on neuronal differentiation, and the response to environmental stress, such as the addition of a neurotoxic compound (*e.g.* excess of glutamate).

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Chapter III

Developmental delay in myelination and oligodendroglia lineage progression in DMSXL mice

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Unpublished data

I. Abstract

Myotonic dystrophy type 1 (DM1) is a trinucleotide repeat expansion disorder that affects multiple tissues, notably the central nervous system. Expanded CTG repeats in DM1 brain lead to debilitating neurological symptoms, histological abnormalities and structural changes, including predominant white matter lesions. However, we still do not know which cell types and molecular pathways are primarily affected in the brain to explain the etiology of the neurological manifestations. Using a transgenic mouse model of DM1, the DMSXL mice, we have studied the molecular and physiological consequences of toxic CUG transcripts in myelination and oligodendrocyte homeostasis. We have found toxic RNA accumulation in oligodendrocytes *in vivo*, as well as missplicing in oligodendrocytes isolated from adult DMSXL brains (two important hallmarks of ongoing RNA toxicity, typical of DM1). The expression of toxic CUG RNA in oligodendrocytes is associated with reduced thickness and lower density of myelinated axons in the corpus callosum in young DMSXL mice. Delayed myelination in young mice is accompanied by a reduced number of mature oligodendrocytes and downregulation of myelin proteins expressed by fully mature oligodendrocytes, which partially recovered in older animals. Abnormal myelin protein expression was also detected in independent MBNL-deficient mouse models of DM1, as well as in human patients, clearly corroborating myelin pathobiology in this condition, and demonstrating the contributing role of MBNL inactivation. Our results demonstrate for the first time myelination defects in DM1, and provide molecular, cellular and structural support for the imaging abnormalities reported in humans.

Key words:

Myotonic dystrophy type I, transgenic mouse model, RNA toxicity, hypomyelination, oligodendroglia lineage.

II. Introduction

Myotonic dystrophy type 1 (DM1, OMIM 160900) is a complex autosomal multisystemic disease with broad clinical manifestations (Udd and Krahe, 2012). Beside muscular and cardiac defects, the central nervous system (CNS) impairment has a profound impact on the quality of life of DM1 patients. The phenomenon of anticipation, characteristic of this disease, is particularly reflected in the CNS and characterized by increased severity of the symptoms appearing at earlier ages from one generation to the next (Ashizawa and Sarkar, 2011). DM1 brain dysfunction ranges from typical executive dysfunction, disruptive behavior (such as apathy and lack of motivation), hypersomnia and impaired social cognition in adult patients, to pronounced intellectual disability with reduced intelligence quotient (IQ) and speech impairment in the congenital cases (Gourdon and Meola, 2017). DM1 brains show anatomic defects, such as ventricular enlargement, global cortical atrophy and decreased white matter fractional anisotropy, in both motor and non-motor related regions (Okkersen et al., 2017, Minnerop et al., 2018), together with connectivity defects (Schneider-Gold et al., 2015, Serra et al., 2016). Imaging studies revealed that DM1 neuropathology affects primarily white matter, while grey matter is affected to a lesser extent. However, we do not know which cell types and pathways contribute to these structural changes and ultimately to CNS dysfunction.

DM1 is caused by the expansion of an unstable non-coding CTG repeat in the 3' untranslated region (UTR) of the DM protein kinase (*DMPK*) gene (Brook et al., 1992). The repeat length correlates with disease severity: larger repeats with more than 1,000 CTG repeats occur in the congenital form, while mild and late-onset patients carry between 50-100 CTG repeats (Harley et al., 1993). Expanded *DMPK* transcripts accumulate in the nucleus, forming RNA foci and perturbing the function of RNA-binding proteins (Braz et al., 2018). Among these, muscleblind-like (MBNL) proteins are directly sequestered by the foci, whereas members of the CUGBP/Elav-like family (CELF) are upregulated. Together, the lack of functional MBNL and excess of CELF proteins disturb the downstream processing of target transcripts, in particular the developmental alternative splicing program of a subset of transcripts (Wang et al., 2012, Sicot et al., 2011, Hernandez-Hernandez et al., 2013b, Hernandez-Hernandez et al., 2013a, Goodwin et al.,

2015, Batra et al., 2014, Braz et al., 2018). Although RNA foci accumulation and consequent dysregulation of MBNL and CELF proteins were previously observed in the brain (Jiang et al., 2004, Dhaenens et al., 2011, Hernandez-Hernandez et al., 2013a), today the cellular distribution of these events, the individual susceptibility to RNA toxicity of each cell type or their contribution to DM1 neuropathogenesis are still unknown. Furthermore, the connection between dysfunctional cellular processes, structural and functional alterations and DM1 neurological symptoms is yet to be uncovered.

To gain insight into the DM1 brain disease, we have investigated the DMSXL mice, a transgenic model of this condition that expresses human *DMPK* transcripts with more than 1,000 CTG repeats in multiple tissues (Seznec et al., 2001, Gomes-Pereira et al., 2007). Expression of toxic transcripts in homozygous DMSXL is sufficient to trigger the accumulation of RNA foci and the dysregulation of alternative splicing, affecting muscular, cardiac and respiratory function (Huguet et al., 2012, Algalarrondo et al., 2014, Panaite et al., 2013). In the CNS, CUG RNA toxicity results in cognitive and behavioral deficits, associated with synaptic dysfunction (Hernandez-Hernandez et al., 2013a, Sicot et al., 2017). Additionally, in the peripheral nervous system (PNS) DMSXL mice show myelin alterations, including reduced number of unmyelinated axon and decreased myelin thickness in phrenic nerves (Panaite et al., 2013). Together these findings corroborate ongoing RNA toxicity in the nervous system of DMSXL mice, however, the molecular and cellular mechanisms leading to the neuropsychological impairment typical of DM1 are not entirely known.

Myelination starts in the early postnatal period, by the ensheathment of larger axons in the major fiber tracts of the CNS (Simons and Lyons, 2013). Myelin defects in neurodegenerative and neurodevelopmental disorders are often characterized by hypomyelination, associated with the lack of oligodendrocytes (Bartzokis et al., 2007, Pacey et al., 2013, Zhou et al., 2017, Aranmolate et al., 2017). Given the prevalent white matter lesions reported in patients, and the role of myelin in the neuropsychological functions altered in DM1, we have sought to investigate the implication of oligodendrocytes and myelin in DM1 brain disease. Using DMSXL transgenic mice, we have described for the first time the presence of toxic RNA foci in oligodendrocytes, in

association with a transient hypomyelination and corpus callosum (CC) hypoplasia in young DMSXL brains, likely due to impaired in oligodendrocyte differentiation.

III. Material and methods

A. Transgenic mice

All experimental procedures using animals followed the ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines. This project was conducted under the authorization for animal experimentation n° 75003 in the animal facility with the approval n°: B 91228107 both delivered by Prefecture de Police and the French Veterinary Department. Both DMSXL and DM20 transgenic mice (>90% C57BL/6 background) used in this study carry 45 kb of human genomic DNA from a DM1 patient or a healthy control individual, respectively (Seznec et al., 2000, Gomes-Pereira et al., 2007). In the DMSXL mice the *DMPK* transgene includes >1,000 CTG repeats, while DM20 mice carry a non-pathological expansion with 20 CTG repeats. DM20 transgenic status was assessed as previously described (Hernandez-Hernandez et al., 2013a). DMSXL genotype was determined by multiplex PCR on DNA extracted from mouse tails, using oligonucleotide primers hybridizing within the *DMPK* transgene and the transgene integration site in the mouse *Fbxl7* gene: FBF (forward, TCCTCAGAAGCACTCA TCCG), FBFBR (reverse, AACCTGTATTTGACCCAG) and FBWDR (reverse, ACCTCCATCCTTTCAGCACC). FBF and FBFBR primers hybridize the mouse *Fbxl7* gene, while the FBWDR primer hybridizes specifically in the human *DMPK* sequence. In the wild type (WT) mice the amplicon generated has 167 bp, which is distinguishable from the 236-bp amplicon produced in homozygous DMSXL samples. Both female and male mice were used in this study. *Mbnl1*, *Mbnl2* and *Mbnl1/Mbnl2* double knockout (KO) mice were generated, characterized and genotyped as described elsewhere (Kanadia et al., 2003, Charizanis et al., 2012, Lee et al., 2013).

B. Human tissue samples

Brain samples from DM1 patients and non-DM controls were collected by Dr. Yasuhiro Suzuki (Asahikawa Medical Center, Japan) and Dr. Tohru Matsuura (Okayama University, Japan), with written informed consent for specimen use for research

obtained from all patients or families. The Ethics Committees of the host institutions approved all experiments using human samples. Clinical information relative to patients is presented in Supplementary table 1 and Supplementary table 2.

C. Immunofluorescence (IF) and fluorescence *in situ* hybridization (FISH)

Frozen brains were prepared in cryostat coronal sections (10 µm) and fixed for 15 min in 4% paraformaldehyde (PFA). Ribonuclear inclusions were detected using a 5'-Cy3-labelled (CAG)₅ PNA probe (Huguet et al., 2012) and combined with IF detection as previously described (Hernandez-Hernandez et al., 2013a). For IF only, anesthetized mice were fixed by intra-cardiac perfusion with 4% PFA. The brains were then collected and frozen for posterior cryostat serial coronal section (10 µm). Primary antibodies and dilutions used are listed in

D. Microscope imaging and image analysis

Images were acquired with a Zeiss ApoTome 2 fluorescent microscope (x63 and x40 objectives) or a Hamamatsu Nanozoomer 2.0 slide scanner (x40 objective). RNA foci were counted in Z-stacks, using the Spot Detector plugin of the ICY bioimage analysis open source program (<http://icy.bioimageanalysis.org>). The percentage of foci-positive nuclei was calculated relatively to the total number of nuclei. Cell subpopulation counting was performed using position-matched slices (identified by cresyl-violet coloration) of Nanozoomer images. Mouse brain regions of interest were anatomically identified, and the number of positive immunolabelled cells per area was calculated. IF and FISH images were treated with Fiji - ImageJ software (Schindelin et al., 2012) to create the figures.

E. Transmission electronic microscopy

Animals at post-natal day (P) 15-17 were anesthetized and intra-cardiac perfused with a solution containing 2% glutaraldehyde / 2% PFA in phosphate buffer (pH 7.4). Brains were dissected and post-fixed overnight (ON) at 4°C in the same fixative solution

with 3 mM of CaCl₂ and transferred the day after into 0.1 M PB (pH 7.4). The samples were washed 3 times and then post-fixed with 1% osmium tetroxide in cacodylate buffer for 1 hour (h) at 4°C. After extensive wash with distilled water (3 x 10 min), sections were incubated for 2 h in 2% uranyl acetate solution. Following dehydration in a graded series of 50%, 70%, 80%, 90%, and 100% ethanol solutions (2 x 5 min each), a final dehydration step was performed in 100% acetone (2 x 20 min). The samples were then progressively infiltrated with an epoxy resin, Epon 812® (EMS, Souffelweyersheim, France): first overnight in 50% resin / 50% acetone at 4°C in an airtight container, and then in pure fresh resin at room temperature (RT) (2 x 2 h). The embedding was performed in the bottom of capsules (Beems® size 3, Oxford Instruments, Saclay, France) and the resin was polymerized at 56°C for 48 h in a dry oven.

The resulting blocks were cut in an UC7 ultramicrotome (Leica, Leica Microsystems SAS, Nanterre, France). Ultra-thin sections (70 nm thick) were recovered on copper (conventional morphology) grids and contrasted with Reynold's lead citrate technique (Reynolds, 1963). Images from the ultrathin sections were obtained with a Hitachi HT7700 electron microscope (Elexience, Verrière-le-Buisson, France) operating at 70 kV. Pictures (2048 x 2048 pixels) were taken with an AMT41B camera (pixel size: 7.4 µm x 7.4 µm), with a magnification of x26.0k(x3.0k) for g-ratio calculation and for myelin sheath counting.

CC images were used to determine the number of myelinated axons, axon caliber and g-ratios. The images were segmented using morphological filters from the MorphoLibJ plugin for ImageJ (Legland et al., 2016), after reducing the background using the rolling ball method. G-ratios of myelinated axons were determined by dividing the inner myelin sheath diameter (Feret diameter) by the outer myelin diameter.

F. Adult oligodendrocyte isolation

Brains from 1-month-old mice were dissected and the cortices were enzymatically and mechanically dissociated using the adult brain dissociation kit (Miltenyi, ref. 130-107-677), following the manufacturer's protocol. The oligodendrocytes were isolated from the resulting single cell suspension using magnetic anti-O4 labeled microbeads (Miltenyi, ref. 130-094-543) for positive selection, following the manufacturer's protocol. The cells in the O4-positive (retained in the column) and

O4-negative cell fraction (flow through) were counted and a cell pellet $<1 \times 10^6$ was collected by centrifugation at 300 g for 5 min.

G. RNA isolation, cDNA synthesis and RT-PCR analysis

RNA extraction was performed with the RNeasy Mini kit (QIAGEN, ref. 74104) following the manufacturer's protocol, with an additional DNase digestion step (RNase-Free DNase Set, QIAGEN, ref. 79254) after the first wash with RW1 buffer. RNA concentration was assessed by NanoDrop spectrophotometry and RNA quality was evaluated by electrophoresis on an agarose gel. cDNA was prepared using SuperScript™ III reverse transcriptase (Invitrogen, ref. 18080044) and a mix of 100 μ M oligo(dTs), random hexamers (50 μ M) and 10 μ M dNTPs, with 250-500 ng of total RNA input. The reaction was performed following the manufacturer's guidelines. Semi-quantitative RT-PCR analysis of alternative splicing and quantitative RT-PCR of expression levels were performed as described elsewhere (Hernandez-Hernandez et al., 2013a, Huguet et al., 2012), using the oligonucleotide primers listed in Supplementary table 4. All samples were normalized to RNA Polymerase II Subunit A (*Pol2ra*).

H. Western blots

Proteins from mouse and human brain tissues were extracted using RIPA buffer (Thermo Scientific, ref. 89901), supplemented with 0.05% CHAPS (Sigma, ref. C3023), 1x complete protease inhibitor (Sigma-Aldrich, ref. 04693124001) and 1x PhosSTOP phosphatase inhibitor (Sigma, ref. 04906845001). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, 23227). 10 – 40 μ g of total protein were mixed with 2X Laemmli Sample Buffer (Sigma, ref. S3401), denatured for 5 min at 95°C and resolved in 4-20% TGX Stain-Free polyacrylamide gels (Bio-Rad 1610183). After electrophoresis, gels were activated for 5 min under UV light, proteins were transferred onto nitrocellulose membranes using Trans-Blot® Transfer System (Bio-Rad) and the total amount of protein on the membrane was imaged using the ChemiDoc Imaging System (Bio-Rad). Membranes were then blocked in Blotto non-fat dry milk (Santa Cruz Biotech; ref. sc2325) or bovine serum albumin (BSA) (Sigma, ref. A7906-50G) in 1x TBS-T (10 mM Tris-HCl, 0,15 M NaCl, 0.05% Tween 20), during 1

h at RT and incubated with the primary antibody overnight at 4°C. Blocking and primary antibody dilutions are indicated in Supplementary table 3. Following three washes in TBS-T, membranes were incubated with IRDye® 800CW donkey anti-rabbit (LI-COR Biosciences, ref. P/N 926-32213) or 680RD donkey anti-mouse (LI-COR Biosciences, ref. P/N 926-68072) for 1 h at RT, washed three times and imaged using LI-COR Odyssey® CLx Imaging System. The intensity of immunodetected bands was quantified using Image Studio Lite software (Bio-Rad), and normalized to the total protein loaded, quantified using the Image Lab software (Bio-Rad).

I. Statistical analysis

Prism software (GraphPad Software, Inc) was used for statistical analyses. After performing a normality test on the numeric variables, we used either a parametric two-tailed Student's t-test, or a non-parametric two-tailed Mann-Whitney U test to compare two groups. When the sample size was too small to test for normality, we used a non-parametric statistical test. For one-way ANOVA, if statistical significance was achieved, we performed post-test analysis to account for multiple comparisons. Statistical significance was set at $p < 0.05$. The data are presented as mean \pm standard error of the mean (\pm SEM), or as Tukey box-and-whisker plots.

IV. Results

A. DMSXL oligodendrocytes show signs of CUG RNA toxicity

CNS function is impaired in DMSXL mice, in response to the expression of the CTG repeat expansion, which we have previously reported to occur in neurons and astrocytes (Hernandez-Hernandez et al., 2013a, Dinca et al., in preparation). To assess if toxic CUG RNA are also expressed and accumulate in the oligodendroglia cell lineage of DMSXL brains, we now focused on the corpus callosum (CC, an oligodendrocyte- and myelin-rich brain region). Fluorescence *in situ* hybridization of expanded CUG RNA detected the accumulation of toxic *DMPK* transcripts in these cells (Fig. 1A). Ribonuclear foci were present in 47.2% of oligodendrocytes at post-natal day 15 (P15), confirming ongoing RNA toxicity in this cell type. The percentage significantly decreased throughout

development, with only 20.5% of OLIG2-positive oligodendroglia showing foci at P30 (Fig. 1B). Interestingly, the number of foci per oligodendrocyte nucleus remained constant, with an average of 2 foci per cell at both ages (Fig. 1C). The accumulation of foci was accompanied by the nuclear sequestration of MBNL proteins, demonstrated by the co-localization of both MBNL1 and MBNL2 proteins with nuclear RNA foci (Fig. 1A). Given the prevalent role of MBNL proteins in the regulation of the developmental alternative splicing switch, from embryonic to adult isoforms (Lin et al., 2006), we studied the splicing of alternative exons in oligodendrocytes committed for myelination, through immunopanning isolation of O4-positive cells from brain cortices of DMSXL mice at P30. Oligodendrocyte enrichment, following magnetic cell sorting, was tested by RT-PCR analysis of the expression of genes specific of neurons or astrocytes (negative fraction), and genes specific of oligodendrocytes (positive fraction) (Supplementary Fig. 1). RT-PCR analysis showed a variable degree of spliceopathy of in DMSXL oligodendrocytes (Fig. 1D). Sorbin and SH3 domain containing 1 (*Sorbs1*), Integrin alpha 6 (*Itga6*) and *Mbnl1* displayed significant missplicing, with a pronounced 73% reduction in the inclusion of alternative exon 27 of *Sorbs1*, a 31% decrease in the inclusion of alternative exon 27 of *Itga6*, and a 50% increase in the inclusion of *Mbnl1* exon 7. Other transcripts, usually dysregulated in DMSXL mouse brains, showed either very mild splicing dysregulation or remained unchanged in DMSXL oligodendrocytes, such as microtubule-associated protein tau (*Mapt/Tau*) and *Mbnl2*, respectively.

Together, these data indicate that oligodendrocytes accumulate nuclear toxic RNA foci in a relevant mouse model of DM1, which sequester MBNL proteins, leading to a mild spliceopathy of alternative exons in this cell type.

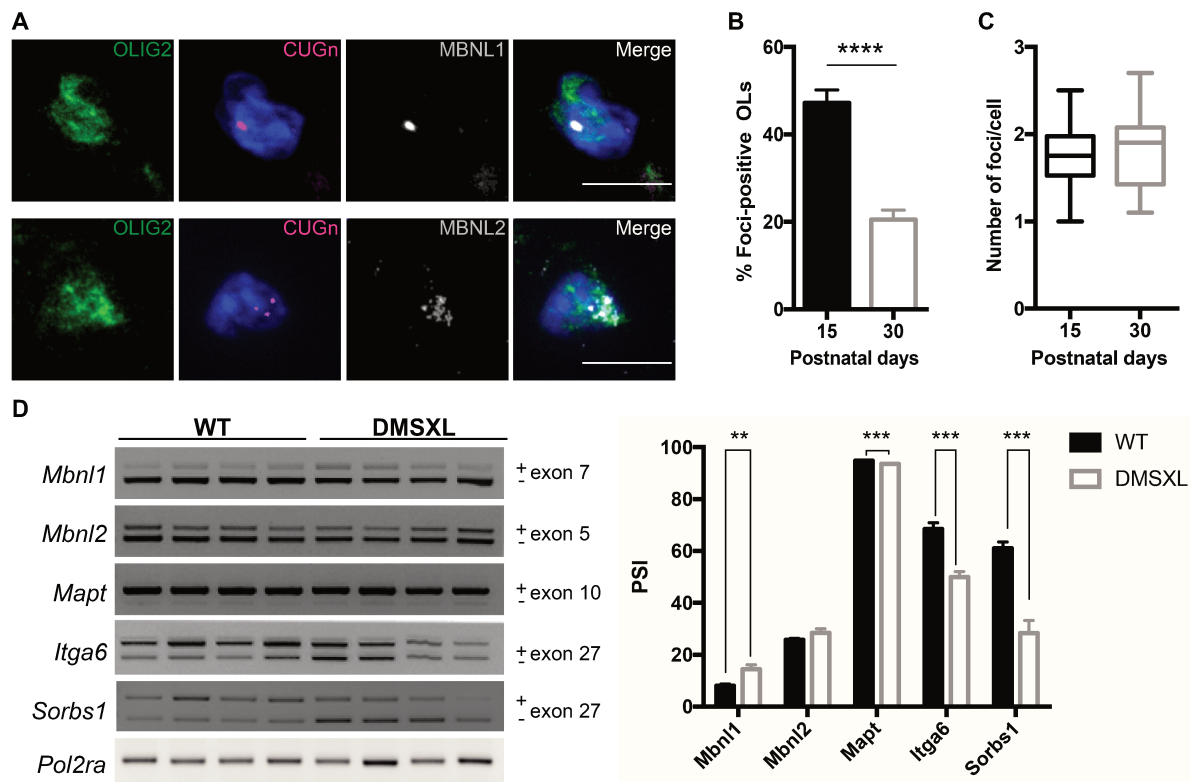


Figure 1 - Expanded CUG transcripts in DMSXL oligodendrocytes accumulate into RNA foci, sequester MBNL proteins and dysregulate alternative splicing.

(A) Representative images of combined FISH and IF showing expanded CUG RNAs (magenta) in OLIG2-expressing oligodendrocytes (green), co-localizing with MBNL1 and MBNL2 (grey) in CC from P30 DMSXL mice. DAPI was used for nuclear staining. The scale bar represents 10 μ m. (B) Quantification of the percentage (\pm SEM) of DMSXL oligodendrocytes containing RNA foci in the nucleus and (C) the average number of foci per nucleus at developmental stages P15 (n = 643) and P30 (n = 676). (D) Representative RT-PCR splicing analysis in isolated oligodendrocytes from WT and DMSXL mice (n=4) at P30, and percentage of splicing inclusion (PSI \pm SEM) of candidate alternative exons. ** p <0.01, *** p <0.001, **** p <0.0001. OL - oligodendrocytes; WT - wild-type.

B. DMSXL mice exhibit transient hypomyelination and corpus callosum hypoplasia

Following the confirmation of hallmarks of RNA toxicity in oligodendroglia cell lineage, we investigated the consequences of the DM1 mutation on brain myelination, through the analysis of myelin-rich brain regions. To this end, we performed myelin basic protein (MBP) immunodetection of myelinated tracts and inferred the thickness of the corpus callosum, the major fiber tract connecting brain hemispheres (Fig. 2A). The results showed that young DMSXL have a 34% thinner CC at P15, when compared with

WT controls. Interestingly, the thickness of the CC recovered to normal levels at 4 months, demonstrating the transient nature of this defect.

To investigate the consequences of the disease mutation on myelin ultrastructure, we performed electronic microscopy analysis in the CC of 2-week-old mice. The analysis of the micrographs revealed normal myelin thickness at axonal level, as shown by the unchanged myelin g-ratios in CC axons (an measurement of myelin thickness relative to the caliber of individual axons) (Fig. 2B). However, a lower number of myelinated axons was observed in DMSXL mice, and among those, the bigger-caliber axons (diameter $\geq 1.3 \mu\text{m}$) were preferential myelinated ($p = 0.003$) (Fig. 2C).

Together, these findings suggest that toxic RNA expression in oligodendrocytes impairs myelin development and structure, particularly in young animals, leading to

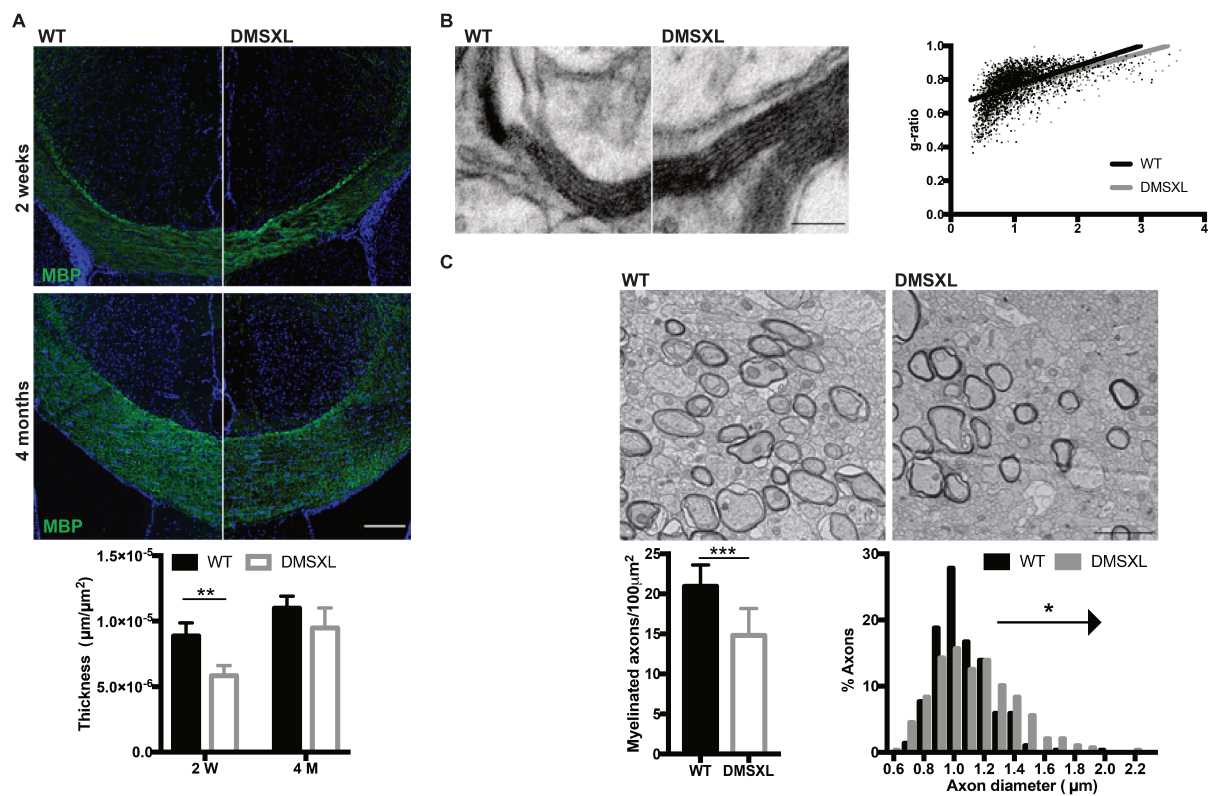


Figure 2 - Reduced thickness and myelination of CC axonal tract in DMSXL mice.

(A) Representative IF images of MBP (green) with nuclear counterstain DAPI (blue) in the CC of DMSXL and WT mice at 2 weeks and 4 months. The scale bar represents $200 \mu\text{m}$. CC thickness, relative to total section area, determined at midline in brain coronal sections from DMSXL and WT littermates. The data was normalized to the total brain section area ($\pm\text{SEM}$; $n = 5$, 2 weeks; $n = 4$, 4 months). (B) Electron micrographs of axons and scatter plot of g-ratio values in CC of DMSXL and WT mice, at 2 weeks ($n \geq 1614$ axons, total of 6 mice per genotype). The scale bar represents 100 nm . (C) Electron micrographs of axons in CC and quantification of the number of myelinated axons and percentage of axons found at each diameter range of DMSXL and WT brains at 2 weeks ($n=6$). Scale bar represents 200 nm . $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

transient hypoplasia of the corpus callosum in association with hypomyelination.

C. Impaired oligodendrocyte lineage progression in DMSXL CNS

We next asked whether transient CC hypoplasia reflected changes in oligodendrocyte number or defective oligodendrocytes maturation. To test this hypothesis, we immunolabeled brain sections of young mice (P15) that show significant myelination defects, using antibodies against markers specific of individual oligodendroglia differentiation stages: OLIG2 (oligodendrocyte transcription factor 2, pan oligodendroglia marker) detects the entire oligodendroglia cell lineage cells; PDGFRA (platelet-derived growth factor receptor alpha) specifically detects oligodendrocyte progenitor cells (OPC); CC1 (adenomatous polyposis coli (APC) clone CC1) only labels mature oligodendrocytes. The specificity of the protein markers for different oligodendroglia subpopulations, allows for an estimation of oligodendroglia cell distribution between different differentiation stages *in vivo* (Fig. 3A).

The quantification of relative cell densities showed a significant reduction of 24.5% in the global population of OLIG2-positive oligodendroglia in the CC of DMSXL mice, relative to WT littermates (Fig. 3B). Furthermore, although the pool of PDGFRA-positive OPC remains unchanged between genotypes, the number of CC1-positive mature oligodendrocytes decreased by 24% in DMSXL (Fig. 3B). The reduction of mature oligodendrocytes in CC, together with the decrease of the global oligodendroglia cell lineage points to a blocking/delay of the oligodendrocyte differentiation program in

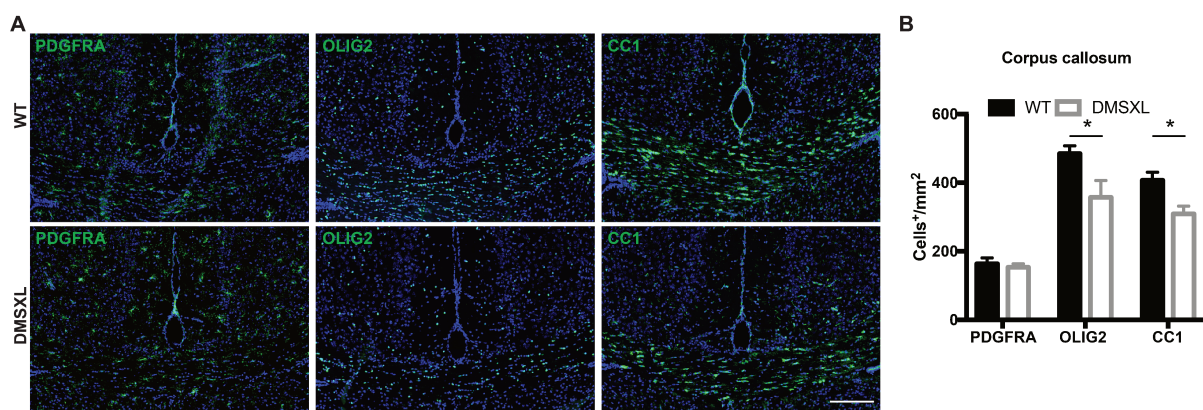


Figure 3 - DMSXL CC display defective oligodendrocyte differentiation.

(A) Representative images of P15 DMSXL and WT CC stained for OPC (PDGFRA; left) oligodendroglia (OLIG2; middle) and mature OLs (CC1; right). DAPI was used for nuclear staining. The scale bar represents 200 μm . (B) Quantification of PDGFRA, OLIG2 and CC1 cell densities in CC ($\pm\text{SEM}$; n=6-8).

DMSXL mice.

In conclusion, developing young DMSXL mice lack mature oligodendrocytes in myelin rich brain structures, most likely due to the impairment of cell differentiation.

D. CTG repeats in oligodendrocytes affects myelin protein expression in young DMSXL mice

We then extended the myelin analysis to frontal cortex, a brain region typically associated with DM1 brain neuropathophysiology. The implication of the frontal cortex in DM1 brain disease has been repeatedly suggested by neuropsychological assessment and imaging studies (Minnerop et al., 2018).

We first quantified myelin-specific proteins (produced by mature oligodendrocytes) throughout mouse development and ageing: MBP, 2',3'-Cyclic Nucleotide 3' Phosphodiesterase (CNP) and myelin oligodendrocyte glycoprotein (MOG).

Western blot analysis confirmed significant downregulation of MBP and CNP in DMSXL frontal cortex. DMSXL mice showed a pronounced 70% reduction in the steady-state protein levels of MBP at P15, when compared with the WT littermates. Although still significant at 1 month of age, the reduction of MBP was of only 45% in DMSXL mice (Fig 4A). Different MBP protein isoforms are encoded by alternative splicing of the *Mbp* pre-mRNAs throughout development (Campagnoni, 1988, Kamholz et al., 1988), as illustrated in Figure 4A. Importantly the pronounced downregulation of MBP protein in DMSXL frontal cortex at P15 is not accompanied by significant protein isoform redistribution, or missplicing of known alternative exons studied by RT-PCR (Fig. 4B). Likewise, CNP is significantly reduced by 50% in DMSXL brains at P15, relative to WT littermates (Fig. 4C).

Similar to the structural defects in CC in these mice, the expression levels of these two proteins in frontal cortex are normalized from 4 months onwards, in line with an early and transitory nature of the myelin defect. MOG, however, showed an intriguing expression profile: MOG steady-state levels were not significantly altered in young mice, but this protein was upregulated in 4-month-old DMSXL brains, followed by significantly decrease levels in aged mice at 10 months (Fig. 4D).

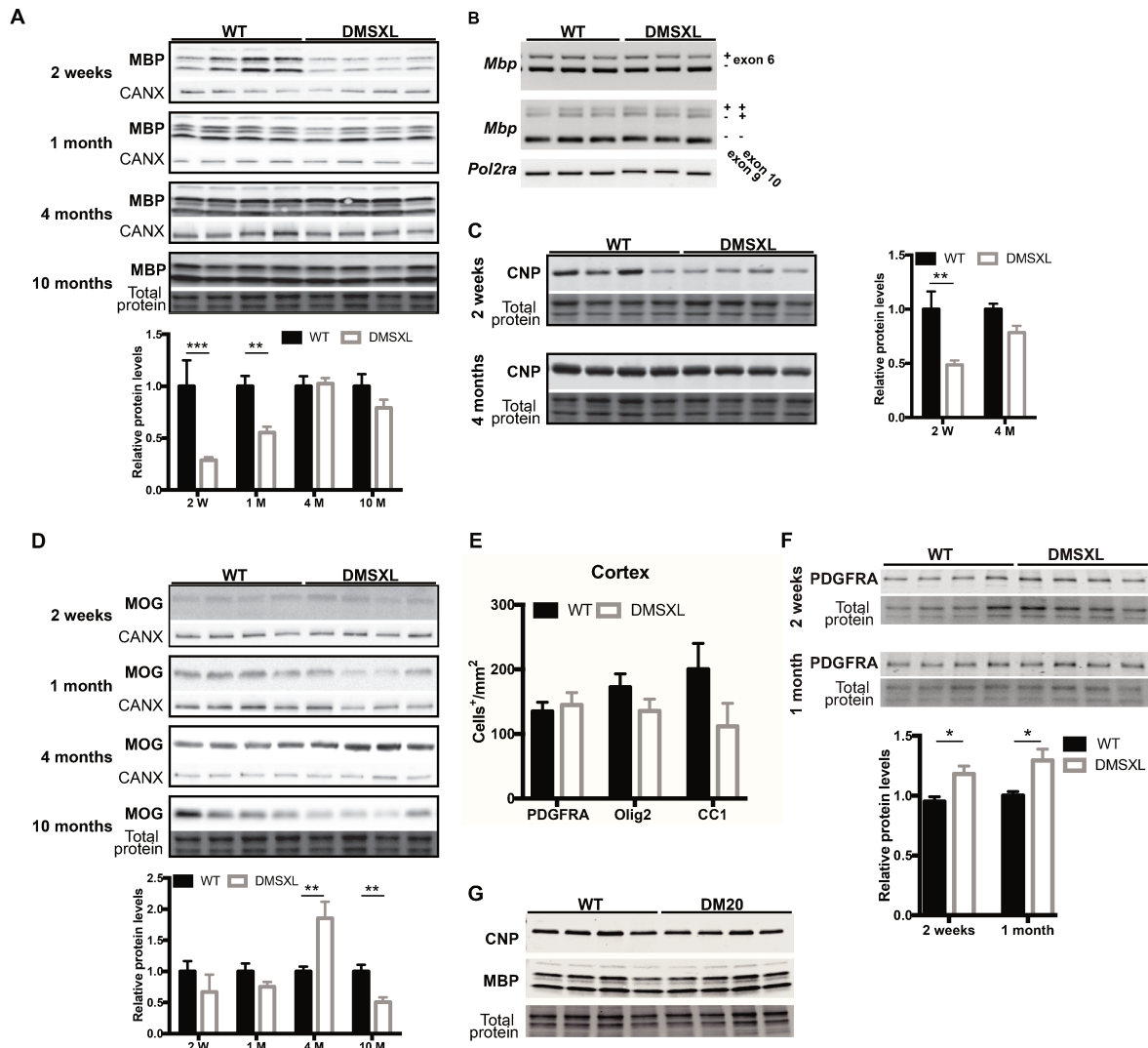


Figure 4 - Abnormal expression of myelin proteins in the frontal cortex of DMSXL mice.

(A) Representative western blot analysis of MBP in DMSXL mice aged 2-weeks, 1, 4 and 10 months, as well as in age-matched WT controls (n=6-8 per genotype). (B) Representative RT-PCR splicing analysis of *Mbp* development-regulated exons 6, 9 and 10 in 2-week-old DMSXL mice (n=6), compared with age-matched WT littermates. (C) Western blot analysis of CNP in 2-week- and 4-month-old DMSXL mice and age-matched WT controls (n=6 per genotype). (D) Western blot analysis of MOG in 2-week-, 1-, 4- and 10-month-old DMSXL mice and age-matched WT controls (n=6-8 per genotype). (E) Quantification of PDGFRA, OLIG2 and CC1 cell densities in frontal cortex at 2 weeks (n = 6-8) (F) Western blot analysis of PDGFRA protein levels in 2-week- and 1-month-old DMSXL and WT mice (n = 6). (G) Western blot analysis of CNP and MBP protein levels in 1-month-old DM20 and wild-type mice (n = 6 per genotype). Western blot quantification graphs represent average steady-state protein levels (\pm SEM), relative to normalized WT controls. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

It is interesting to note that DMSXL cerebellum did not show altered levels of MBP or MOG protein levels (Supplementary Fig. 2A), suggestive of a region-specific deregulation of myelination in young DMSXL mice. Similar to CC, abnormal myelin

biology in frontal cortex is associated with a trend towards a decrease in the density of OLIG2- ($p = 0.2$) and CC1-expressing ($p = 0.1$) oligodendroglia at P15 (Fig. 4E), together with a significant upregulation of ~20% in PDGFRA protein levels in DMSXL (Fig. 4F), relative to WT controls.

To verify if myelin protein deficits could be accounted for by the overexpression of *DMPK* transcripts alone, we analyzed MBP and CNP expression in the frontal cortex of DM20 mice, which overexpress control *DMPK* RNA containing short 20 CTG repeats (Seznec et al., 2001). DM20 mice show normal MBP and CNP levels (Fig. 4G), indicating that the protein defects in DMSXL frontal cortex are likely due to the expression of expanded CUG RNA repeats, rather than the overexpression of the *DMPK* gene alone.

E. Abnormal MBP expression and splicing in *Mbnl*-deficient mice

To assess the contribution of MBNL dysfunction to the dysregulation of myelin proteins, we studied the impact of *Mbnl* gene inactivation on the expression of MBP, the myelin protein most severely deregulated in DMSXL mice. To this end we took advantage of knock-out mice deficient for *Mbnl1* and/or *Mbnl2* (Kanadia et al., 2003, Charizanis et al., 2012, Goodwin et al., 2015). Western blot analysis showed that inactivation of *Mbnl2* was sufficient to downregulate MBP in the frontal cortex of adult knock-out mice by 78%, while MBNL1 depletion leaves MBP levels unaltered (Fig. 5A). Interestingly, and in agreement with DMSXL mice, the decreased levels of MBP protein were not associated with major change in protein isoform distribution, as detected by western blot analysis (Fig. 5B). In contrast, the simultaneous deletion of *Mbnl1* and *Mbnl2* resulted not only in a pronounced downregulation of MBP protein levels by 84%, but also in a dramatic redistribution of protein and transcript isoforms in mouse frontal cortex, with pronounced missplicing alternative exons 9 and 10 (Fig. 5A and 5C). Our data suggest that MBP downregulation in DMSXL and *Mbnl2* knock-out mice may result from mechanisms that are not exclusively driven by missplicing.

In summary, these results suggest that myelin proteins are dysregulated in developing mice, as a consequence of the DM1 repeat expansion mutation, possibly through a combination of different mediating mechanisms, including missplicing regulated by MBNL proteins.

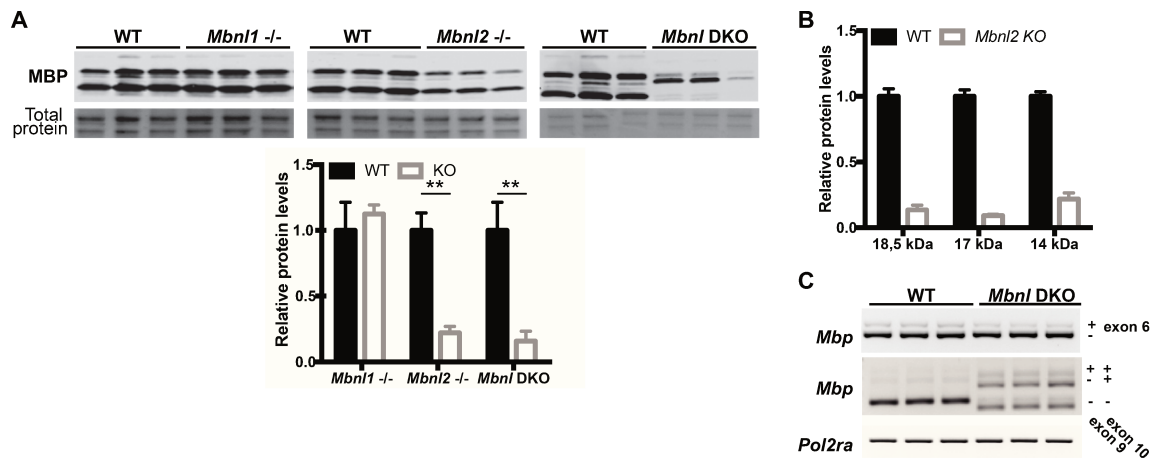


Figure 5 – MBP downregulation in *Mbnl2*-inactivated mice and dysregulated *Mbp* isoforms in *Mbnl1* and *Mbnl2* double knockout.

(A) Western blot analysis of MBP protein levels in mice inactivated for *Mbnl1*, *Mbnl2* or *Mbnl1/Mbnl2* double knockout (DKO) at 3 months. The graph represents the average MBP steady state-levels (\pm SEM) in MBNL-deficient mice, relative to normalized age-matched WT controls ($n = 3$ per genotype). (B) Densitometric analysis of MBP protein isoforms in *Mbnl2* KO mice and WT controls (\pm SEM) (C) Representative RT-PCR splicing analysis of development-regulated exons 6, 9 and 10 of *Mbp* in *Mbnl* DKO and WT mice ($n=3$, per genotype). $**p < 0.01$.

F. Human DM1 brains accumulate RNA foci in oligodendrocytes and myelin protein expression abnormalities

To investigate whether myelin protein deficits in mice reflect a pathophysiological event in DM1 brain disease, we studied post-mortem samples of human frontal cortex. Like in DMSXL mice, expanded *DMPK* transcripts accumulate in human OLIG2-positive cells in frontal cortex (Fig. 6A), confirming the toxicity of CUG RNA in oligodendrocytes. We then studied the dysregulation of myelin proteins and other protein markers of oligodendroglia cell lineage differentiation by western blot immunodetection (Fig. 6B). We found great variability in myelin and oligodendroglia protein content between samples, most likely due to the heterogeneity of tissue composition and variability in the cell type content of post-mortem samples. To reduce this confounding effect, we quantified PDGFRA, MBP and MOG steady-state levels relative to OLIG2, a pan protein marker of the oligodendroglia cell lineage. The results revealed a two-fold increase in the protein levels of PDGFRA in the frontal cortex of DM1 patients. While MBP levels remained unaltered in DM1 patients, the steady state levels of MOG exhibited a trend towards mild upregulation ($\sim 30\%$ increase, $p=0.13$) (Fig. 6C). To estimate the differentiation of oligodendroglia in DM1 patients we calculated the

expression of late markers of oligodendroglia cell lineage (MBP and MOG), relative to oligodendroglia-specific proteins expressed in earlier stages of maturation (PDGFRA). Similar to DMSXL mice, the analysis revealed a significant decrease of ~70% in the relative expression of MBP, but no change in the relative expression of MOG (Fig. 6D). Like DMSXL and *Mbnl2* KO mice, adult DM1 patients do not show abnormal splicing of candidate *MBP* alternative exons, consistent with a pathological defect that is independent of dysregulated missplicing (Fig. 6E).

In summary, in agreement with DMSXL results, DM1 oligodendrocytes accumulate toxic RNA foci in association with MBP protein deficits, without overt defects in alternative splicing.

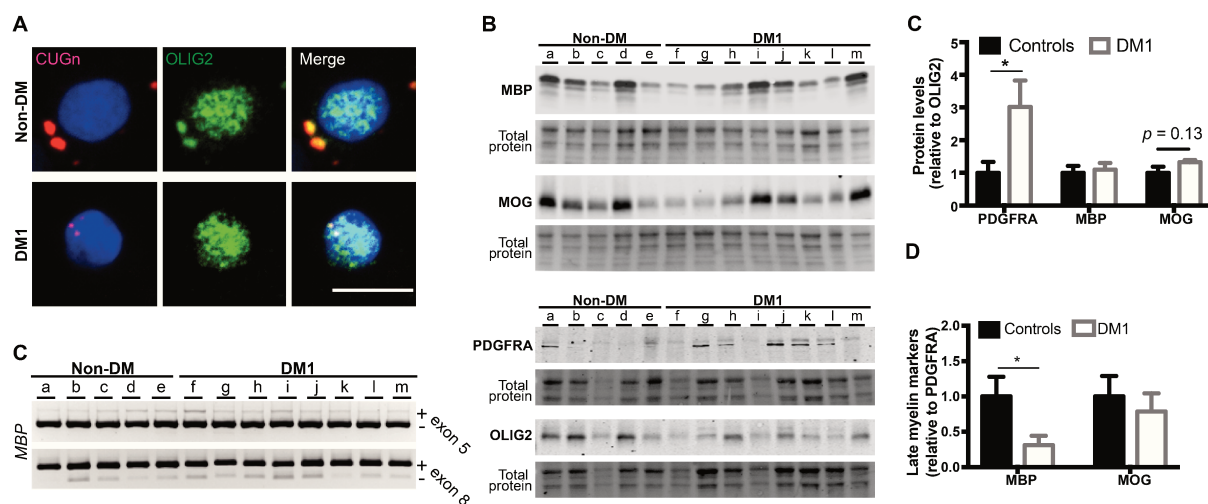


Figure 6 - DM1 frontal cortex oligodendrocytes show RNA foci and myelin protein deficits.

(A) FISH combined with IF showing expanded CUG RNAs (red) in the nucleus OLIG2-expressing oligodendrocytes (green) from DM1 post-mortem frontal cortex. DAPI was used for nuclear staining. The scale bar represents 10 μ m. (B) Western blot analysis of MBP, MOG, PDGFRA and OLIG2 protein levels in DM1 patients compared with non-DM controls. (C) Densitometric quantifications of PDGFRA, MBP and MOG levels relative to OLIG2-pan marker, in DM1 and control individuals. (D) Ratios between late myelin proteins and PDGFRA levels. Graphs represent mean (\pm SEM) ($n = 5$, non DM controls; $n = 8$, DM1 patients) (E) Representative RT-PCR splicing analysis of alternative *MBP* exons 5 and 8 in DM1 and controls subjects. * $p < 0.05$.

V. Discussion

RNA toxicity in oligodendroglia and myelin phenotypes

In the present work we report the presence of molecular hallmarks of DM1 in oligodendrocytes of a transgenic mouse model of the disease, and in the brain of human patients. In DMSXL transgenic mice, RNA foci accumulation is associated with significant missplicing in isolated oligodendrocytes, as well as abnormal patterns of myelination. In particular, we have found that the normal progression of myelination is delayed during the first postnatal month in the CC and frontal cortex: there is a reduced number of myelinated axons and among these, the larger caliber are preferentially myelinated and transient downregulation of myelin proteins. Since larger axons are myelinated first under physiological conditions (Simons and Lyons, 2013), the preferential myelination of larger axons detected in young DMSXL mice provides additional indirect evidence of delayed myelination. Myelination defects are accompanied by abnormal myelin protein expression and by a reduced number of mature oligodendrocytes, particularly in the CC.

The myelination phenotypes of DMSXL mice were more pronounced between 2 weeks and 1 month, being later resolved at 4 months. Similarly, the reduced expression of myelin proteins was evident from the second postnatal week until the age of one month, being later corrected. Together these findings point to a transient delay in myelination, in response to toxic CUG RNA. The age-dependent nature of the myelin phenotypes in DMSXL mice may be accounted for by variations in RNA foci content, which decreases during the first postnatal month.

Delayed oligodendroglia cell differentiation in DMSXL mice.

The investigation of the cellular abnormalities behind myelin defects revealed a reduction in the number of OLIG2-expressing cells and CC1-positive oligodendrocytes in DMSXL brains, suggestive of perturbed homeostasis of pre- and myelinating oligodendrocytes committed to undergo full differentiation and myelination.

The initiation of oligodendrocyte differentiation depends on a fine-tuned balance between pro-myelinating and inhibitor factors. Among these, PDGF signaling through PDGFRA is important for OPC proliferation and needs to be shut down (for instance by degradation) to initiate differentiation (Hart et al., 1989, Zhu et al., 2014, Calabretta et

al., 2018). The blockade in oligodendrocyte maturation may be explained, at least partly, by the upregulation of PDGFRA in DMSXL mice. If PDGFRA is not correctly downregulated, it inhibits lineage progression, maintains the progenitor phenotype for longer and delays the onset of myelination, ultimately causing myelin defects (Hart et al., 1989, Zhu et al., 2014, Calabretta et al., 2018).

In agreement with myelin abnormalities and impaired oligodendroglia differentiation, brains of adult DM1 patients also present increased steady-state levels of PDGFRA, associated with lower relative expression of late markers of myelination. Therefore, it is possible that, contrary to DMSXL mice, myelination defects persist in adult human patients. To confirm whether myelin defects are more pronounced during early human development, it would be interesting to study myelin protein expression and myelin ultrastructure in brains of young congenital patients, during periods of active myelination. More thorough studies, with suitable sets of tissue samples must be pursued, ideally with combined imaging analyses.

In summary, we gathered mouse and human evidence to support the hypothesis that a reduced number of pre- and myelinating oligodendrocytes likely contribute to the delayed myelination found in DM1 mice.

Contributing role of MBNL proteins towards myelin abnormalities

In addition to CUG-expressing DMSXL mice, independent MBNL-deficient mouse models of DM1 have also displayed abnormal expression of myelin proteins pointing to the critical role of these RNA-binding proteins in myelin pathobiology. As a consequence of RNA foci accumulation, MBNL1 and MBNL2 splicing regulators are sequestered in the nucleus of oligodendrocytes, and their partial inactivation is associated with splicing abnormalities of relevant target transcripts, such as *Sorbs1*, *Itga6* or *Mbnl1*.

We studied the role of MBNL proteins in the regulation of alternative splicing of myelin-specific transcripts. Although it is known that alternative splicing of *Mbp* regulates the expression of different MBP protein isoforms throughout development (Campagnoni, 1988), RNA missplicing does not appear to underlie MBP misregulation in DMSXL and human tissues. This is consistent with a possible contribution of MBNL2 inactivation to MBP downregulation, in agreement with the absence of *Mbp* splicing abnormalities detected in *Mbnl2* knock-out mice (despite pronounced MBP protein

downregulation). The more severe molecular abnormalities of MBP protein and transcripts in *Mbnl1/Mbnl2* DKO animals, relative to DMSXL mice, may be accounted for the different degree of MBNL inactivation in the two mouse lines: while *Mbnl1/Mbnl2* DKO mice lack MBNL proteins completely throughout development, in adult DMSXL mice only 20% of oligodendrocytes exhibit RNA foci, suggesting partial inactivation of MBNL proteins.

Non-cell autonomous mechanisms of delayed myelination

In addition to the perturbation of mechanisms intrinsic to oligodendroglia, non-cell autonomous mechanisms may also contribute to hypomyelination in DMSXL mice.

Motor-dependent neuronal activity influences adaptive myelination (Gibson et al., 2014), thus the lower exploratory activity and motor impairment of DMSXL mice (Hernandez-Hernandez et al., 2013a, Sicot et al., 2017) might also contribute to myelin deficits. On the other hand, vesicular release provides guidance clues to target oligodendrocytes towards the axons to be myelinated (Hines et al., 2015). The dysregulation of synaptic proteins in DMSXL mice (Hernandez-Hernandez et al., 2013b) may perturb myelination in young animals, and contribute to the preferential ensheathment of larger axons. In particular, glutamate release from unmyelinated callosal axons is crucial for the progression of myelination of this structure (Ziskin et al., 2007). Given the alterations in the expression of the GLT1/SLC1A2 glutamate transporter and glutamate uptake in DMSXL mice (Sicot et al., 2017), it is conceivable that glutamate imbalance may impair CC myelination in DMSXL mice. For all these reasons, neuronal integrity and activity, as well as the neuron-oligodendrocyte crosstalk must be carefully studied in this model.

Implications of transient hypomyelination to brain function

Even if transient, lower MBP and CNP expression, reduced CC thickness and lack of myelinated axons in DMSXL young mice, may translate into neurological damage, WM lesions, impaired cognitive function and susceptibility to neuropsychological disorders later in life (van Tilborg et al., 2018). In support of this view, the early myelin phenotype of DMSXL exhibit is accompanied by relevant behavioral and cognitive impairments later in life (Hernandez-Hernandez et al., 2013a). Similarly, mouse models of autism

spectrum disorder (Graciarena et al., 2019) or social isolated animals (Liu et al., 2012), where myelin and oligodendrocyte are impaired, induce abnormal behavior that persists in time.

The period of pronounced myelin phenotype in DMSXL mice corresponds approximately to childhood and adolescence in humans (Dutta and Sengupta, 2016). The onset of DM1 within this period leads to clinical forms of the disease that are characterized by marked neurological symptoms (De Antonio et al., 2016), and pronounced neuroimaging abnormalities in major myelinated tracts (Wozniak et al., 2013, Wozniak et al., 2011). Juvenile DM1 patients present characteristic behavioral traits, including social avoidant personality and autism spectrum disorder, which may be related to myelin changes.

Defective myelination of CC may also result in impaired brain connectivity, a feature previously described in DM1 brains (Serra et al., 2016, Serra et al., 2014). Proper brain connectivity is essential for neuronal maturation synchronicity occurring through adolescence, in association with the acquisition of higher cognitive and executive functions (James et al., 2008), known to be primarily affected in DM1.

Myelin and oligodendroglia abnormalities in trinucleotide repeat expansion disorders

Similar myelin phenotypes have been reported in other triplet repeat expansion diseases. Transient hypomyelination is a shared feature between mouse models of Fragile X Syndrome (FXS) and Huntington Disease (HD). The *Fmr1* knockout model shows reduced number of myelinated axons and myelin thickness, associated with reduced expression of myelin proteins at 7 days postnatally, in both cerebellum and cortex. Interestingly these defects are recovered after 15 days (Pacey et al., 2013, Giampetruzzi et al., 2013). Similar to MOG expression in DMSXL, myelin over-compensation is observed around 2 months in cerebellum (Pacey et al., 2013) and medial CC (Lee et al., 2019), and translated by the abnormal upregulation of MBP levels.

Transgenic mice expressing human mutant huntingtin (*mHTT*) show early hyperintensity changes in CC tracts, prior to axonal loss (Teo et al., 2016), and these abnormalities are associated with progressive motor impairment and depressive-like behavior. Importantly, these defects are also observed in mice in which *mHtt* expression

is restricted to mature OL (Jin et al., 2015, Huang et al., 2015, Ferrari Bardile et al., 2019). Transcriptional defects of myelin genes are transient being recovered, to some extent, at later stages in certain HD mouse models (Jin et al., 2015). However reduced number of myelinated axons and thinner myelin in CC are not corrected in HD mice (Jin et al., 2015, Teo et al., 2016, Ferrari Bardile et al., 2019), unless mHTT expression is specifically knocked-down in OPCs (Ferrari Bardile et al., 2019). Importantly, mHTT knocking-down rescues not only the myelin phenotypes, but also the behavioral and motor function of these animals. Together these results support the view that myelination defects trigger pathogenic events in the CNS that are relevant for the etiology of expanded trinucleotide diseases.

Interestingly, deficits in oligodendroglia differentiation may account for the myelin phenotypes reported in FXS and HD. While in a FXS mouse model there is a regional deficit of OPCs in cerebellum (Pacey et al., 2013), early stages human (Myers et al., 1991) and mouse HD brains (Jin et al., 2015) display increased OPC proliferation in response to myelin loss in striatum and CC, before disease onset. Similar to DMSXL mice, differentiation is also impaired in mHTT-expressing oligodendrocytes *in vivo* (Huang et al., 2015).

VI. Conclusion

The association between structural, histological and molecular myelin defects in mouse models of DM1 and in the brain of patients strongly supports a deleterious delay in oligodendrocyte differentiation in DM1 neuropathophysiology. To obtain a broader view of the oligodendroglia impairments contributing to myelination phenotypes, other parameters, such as cell proliferation, cell death and morphological differentiation must be further explored in response to the DM1 repeat expansion. Further research must be conducted to assess the direct contribution of myelin pathobiology to the development of DM1 neurological symptoms, and to help design of future therapeutic interventions targeting WM abnormalities and myelin-rich structures.

VII. Supplementary Information

A. Supplementary tables

Supplementary table 1 - Clinical data of control individuals (related to Figure 6).

| | Non-DM controls | | | | |
|----------------------------|------------------|------------------------------|----------------------------|-----------------|------------------------------------|
| | a | b | c | d | e |
| Sex | M | M | M | M | F |
| Diagnosis | Brain metastasis | Limb-Girdle dystrophy | Myelitis | Distal myopathy | Myasthenia Gravis, B cell lymphoma |
| Age; cause of death | 64; pneumonia | 66; pneumonia, heart failure | 79; Pneumocystis pneumonia | 78; pneumonia | 81, heart failure |

Supplementary table 2 - Clinical data of DM1 individuals (related to Figure 6).

| | DM1 samples | | | | | | | |
|------------------------------|--------------------|-----------------------------------------|------------------|------------------|------------------|-------------------------|------------------------------------------------------------|------------------------------------------------------------|
| | f | g | h | i | j | k | l | m |
| Sex | M | F | F | M | F | F | F | M |
| CTG length (in blood) | >2000 | 2700-2900 | 1300-1400 | 1600-1800 | 700-1100 | N/D | 1600-1750 | 1100-2100 |
| Age of onset | 0 | N/D | 40 | 40 | 30 | 34 | 12 | 8 |
| Clinical form | Congenital | N/D | Adult | Adult | Adult | Adult | Adult | Adult |
| DM main symptoms | Mental retardation | Gait disturbance | Gait disturbance | Gait disturbance | Gait disturbance | Gait disturbance | Gait disturbance | Gait disturbance |
| Neuroimaging | N/D | Diffuse atrophy | Diffuse atrophy | Normal | N/D | N/D | Diffuse atrophy, high intensity in bilateral temporal pole | Diffuse atrophy, high intensity in bilateral temporal pole |
| Age; cause of death | 32; pneumonia | 64; Acute respiratory distress syndrome | 69; pneumonia | 62; pneumonia | 67; pneumonia | 64, respiratory failure | 57, respiratory failure | 55, respiratory failure |

Supplementary table 3 - Primary antibodies for IF and WB (related to Figures 1, 2, 3 and 4).

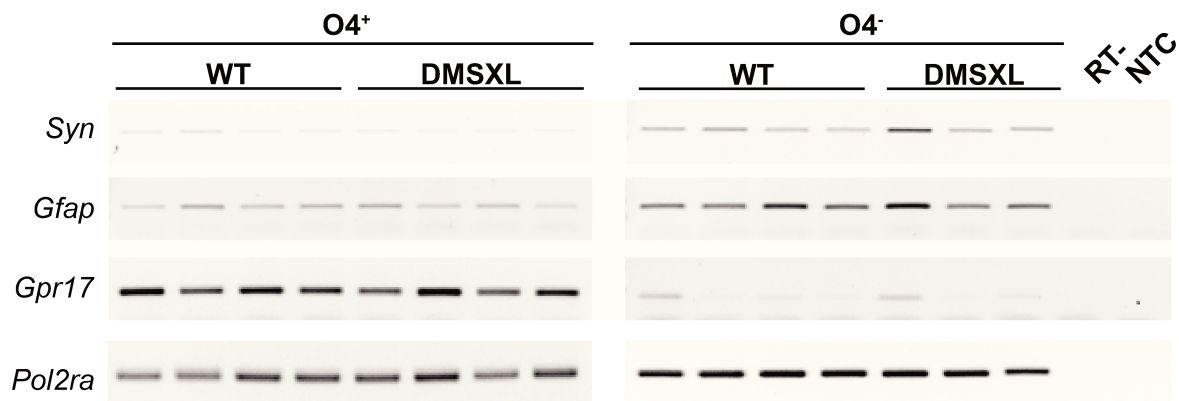
| Antigene | Supplier; catalogue number | IF blocking and incubation conditions | WB blocking and incubation conditions | Dilution (IF; WB) |
|----------------|-------------------------------|------------------------------------------|------------------------------------------|----------------------|
| OLIG2 | Millipore; AB9610 | 0.2% BSA, 10% NGS, 0.3% TX-100; 1h, RT | 5% blotto, 1h, RT | 1/250; 1/1000 |
| MBNL1 | MB1A from Glen Morris (gift) | 0.2% BSA, 10% NGS; 1h, RT | - | 1/10 |
| MBNL2 | MB2A from Glen Morris (gift) | 0.2% BSA, 10% NGS; 1h, RT | - | 1/10 |
| MBP | Abcam; ab40390 | 0.2% BSA, 10% NGS, 0.3% TX-100; 1h, RT | 5% blotto, 1h, RT | 1/500 |
| MOG | Abcam; ab109746 | - | 5% blotto, 1h, RT | 1/1000 |
| CNP | Abcam; ab6319 | - | 5% blotto, 1h, RT | 1/500 |
| PDGFR α | Santa Cruz; sc-338 | 0.5% BSA, 0.2% TX-100; 1h, RT | 5% blotto, 1h, RT | 1/200; 1/100 |
| APC (CC1) | MERK; OP80 | 10% NGS, 0.3% TX-100; 1h, RT | - | 1/20 |
| MYEF2 | Santa Cruz; sc-102031 | - | 2% BSA, 1h, RT | 1/400 |

NGS, normal-goat serum

Supplementary table 4 - Oligonucleotide primers sequences used for RT-PCR analysis in mouse oligodendrocytes (related to Figure 1, 4, 5 and 6).

| Gene | Species | Spliced exon | Forward primer | Reverse primer | Product size (bp) |
|---------------|---------|-----------------|------------------------------|--------------------------------|----------------------|
| <i>Mbnl1</i> | Mouse | 7 | TGGTGGGAGAAATGCTGTATGC | GCTGCCCAATACCAGGTCAAC | 270/216 |
| <i>Mbnl2</i> | Mouse | 5 | CTTTGGTAAGGGATGAAGAGCAC | ACCGTAACCGTTTGTATGGATTAC | 255/201 |
| <i>Mapt</i> | Mouse | 10 | CTGAAGCACCAGCCAGGAGG | TGGTCTGTCTTGGCTTTGGC | 367/274 |
| <i>Capzb</i> | Mouse | 8 | GCACGCTGAATGAGATCTACTTTG | CCGGTTAGCGTGAAGCAGAG | 280/192 |
| <i>Sorbs1</i> | Mouse | 27 | CCAGCTGATTACTTGGAGTCCACAGAAG | GTTACACCTTCATACCAGTTCTGGTCAATC | 349/181 |
| <i>Tanc2</i> | Mouse | 23 | GCCATGATTGAGCATGTTGACTACAGT | CCTCTTCCATCAGCTTGCTCAACA | 214/184 |
| <i>Mbp</i> | Mouse | 6 | CCATCCAAGAAGACCCCAACA | TGGGTAGTTCTCGTGTGTGA | 238/316 |
| <i>Mbp</i> | Mouse | 9/10 | CTCGAACACCACCTCCATCC | ATTAAGAAGCCGAGGGCAGG | 155/278/344 |
| <i>Gpr17</i> | Mouse | - | TCACAGCTTACCTGCTTCCC | GACCGTTCATCTTGTGGCTCT | 78 |
| <i>MBP</i> | Human | 5 | CCGAGAGCCTGGATGTGATG | CGTAGTGAGCAGTCTTGCC | 232/306 |
| <i>MBP</i> | Human | 8 | GCACACCACCCCGTC | GGGCATCGACTCCCTTGAAT | 117/150 |
| <i>Pol2ra</i> | Mouse | - | GGCTGTGCGGAAGGCTCTG | TGTCCTGGCGGTTGACCC | 155 |

B. Supplementary figures



Supplementary figure 1 - Oligodendrocyte enrichment with magnetic cell sorting.

RT-PCR analysis of the two cell fractions collected following magnetic cell sorting with O4-labeled beads showing high expression of oligodendrocyte-specific *Gpr17* (G Protein-Coupled Receptor 17) and low expression of neuron-specific *Syn* (Synapsin) and astrocyte specific *Gfap* (Glial Fibrillary Acidic Protein) in the O4-positive fraction. Oligodendrocytes were depleted from the negative cell fraction, which contained mainly *Syn*-expressing neurons and *Gfap*-expressing astrocytes.

VIII. Author contribution

Sandra O. Braz: mouse breeding and genotyping; analysis of RNA toxicity *in vivo* (foci accumulation, MBNL sequestration and missplicing); immunofluorescent analysis of myelin and oligodendroglia cell lineage throughout mouse brain development; EM data collection and post-acquisition analysis; analysis of myelin protein expression in mouse and human tissue samples; study design; data collection and analysis; preparation of figures; manuscript writing.

Alain Schmitt: tissue processing for EM; EM image collection.

Dominique Langui: tissue processing for EM; EM image collection.

Aline Huguet: mouse colony maintenance and genotyping.

Geneviève Gourdon: study design; data analysis.

Mário Gomes-Pereira: study design; data analysis; manuscript revision.

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Chapter IV

GUG RNA toxicity is associated with differentiation deficits in oligodendrocytes

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Unpublished data

I. Abstract

Myotonic dystrophy type 1 (DM1) is a trinucleotide repeat disorder caused by an abnormal expanded CTG repeat. The neurological manifestations are very debilitating, but the contribution of each cell type for brain cell dysfunction is still poorly understood. We have previously observed myelin deficits in a transgenic mouse model of DM1, which together with the prevalent white matter lesions in the brains of human patients prompted us to study the impact of expanded CUG RNAs on oligodendrocyte biology. Taking advantage of cell culture models derived from DM1 transgenic mice, we found progressive CUG RNA toxicity from precursor to differentiated oligodendrocytes, illustrated by higher expression of transgenic CUG transcripts and greater foci content in more mature stages. Increased RNA toxicity during differentiation was accompanied by abnormal oligodendroglia cell biology: despite normal proliferation and migration of oligodendrocyte progenitor cells in culture, the expanded repeats were associated with impaired differentiation into ramified oligodendrocytes. To gain insight into the molecular pathways dysregulated by the repeat expansion in the oligodendroglia cell lineage, we performed RNA sequencing analysis in homogenous cultures of both oligodendrocytes progenitor cells and differentiated oligodendrocytes. Gene ontology suggested abnormal expression and splicing of structural and regulatory components of the cytoskeleton, as well as missignaling in pathways involved in cell differentiation. Importantly, we found that the majority of splicing defects in mature oligodendrocytes recreate the splicing profiles of oligodendrocyte progenitor cells, in line with defective differentiation of the oligodendroglia cell lineage in response to the DM1 repeat expansion. Through the identification of disease-specific mechanisms operating in oligodendroglia, we demonstrate the implication of oligodendrocyte defects to the myelin abnormalities in a DM1 mouse model.

Key words:

Oligodendrocytes, RNA toxicity, cell phenotypes, real-time videomicroscopy; primary cell cultures.

II. Introduction

White matter (WM) abnormalities are a pathological feature observed in nearly all neurodegenerative, neurodevelopmental and in some neuromuscular disorders, including in myotonic dystrophy type I (DM1, OMIM 160900). DM1 is a multisystemic disease that affects primarily skeletal muscles, heart and brain with great variability in symptom manifestations, disease severity and age of onset (even among individuals of the same family) (Udd and Krahe, 2012).

DM1 is included in a group of disorders associated with the abnormal expansion of trinucleotide repeats (Harper, 2001). DM1 is caused by the expansion of a CTG trinucleotide repeat in the 3'UTR of the dystrophin myotonia-protein kinase (*DMPK*) gene (Brook et al., 1992). Disease pathogenesis is mainly mediated by a gain of function of expanded *DMPK* transcripts, which accumulate in nuclear RNA foci, perturbing the function of at least two families of alternative splicing regulators: the muscleblind-like (MBNL) and CUGBP/Elav-like (CELF) proteins (Sicot et al., 2011). In consequence, downstream RNA processing is disrupted, particularly the splicing of target transcripts that undergo a developmentally regulated program (Wang et al., 2012, Sicot et al., 2011, Hernandez-Hernandez et al., 2013b, Hernandez-Hernandez et al., 2013a, Goodwin et al., 2015, Batra et al., 2014, Braz et al., 2018).

Central nervous system (CNS) impairment is among the most debilitating symptoms, with a profound impact on the quality of life of patients. The neurological symptoms are usually more frequent and severe in patients with a younger age of onset (Harper, 2001). In particular, congenital DM1 patients present pronounced intellectual disability with reduced intelligence quotient and speech impairment, while adult patients have typical executive dysfunction and visuoconstructive impairment, as well as some characteristic behavior abnormalities (Gourdon and Meola, 2017).

WM changes are transversal to all disease forms: structural imaging techniques consistently reveal WM hyperintensities, general atrophy and abnormal tract connectivity in diverse white matter regions (Wozniak et al., 2011, Wozniak et al., 2013, Minnerop et al., 2011, Serra et al., 2016). WM loss has been associated with myelin alterations in other trinucleotide repeat disorders, such as Huntington disease Fragile X syndrome; neurodegenerative diseases, such as amyotrophic lateral sclerosis, and other

neuromuscular conditions, such as Duchenne muscular dystrophy (Bartzokis et al., 2007, Pacey et al., 2013, Zhou et al., 2017, Aranmolate et al., 2017).

In support of myelin defects in DM1, we have previously found that the expression of expanded *DMPK* transcripts induced RNA toxicity in oligodendrocytes and myelin alterations in the brain of a DM1 mouse model, including transient hypomyelination (characterized by a reduced number of myelinated axons and corpus callosum thickness), lack of mature oligodendrocytes, in association with misregulation of myelin proteins.

The previous observation of toxic RNA accumulation in oligodendroglia cells from transgenic DM1 mice and human patients (Chapter III), raises the hypothesis of a deleterious impact of the toxic repeats in the oligodendrocyte physiology. To investigate the cellular and molecular mechanisms dysregulated by the CTG expansion in the oligodendroglia cell lineage, we have used the DMSXL mouse model as a renewable source of primary and homogenous cultures of oligodendrocytes. This transgenic line ubiquitously expresses human *DMPK* transcripts with more than 1,000 CTG repeats, showing RNA foci accumulation and missplicing in several tissues (Gomes-Pereira et al., 2007, Michel et al., 2015, Huguet et al., 2012). In particular, the expression of expanded CUG RNA in the brain leads to relevant behavioral and electrophysiological abnormalities, in association with altered expression of neuronal and astrocyte proteins (Hernandez-Hernandez et al., 2013b, Hernandez-Hernandez et al., 2013a, Sicot et al., 2017).

To explore the involvement of oligodendrocytes in DM1 neuropathology, we have combined molecular, cellular and transcriptomics approaches to analyze primary DMSXL oligodendrocytes. We have found that oligodendrocyte progenitor cells (OPC) are relatively spared from RNA toxicity, when compared with differentiated oligodendrocytes (OL). In contrast, mature DMSXL oligodendrocytes showed impaired morphological differentiation in association with expression and alternative splicing defects of transcripts related to cytoskeleton dynamics, which recreated the molecular signature of immature OPC. Together these data point to a differentiation phenotype of the oligodendroglia cell lineage, driven by transcriptomics abnormalities. Understanding the contribution of this cell type to the overall DM1 WM changes and brain pathology will help develop future rational therapies.

III. Material and methods

A. Transgenic mice

Primary dissociated cell cultures of cortical OPC were prepared from postnatal day (P) 0 to P2 DMSXL and WT mouse littermates. The protocol used was adapted from Dincman *et al.* (Dincman et al., 2012). Briefly, the brains were dissected and placed in Hank's balanced salt solution without (HBSS) Ca²⁺ and Mg²⁺, and the cortices free of meninges were isolated. A single cell suspension from each brain was then prepared using the neural tissue dissociation kit (130-092-628, Miltenyi) and the OPC were isolated by magnetic cell sorting with magnetic anti-CD140a-labeled microbeads (130-092-628, Miltenyi), following the manufacturer's protocols. The isolated cells were plated in OPC medium containing DMEM F-12, 1% N2 supplement, 2% B27 supplement, 50 units/mL penicillin and 50 µg/mL streptomycin, 0.01% bovine serum albumin, 40 ng/mL FGF2 and 20 ng/mL PDGF-AA; at initial cell densities ranging between 9,000-15,000 cells/cm². OPC were kept in plates coated with 0.1 mg/mL of poly-D-lysine (PDL) and 10 µg/mL laminin (unless stated otherwise) in OPC medium to proliferate. Two days later the growth factors were withdrawn from the medium and replaced by 50 µg/mL insulin, 40 ng/mL triiodo-thyronin and 1 ng/mL CNTF replaced (differentiation medium), in order to induce the differentiation of OPC into oligodendrocytes. For experiments requiring greater numbers of cells (*e.g.* protein and RNA extraction) the OPC were left to expand in OPC medium for 5-7 days until reaching approximately 70% confluence before splitting.

B. Primary OPC cell culture and differentiation

Primary dissociated cell cultures of cortical OPC were prepared from postnatal day (P) 0 to P2 DMSXL and WT mouse littermates. The protocol used was adapted from Dincman *et al.* (Dincman et al., 2012). Briefly, the brains were dissected and placed in Hank's balanced salt solution without (HBSS) Ca²⁺ and Mg²⁺, and the cortices free of meninges were isolated. A single cell suspension from each brain was then prepared using the neural tissue dissociation kit (130-092-628, Miltenyi) and the OPC were isolated by magnetic cell sorting with magnetic anti-CD140a-labeled microbeads (130-

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C. CG4 cell culture and transfection

Rat CG4 oligodendrocyte cell line, kindly gifted by Dr. Catherine Lubetzki and Dr. Bruno Stankoff laboratory, were maintained grown on poly-d-lysine-coated (0.1 mg/mL) plates in a mixture of N1 medium supplemented with B104 medium (30%) and biotin (10 ng/ml), at 2,500 cells/cm² as previously described (Franklin et al., 1995). For cell differentiation into mature oligodendrocytes, the B104 conditioned medium was removed. CG4 cells were transfected with no-CTG control DMPKS expressing constructs, or DT960 plasmids that expressing 960-CTG repeats (kindly provided by Dr. Thomas Cooper) at OPC stage, using Lipofectamine™ 3000 transfection Reagent following the manufacturer's protocol (Invitrogen, ref. L3000008).

D. Induced pluripotent stem cells (iPSC) differentiation

Human iPSC were generated in the Dr. Cecile Martinat laboratory (I-STEM, France). DM1 lines derived from three different patients, carrying ≥1,000 CTG repeats and respective control lines, derived from two unaffected relatives. Human iPSC were differentiated into oligodendrocytes as previously described (Douvaras and Fossati, 2015). Briefly, human iPSC lines were differentiated for 92 days, through a multistep protocol of expansions of neural stem cells, resuspension and aggregation of

oligodendrocyte transcription factor 2 (OLIG2)-enriched spheres. The cells were plated in a combination of cocktails of pro-differentiation small molecules and growth factors (Douvaras and Fossati, 2015), to perform assays of oligodendrocyte migration out of the spheres and subsequent differentiation.

E. Immunofluorescence (IF) and fluorescent in situ hybridization (FISH)

Primary mouse cells and CG4 cells were fixed at 0, 3, 5-7 days of *in vitro* differentiation (div), for 15 min in 4% PFA, and ribonuclear inclusions were detected using a 5'-Cy3-labelled (CAG)₅ PNA probe, as previously described (Huguet et al., 2012). In the case of differentiated human iPSC, cells were fixed after 92 div. Combined FISH and IF were performed as previously described in (Hernandez-Hernandez et al., 2013a). Primary antibodies and dilutions used are listed in Supplementary table .

F. Microscope imaging and image analysis.

Confocal images were acquired as z-stacks with either a Leica TSC SP8 SMD or Zeiss spinning disk confocal microscopes at 40x or 63x magnification. For foci quantification, Z projections were analyzed using the Spot Detector plugin of the ICY bioimage analysis open source program (<http://icy.bioimageanalysis.org>). The percentage of foci-positive nuclei was calculated relative to the total number of nuclei. The percentage of OLIG2- and O4-positive cells per area was calculated. MBNL1 and MBNL2 nucleo-cytoplasmic ratio was quantified on confocal images using nuclear DAPI or MBNL signal to create a mask of the nucleus and cytoplasm respectively, and to measure the MBNL1 and MBNL2 intensity inside the mask. CG4 process length and branching were measured by semi-manual tracing of oligodendrocyte actin cytoskeleton processes using the NeuronJ plugin for ImageJ (<http://imagescience.org/meijering/software/neuronj/>). Sholl analysis plugin was used to measure oligodendrocyte ramification complexity in cultures stained with O4 using ImageJ (Ferreira et al., 2014) IF and FISH images were treated with Fiji - ImageJ software (Schindelin et al. 2012) to create representative figures of the data collected.

G. Videomicroscopy monitoring of cells dynamics

OPC proliferation was monitored using the IncuCyte Zoom video- microscope (Essen BioScience) after seeding 5,000 cells/well in 96-well plates and taking phase-contrast pictures every 1-2 hours. The percentage of cell confluence was determined using the IncuCyte ZOOM basic analysis method. For cell migration, 10,000 cells/well were seeded on 96-well plates 2-3 days prior to the beginning of the assay. The Cell Migration Assay kit and migration module of the IncuCyte were used to monitor cell migration towards an empty space. Oligodendrocyte process outgrowth was monitored in 5,000 cells/well, in 96-well plates, using the Neurotrack module of the IncuCyte on phase-contrast images taken each 2 hours. Detailed protocols are described in Chapter II.

H. RNA isolation, cDNA synthesis and RT-PCR analysis

RNA extraction was performed with the RNeasy Mini kit (QIAGEN, ref. 74104) following the manufacturer's protocol, with an additional DNase digestion step (RNase-Free DNase Set, QIAGEN, ref. 79254) after the first wash with RW1 buffer. RNA concentration was assessed using NanoDrop spectrophotometry, and RNA quality was verified by electrophoresis on an agarose gel. cDNA was prepared from an input of 250-500 ng of total RNA, using SuperScript™ III reverse transcriptase (Invitrogen, 18080044) and a mix of 100 μM oligo(dTs), random hexamers (50μM) and 10 μM dNTPs. The reaction was performed following the manufacturer's guidelines. Semi-quantitative RT-PCR analysis of alternative splicing and quantitative RT-PCR of total human *DMPK*, murine *Dmpk*, Annexin V (*Anxa5*), Annexin VI (*Anxa6*), Integrin alpha 3 (*Itga3*) and Serine (or cysteine) peptidase inhibitor, clade A, member 3n (*Serpina3n*) expression levels were performed as described elsewhere (Hernandez-Hernandez et al., 2013a, Huguet et al., 2012), using oligonucleotide primers listed in Supplementary table . All samples were normalized relative to the expression of an internal control: RNA Polymerase II Subunit A (*Pol2ra*).

I. RNA sequencing and bioinformatics analysis

RNA samples were prepared from WT and DMSXL OPC and oligodendrocytes differentiated for 7 days *in vitro*. Illumina-compatible precapture barcoded mRNA libraries were constructed, and a series of 24 barcoded libraries was pooled at equimolar concentrations. The capture process was performed according to the manufacturer's protocols for TruSeq Stranded mRNA (Illumina) and sequencing on an Illumina HiSeq2500.

Alternative splicing analyses were performed using the open source FARLINE pipeline (http://kissplice.prabi.fr/pipeline_ks_farline) as previously described (Fontrodona et al., 2019). Significant expression changes were considered for further analysis if fold change between genotypes > 1.4 , and P (corrected for multiple comparisons) < 0.05 . Significant missplicing events were selected for further analysis if the percentage of splicing inclusion between genotypes > 10 and P (corrected for multiple comparisons) < 0.05 . Percentage of splicing inclusion (PSI) was calculated as $(\text{inclusion of alternative exon}) / [(\text{inclusion of alternative exon}) + (\text{exclusion of alternative exon})]$. Gene ontology (GO) analysis were performed using the freely available GOrilla software analysis tool (Eden et al., 2009)

J. Western blot protein detection

Proteins from mouse and human brain tissues were extracted using RIPA buffer (Thermo Scientific, ref. 89901) supplemented with 0,05% CHAPS (Sigma, C3023), 1x complete protease inhibitor (Sigma-Aldrich, ref. 04693124001) and 1x PhosSTOP phosphatase inhibitor (Sigma ref. 04906845001). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, ref. 23227). Between 10 and 40 μ g proteins were mixed with 2X Laemmli Sample Buffer (Sigma, ref. S3401), denatured for 5 minutes at 95°C and resolved in 4-20% TGX Stain-Free polyacrylamide gels (Bio-Rad, ref. 1610183). After electrophoresis, gels were activated for 5 minutes under UV light, proteins were transferred onto nitrocellulose membranes using Trans-Blot® Transfer System (Bio-Rad) and total protein on the membrane was imaged using the ChemiDoc Imaging System (Bio-Rad). Membranes were then blocked in Blotto non-fat dry milk (Santa Cruz Biotech; sc2325) or bovine serum albumin (BSA) (Sigma, ref. A7906-50G) in 1x TBS-T (10mM Tris-HCl, 0.15M NaCl, 0.05% Tween 20),

during 1 h at room temperature (RT) and incubated with the primary antibody ON at 4°C. Blocking and primary antibody dilutions are indicated in Supplementary table . After three washes in TBS-T, membranes were incubated with IRDye® 800CW donkey anti-rabbit (LI-COR Biosciences, ref. P/N 926-32213) or 680RD donkey anti-mouse (LI-COR Biosciences, ref. P/N 926-68072) for 1 h at RT, washed three times and imaged using LI-COR Odyssey ® CLx Imaging System. Band intensity was quantified using Image Studio Lite and the values were normalized to the total protein quantified using the Image Lab software (Bio-Rad).

K. Statistical analysis

The software Prism (GraphPad Software, Inc) was used for statistical analyses. After performing a normality test on the numeric variables, we used two-tailed Student's t-test for parametric data and Mann-Whitney U test for non-parametric data, when two groups were compared. For one-way ANOVA, if statistical significance was achieved, we performed post-test analysis to account for multiple comparisons. Statistical significance was set at $P < 0.05$. The data are presented as mean \pm standard error of the mean (\pm SEM), or as or as Tukey box-and-whisker plots.

IV. Results

A. CUG RNA foci and expression increases throughout oligodendroglia differentiation in culture

To investigate the extent of the toxic effects of the expanded CUG RNA in the oligodendroglia cell lineage throughout differentiation, we have isolated cortical OPC from DMSXL newborn mice, which we grew and differentiated in culture. We then monitored the accumulation of expanded *DMPK* transcripts in nuclear foci over time (Fig. 1A). The percentage of cells with nuclear foci increased significantly from OPC stage (platelet-derived growth factor receptor alpha (PDGFRA)-expressing cells), to the immature (3 div, O4-expressing cells) and mature oligodendrocytes (OL, 7div, myelin basis protein (MBP)-expressing cells), with only 22% of OPC presenting foci in the nucleus compared with 48% of mature oligodendrocytes. Moreover, the mean number

of foci per cell was 2-fold higher in differentiated oligodendrocytes than in OPC: 2.7 ± 1.19 in OL vs 1.3 ± 0.07 in OPC (Fig. 1B). This higher foci content was associated with a 8-fold higher expression of the expanded *DMPK* transgene in DMSXL mature OL relative to precursor OPC. The expression profile of the human *DMPK* transgene follows the developmental variation in the expression of the endogenous *Dmpk* (Fig. 1C).

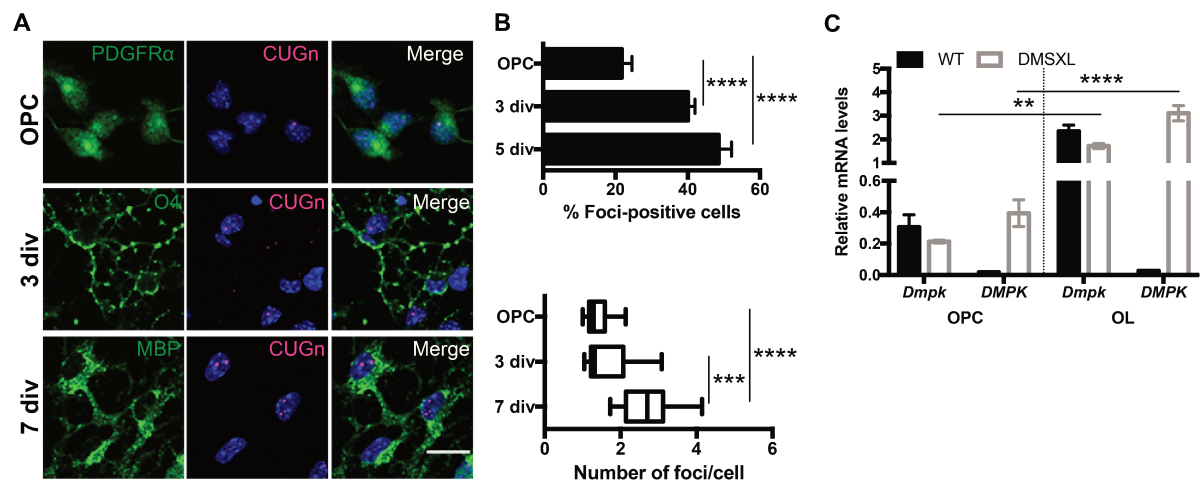


Figure 1 - DMSXL OL show higher accumulation of toxic transcripts compared to OPC.

(A) Representative images of combined FISH and IF showing expanded CUG RNAs (magenta) in OPC (PDGFR α -positive cells, green), immature oligodendrocytes (O4-positive cells, green) and mature oligodendrocytes (MBP-positive cells, green), at 3 and 7 div respectively, derived from DMSXL newborn mice. DAPI was used for nuclear staining. The scale bar represents 20 μ m. (B) Quantification of the percentage of cells containing RNA foci in the nucleus and the average number of foci per nucleus throughout oligodendroglia differentiation ($n \geq 958$ cells per stage). (C) Expression of mouse endogenous *Dmpk* and human *DMPK* transgene, relative to *Pol2ra* internal control in OPC and 7 div oligodendrocytes (OL). Data represent mean \pm SEM ($n=3$, OPC; $n=7$, OL). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

To further characterize the extent of RNA toxicity in OPC and OL, we assessed the sequestration of MBNL proteins by nuclear foci. Although scarce in OPC (data not shown), the co-localization of both MBNL1 and MBNL2 with RNA foci was apparent in differentiated oligodendrocytes (Fig. 2A). Since higher levels of CUG RNA and MBNL co-localization is predictive of pronounced spliceopathy, we then evaluated missplicing events in oligodendroglia. We have previously shown that *Mbnl1* and *Mbnl2* transcripts serve as robust markers of spliceopathy in our mouse model (Hernandez 2013; Sicot 2017). As predicted, the pronounced signs of RNA toxicity in DMSXL OL produced marked dysregulation of both transcripts, with an increased inclusion of the *Mbnl1* exon 7 and *Mbnl2* exon 5. In contrast, the lower levels of CUG RNA and toxic foci in DMSXL

OPC were insufficient to alter splicing profiles at this earlier differentiation stage (Fig. 2B). *Mbnl1* and *Mbnl2* transcripts including exons 7 and 5, respectively have been correlated with lower global levels of MBNL mRNA, especially in brain tissue (Konieczny et al., 2014). To investigate the impact of *Mbnl1* and *Mbnl2* missplicing on protein levels we used quantitative western blot and found significant downregulation of MBNL1 steady-state levels during the later stages of oligodendrocyte differentiation, relative to WT controls, but not in OPC. Surprisingly, we found reduced MBNL2 protein levels already in precursor cells (OPC), in which exon 5-containing isoform is predominant in both genotypes (Fig. 2C). It has also been suggested that alternative splicing plays a determinant role in the nucleus/cytoplasm shuttle of MBNL proteins (Tran et al., 2011). To address this question, we studied the consequences of the pronounced missplicing of *Mbnl1* exon 7 and *Mbnl2* exon 5 in differentiated OL, through the quantification of the intracellular distribution of MBNL proteins. Careful quantification of MBNL fluorescent signal by confocal microscopy failed to detect marked protein misdistribution in DMSXL cells, relative to WT controls (Supplementary Fig. 1).

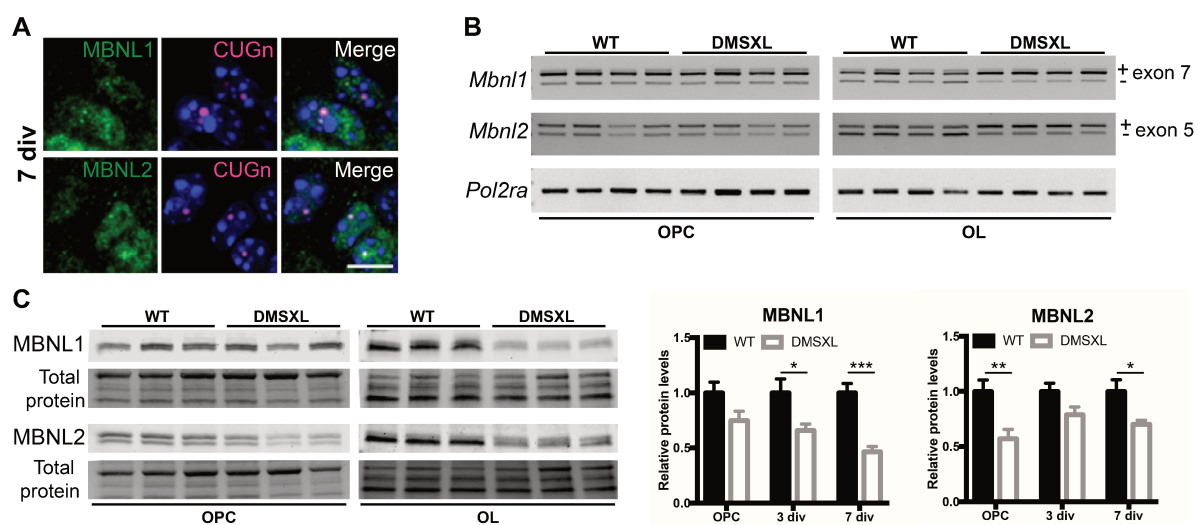


Figure 2 - DMSXL OL show MBNL sequestration into RNA foci associated with splicing and protein abnormalities.

(A) Representative images of combined FISH and IF showing expanded CUG RNAs (magenta) co-localizing with MBNL1 and MBNL2 (green) in OL derived from DMSXL brains. Diamidino-2-phenylindole was used for nuclear staining. The scale bar represents 10 μ m. (B) Representative RT-PCR splicing analysis in OPC and OL from WT and DMSXL mice (n = 4 independent cultures per group) *Mbnl1* exon 7 and *Mbnl2* exon5. (C) Western blot analysis of MBNL1 and MBNL2 protein levels in OPC and OL from DMSXL and WT mice (n=7 independent cultures per group). Data represents means \pm SEM. * p <0.05, ** p <0.01, *** p <0.001.

B. Splicing dysregulation in DMSXL OL leads to the abnormal production of OPC-associated splicing profiles

In order to gain insight into the extent of global transcriptome dysregulation in oligodendroglia cell lineage, we performed RNA sequencing of primary DMSXL cell cultures to identify significant alterations in total transcript levels and alternative splicing in OPC and OL.

Not surprisingly, given the lower toxicity, DMSXL OPC showed no significant changes in total transcript levels (other than *Fbxl7*, the mouse gene disrupted by the insertion of the *DMPK* transgene) and only six mild missplicing events. In contrast, DMSXL OL displayed 85 significant changes in total RNA levels, and 72 splicing defects. Splicing defects affected single alternative exons, multiple alternative exons and mutually exclusive alternative exons.

To shed light onto the biological meaning of transcriptome abnormalities in DMSXL oligodendroglia, we investigated how many of the RNA events affected in DMSXL OL were under cell differentiation-dependent regulation. The comparative analysis of the transcriptome of WT OPC and WT OL revealed significant changes in RNA expression and alternative splicing that occur during cell differentiation, under physiological conditions: oligodendroglia differentiation was accompanied by 8140 significant changes in total transcript levels, as well as 959 changes in the splicing of alternative exons.

Interestingly, out of the 85 transcripts that show altered total levels in DMSXL OL relative to WT OL controls, 80 transcripts undergo physiological changes in gene expression during WT cell differentiation (Fig 3A). The remarkable overlapping corresponds to a highly significant 2.4-fold increase in the sharing than expected by chance ($p < 1 \times 10^{-26}$, hypergeometric test). Similarly, 38 of the 72 exons that are misspliced in DMSXL OL are subjected to a physiological splicing transition during WT cell differentiation (Fig 3B), corresponding to a significant 12-fold increase in the representation factor ($p < 1 \times 10^{-32}$, hypergeometric test).

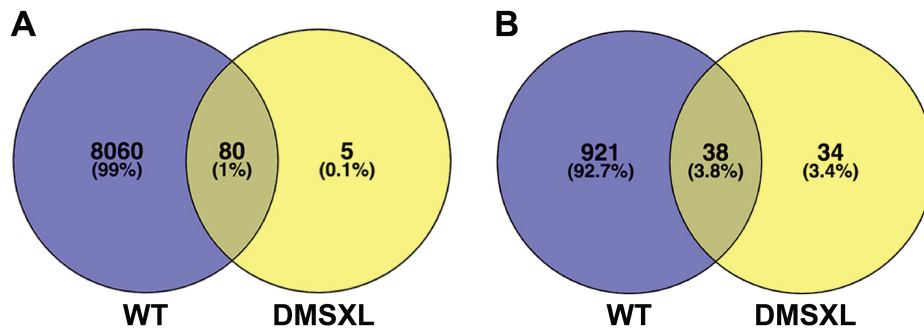


Figure 3 - Summary of the number of physiological and repeat-associated transcriptome changes in oligodendroglia in culture.

The number of physiological changes in total RNA levels (A) and alternative splicing events (B) during the differentiation of WT OPC into WT OL is represented in blue. The number of expression (A) and splicing (B) defects in DMSXL OL relative WT controls is represented in yellow. The diagrams highlight the number of events shared between WT differentiation and repeat-associated RNA abnormalities.

When we analyzed the RNA sequencing data in further detail, we found that in 72 out of the 80 shared events of gene expression dysregulation, the defects detected in DMSXL OL recreated (to a certain extent) the expression profiles of undifferentiated OPC, suggesting an expression profile that resembles that of immature cells. We have validated the expression changes of 3 genes, whose expression is reported to increase during cell differentiation. Quantitative RT-PCR confirmed significant downregulation of *Anxa6* (43%), *Anxa5* (53.8%), and *Itga3* (53.3%) in mature DMSXL OL, relative to WT controls cells (Fig. 4).

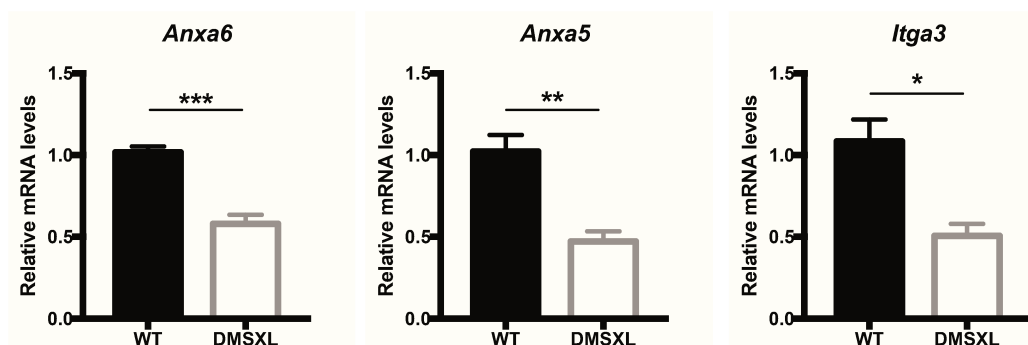


Figure 4 - Gene expression abnormalities in DMSXL OL.

Real-time PCR quantification of *Anxa5*, *Anxa6* and *Itga3* transcripts in DMSXL OL compared with WT cells (n=7). Data represents means \pm SEM. * $p < 0.05$, *** $p < 0.001$.

Similarly, the analysis of the RNA sequencing data showed that, among the 38 missplicing events in DMSXL OL, 33 increased the expression of splicing isoforms that are associated with immature OPC stage. We have confirmed this result by semi-quantitative RT-PCR analysis of candidate splicing events that appear to be markedly dysregulated and/or with known function in OL cell differentiation (studied exons are listed in Supplementary table 2). RT-PCR analysis confirmed that some missplicing events studied in DMSXL OL retained abnormally high levels of typical OPC-specific isoforms. The examples of *Cacna1a* and *Inf2* (among others) illustrate well the absence of splicing transition in DMSXL OL (Fig. 5A). In other cases, splicing analysis revealed a milder defect characterized by an incomplete splicing switch during DMSXL oligodendroglia differentiation. This is the case of *Capzb* and *Magi*: in spite of a splicing transition between DMSXL OPC and DMSXL OL, the PSI of the alternative exons does not reach the levels found in WT OL controls (Fig. 5B).

Surprisingly, some splicing abnormalities detected in DMSXL OL, such as those of *Mbnl2*, *Itga6* and *Arhgef12*, recreated an exaggerated inclusion/exclusion of alternative exons, producing splicing profiles that are even more accentuated than those found in WT OPC (Fig. 5C). For instance, the inclusion of exon 27 of *Itga6* transcripts increases during WT cell differentiation, while it decreases for DMSXL cells, producing an abnormally high number of transcripts depleted of exon 27.

Finally, we have also validated some splicing defects that do not recreate OPC splicing profiles. For instance, the increased inclusion of *Dgki* exon 22 in DMSXL OL produces a splicing isoform that is low abundant in immature OPC cells (Fig. 5D).

All together these results demonstrate that the expanded CUG RNA disrupts the developmental splicing program through oligodendroglia cell lineage progress. In the vast majority of the cases, the spliceopathy leading to the retention of abnormally high levels of splicing isoforms typically associated with immature cell stages.

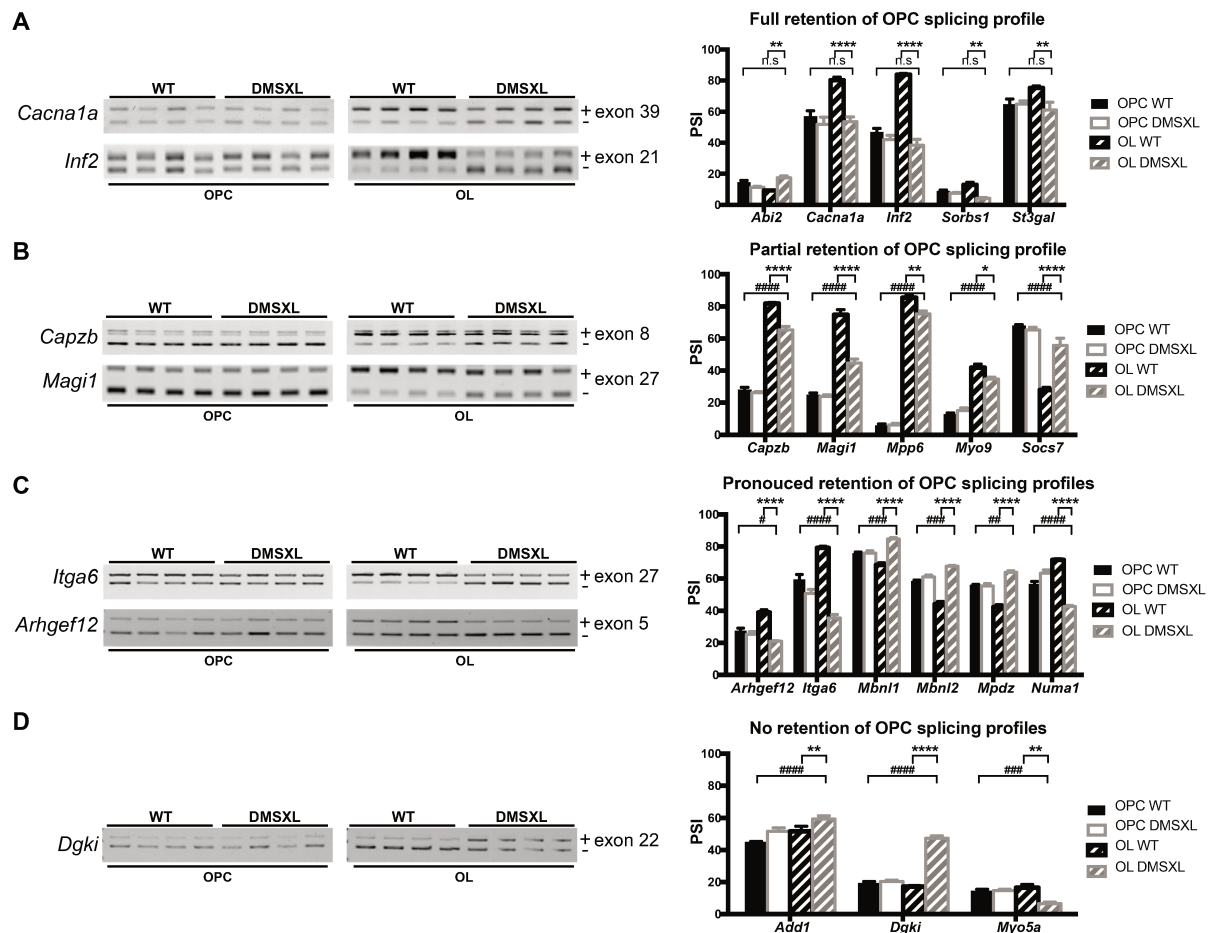


Figure 5 – Most of DMSXL OL splicing abnormalities retain OPC splicing profiles to different extents.

RT-PCR validation of RNA sequencing data showing sets of genes that either (A) full recreate, (B) partially retain or (C) exaggerate OPC splicing pattern. (D) A small subset of gene are misspliced in DMSXL OL to produce isoforms that are not OPC-associated. Data represent means \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

C. Transcriptomic analysis of DMSXL oligodendrocytes indicates overall dysregulation of cell adhesion and cytoskeleton dynamics

We then perform GO analysis to look for enriched “biological processes” and “cell components”, in order to shed light onto the cell processes and pathways that are most significantly dysregulated in the oligodendroglia cell lineage. The transcriptomics profiling revealed no enrichment in DMSXL OPC (as expected, given the lack of a significant number of altered events). In contrast, DMSXL OL are more sensitive to the

deleterious effect of toxic CUG RNA, particularly through changes in relevant clusters of transcripts related to their physiology.

The results suggested that changes in gene expression levels affect preferentially extracellular cell matrix interactions of DMSXL OL, which are critical to regulate cell shape and motility (Fig. 6A). On the other hand, the missplicing events reported in DMSXL OL cells affected more severely cell-cell adhesion pathways and components, mediated by actin cytoskeleton-based structures, such as protrusion outgrowth and polarization or GTPase-mediated signaling (Fig. 6B).

D. DMSXL OPC exhibit normal proliferation and signs of substrate-dependent suboptimal migration

We then investigated the functional consequences of CUG RNA toxicity on the physiology of the oligodendroglia cell lineage. Under physiological conditions, motile OPC engage in guided migratory routes to colonize the brain. Once arrived to the final location, OPC start to proliferate until appropriate cell densities have been reached (van Tilborg et al., 2018). We first tested whether pathological CTG repeats impair the dynamics of OPC by time-lapse videomicroscopy. To this end we first monitored OPC population growth over time, through the quantification of cell confluence, estimated as the percentage of the surface of the well occupied by cells. The analysis showed normal proliferation of DMSXL OPC (Fig. 7A). These results were further confirmed in the CG4 oligodendrocyte cell line transfected with *DMPK* constructs carrying either a CTG expansion (DT960) or no CTG repeats (DMPKS): similar to DMSXL OPC, the expansion had no significant effect on CG4 cell proliferation (Supplementary Fig. 2A).

Then to explore the migratory capacity of OPC, we performed wound-healing assays on DMSXL and WT primary cultures, and quantified the evolution of cell confluence over time inside a wounded space. Videomicroscopy analysis showed suboptimal migration capacity of DMSXL OPC, compared with WT controls (Fig. 7B): DMSXL OPC spend 13.4% more time to fulfill the space left by the wound (Fig. 7C). However, the difference did not reach statistical significance ($p=0.3$).

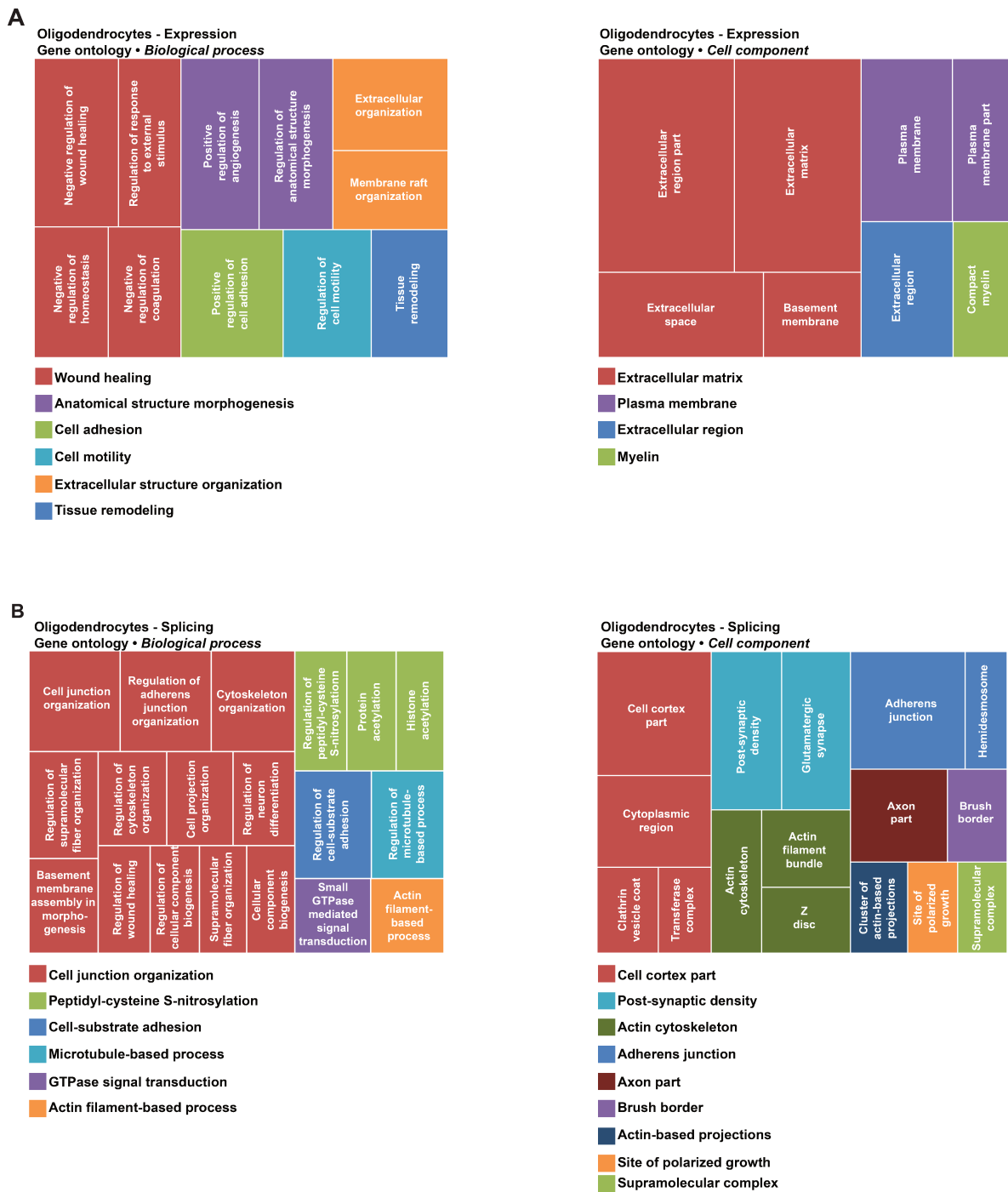


Figure 6 – DMSXL OL show dysregulation of clusters of transcripts relevant for the cellular phenotype observed.

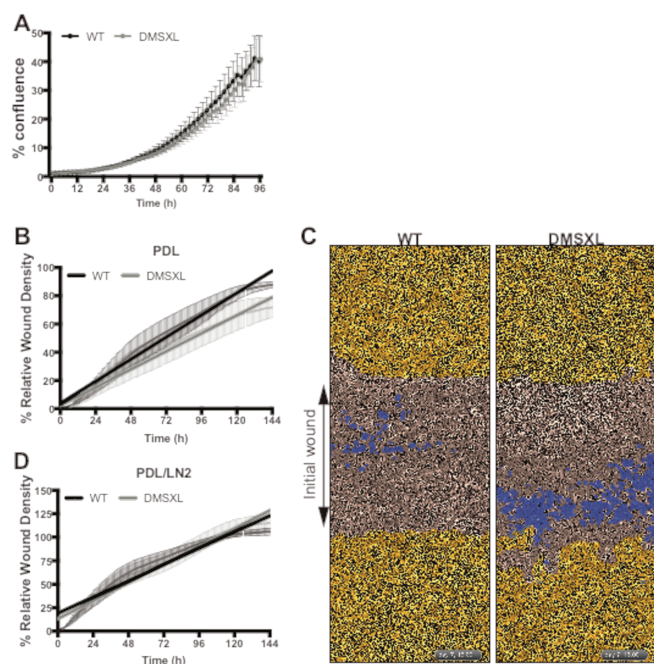
Treemap schematic representation of GO enrichment analysis of biological processes and cellular compartments associated with (A) dysregulated transcript levels and (B) missplicing events in DMSXL OL. The area attributed to each GO term is proportional to the statistical significance of the enrichment (i.e. highly statistical enrichments are represented by larger boxes). Related terms were grouped by similarity, using the Revigo software webtool (Supek et al., 2011) and color-coded.

Since extracellular matrix components influence OPC physiology (Hu et al., 2009), we have tested the effect of different coatings on OPC migration. To this end, we added Laminin 2 (LN2) in addition to standard PDL substrate, and assessed the impact of the extra cellular matrix on DMSXL OPC cell motility. Interestingly, the LN2-coating enhanced DMSXL OPC migration performance, yielding confluence levels within the wounded space that were undistinguishable from those measured in WT control cells (Fig. 7D). Hence, it is conceivable that OPC physiology is modified to a limited extent by a combined effect of the DM1 repeat expansion and extracellular factors.

In summary, the CUG RNAs toxicity in DMSXL OPC is not sufficient to induce significant proliferation or migration cell phenotypes. Nevertheless, signs of suboptimal cell migration might be revealed under particular growth conditions, pointing to a possible role of environmental factors in the modulation of cell phenotypes.

Figure 7 – DMSXL OPC show normal proliferation and poor migration.

(A) Videomicroscopy quantification of cell confluence over 96 h *in vitro* (n=10 cultures per genotype). (B) Quantification of migration speed estimated by the increase in wound confluence over time of DMSXL and WT OPC, plated on PDL-coated plates (n=6 cultures per genotype). (C) Phase contrast image of a scratched OPC monolayer and merged confluence masks used for quantification (yellow) after 7 days of migration. The merged images show the initial wound width (double arrowhead). (D) Quantification of migration speed of OPC plated on PDL/LN2-coated plates (n=6 cultures per genotype). Data represented as means \pm SEM.



E. DMSXL OL is associated with differentiation deficits

We next focused on the oligodendroglia cell stage that shows more pronounced molecular abnormalities (mature OL) to investigate whether CUG RNA toxicity impacts the morphological changes that take place during differentiation. To answer this question we differentiated mouse OPC *in vitro* for 7-8 days, by withdrawing growth factors from the culture medium one day after plating, and assessed process outgrowth

and branching through time by time-lapse videomicroscopy (Fig. 8A). Bright field tracking of process outgrowth and branching revealed that cell processes grew 16.5% slower in DMSXL cells relative to WT controls and showed a significant reduction of 27,1% in branching points over time (Fig. 8B), suggesting a differentiation phenotype in response to the DM1 mutation. Expanded *DMPK* constructs also impaired OL process branching of the CG4 cell line at 3 div, and resulted in a trend for shorter process length at 5 div when compared with cells expressing no repeat control *DMPK* RNA (Supplementary Fig. 2B).

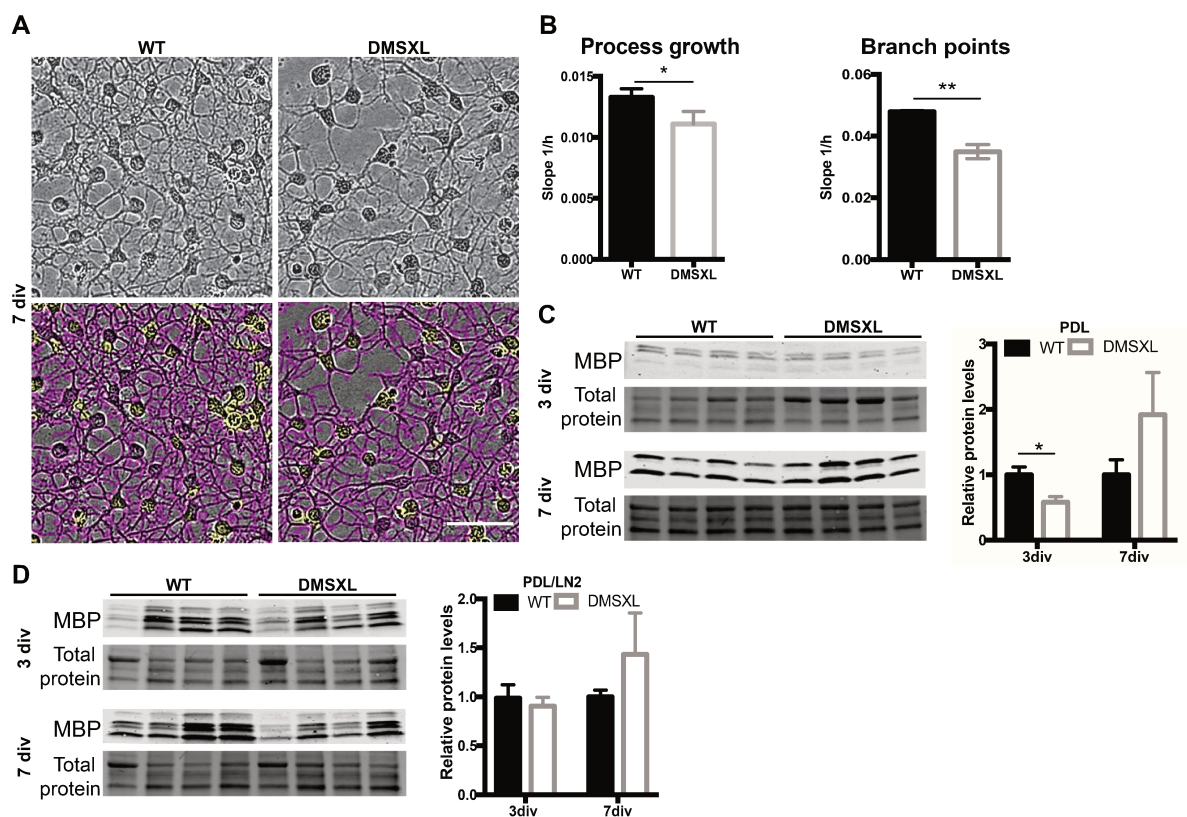


Figure 8 - DMSXL OL display defects in process outgrowth and branching, in association with early MBP downregulation.

(A) Representative bright field images of DMSXL and WT OL at 7 div, illustrating the process of cell arborization in culture. (B) Quantification of the speed of process growth, length and branching over 8 DIV by time-lapse videomicroscopy (n=5 per genotype). Scale bar represents 50 μ m. Experiments were performed in triplicate. (C) Western blot immunodetection of MBP protein levels in OL with 3 and 7 div from DMSXL and WT mice (n=4 independent cultures per group) differentiated in the absence or (D) presence of LN2 (n=7 independent cultures per group). Data represented as mean \pm SEM. * p <0.05; ** p <0.01.

Process extension and branching depend on the reorganization of the cytoskeleton during differentiation. The two phenomena are intrinsically connected with the reprogramming of gene expression required for OL terminal differentiation (Seixas et al., 2019, Zuchero et al., 2015). To test if the reduced process extension and branching during DMSXL OL maturation *in vitro* were accompanied by defective expression of protein markers of fully mature OL, we quantified the levels of MBP at 3 and 7 div. Interestingly, MBP protein expression in DMSXL OL follows a similar profile to that detected *in vivo* (Chapter III), with an early mild MBP downregulation in DMSXL oligodendrocytes at 3 div, and a normalization of protein levels at 7 div, relative to WT OPC cultures (Fig. 8C). Interestingly, the presence of the LN2 (and PDL) in the coating changed the protein isoform distribution of MBP, increasing the expression of the 21,5 kDa and 14 kDa isoform at 3 div, that are typically associated with mature OL. More importantly, the double PDL/LN2 coating abolished the difference in total MBP steady-state protein levels at 3 div between both genotypes (Fig. 8D).

Taken together these results showed impaired morphological maturation of DMSXL OL in culture, in association with substrate-dependent defects in myelin protein expression.

F. Human OL derived from DM1 iPSC show arborization defects

To explore if the DM1 mutation had a similar impact on process outgrowth and branching in human oligodendroglia, we differentiated iPSC from DM1 patients and healthy relative controls into OL. Since cell densities are an important cue for OL differentiation (Rosenberg et al., 2008), and given that some variability between cell lines was described as an intrinsic feature of the protocol (Douvaras and Fossati, 2015), we started by excluding the possible bias introduced by different cell densities on the capacity of iPSC to differentiate into oligodendroglia. To this end, we quantified the percentage of OLIG2-positive cells, a pan marker for all stages of oligodendroglia cell lineage. After 92 div, both DM1 and control cells produced OLIG2-positive cells within the expected range previously reported (Douvaras and Fossati, 2015), and in similar percentages between groups (Fig. 9A and B). Moreover, when we checked the different maturation stages, the percentage of immature O4-positive OL among the total OLIG2 population did not differ significantly between DM1 and controls (Fig. 9B). These results

suggest that once committed to the oligodendroglia lineage, DMSXL cells show a distribution of cells in immature pre-myelinating stages similar to control cells.

However, standard immunofluorescence microscopy of human OL labeled with O4, gave the impression of reduced cell area and complexity in DM1 OL. To confirm this phenotype we performed a Sholl analysis to evaluate cell complexity, through the quantification of the number of intersections between cell processes and concentric Sholl rings aligned from the cell body. Despite some variability detected between controls, the results revealed a significant lower number of intersections in DM1 oligodendroglia cells compared with controls (Fig. 9C), indicating reduced complexity of cell morphology in response to the DM1 expansion mutation.

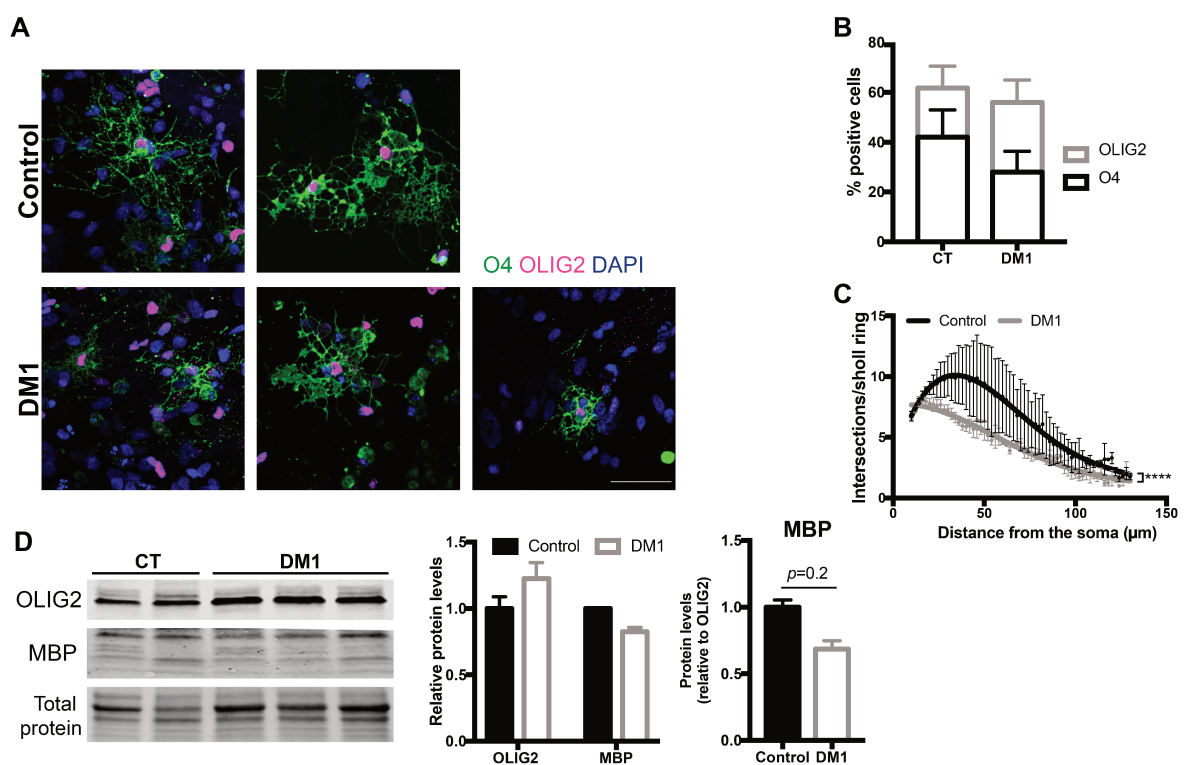


Figure 9 – Reduced morphological complexity in DM1 OL.

(A) Representative confocal images IF showing OLIG2 (magenta) and O4 (green) staining in iPSC-derived OL from healthy controls and DM1 patients, after 92 div. Diamidino-2-phenylindole was used for nuclear staining. The scale bar represents 50 μm. (B) Quantification of percentage of OLIG2- and O4-positive cells. (C) Sholl analysis quantification of intersection number per sholl ring radius ($n_{\text{cells}}=59-93$). (D) Representative western blot of MBP protein levels in iPSC-derived OL after 92 div from DM1 patients and controls. Quantification protein levels relative to total protein and MBP relative to OLIG2 internal control. ($n=2$, non-DM controls; $n=3$, DM1; mean of 3 independent cell cultures). Data represents means \pm SEM. **** $p<0.0001$

We then investigated if the morphological signs of defective maturation were accompanied by reduced expression of myelin proteins in OLIG2-positive DM1 oligodendroglia. Interestingly, similar to the findings reported in DMSXL mice (Chapter III), human OL presented a trend towards lower levels of MBP, a protein usually expressed by mature myelinating OL, relative to OLIG2 (Fig. 9D). These findings corroborate the impaired differentiation of the oligodendroglia in DM1 brain disease.

V. Discussion

DMSXL primary cultures: a cell model of RNA toxicity in oligodendroglia lineage

In this chapter we have investigated the consequences of the expression of DM1 expanded CTG repeats in oligodendroglia throughout cell differentiation. Taking advantage of primary cell culture models, we have described for the first time CUG RNA expression and foci accumulation in DMSXL oligodendroglia cell lineage, from the early OPC cell stage to fully differentiated OL. Interestingly, *DMPK* transgene expression increased with cell differentiation, leading to a higher number of nuclear RNA foci per cell, which predicts more pronounced sequestration and functional inactivation of MBNL proteins in OL relative to OPC. As a result, differentiated DMSXL OL exhibit more severe expression abnormalities and spliceopathy than progenitor cells.

We have previously reported RNA foci accumulation in DMSXL brains, in particular in OL (Chapter III), neurons and astrocytes (Hernandez-Hernandez et al., 2013a, Sicot et al., 2017). *In vivo*, the percentage of foci-containing oligodendrocytes decreases with development, but the average number of foci *per* cell is maintained though. However, in cultured oligodendrocytes the percentage of foci-positive cells increases from OPC to mature OL and it is accompanied by higher average number of foci *per* nucleus. This is in agreement with the increased expression of the human *DMPK* transgene during OL differentiation, following to some extent the pattern observed during DMSXL brain development (Michel et al., 2015). A possible explanation for the contrasting results reported *in vivo* and in culture could be the elimination of damaged OL in mouse brains by the microglia surveillance system (Hanisch and Kettenmann, 2007): if microglia operates in mouse brains to sense and eliminate cells in which RNA

toxicity causes pronounced damage, this process may account for the reduction in the number of foci-positive cells between P15 and P30. We cannot exclude a reduction in transgene expression with age, as a contributing factor towards the lower percentage of OL containing cells at P30. To test this hypothesis, *DMPK* expression should be quantified in cells isolated from DMSXL brains at different developmental time points. Finally, the expression of other factors, which are important for the aggregation of ribonuclear foci, may vary in different settings and developmental stages, contributing to discrepancies observed.

Interestingly both MBNL proteins were downregulated in DMSXL OPC and OL compared to WT. Since the protein extraction protocol used is stringent enough to extract both nuclear and cytoplasmic protein fractions, we believe the lower levels of MBNL proteins detected in DMSXL cells are not explained directly by their sequestration into RNA foci, but rather by a reduced mRNA expression mediated by the missplicing *Mbnl* transcripts. In order to address this question, it would be interesting to measure steady-state levels of *Mbnl1* and *Mbnl2* transcripts in DMSXL cells relative to WT controls.

Transcriptome dysregulation in DMSXL oligodendrocytes: from molecular abnormalities to dysfunctional pathways

Consistent with the more pronounced accumulation of toxic RNA foci in mature DMSXL OL, RNA sequencing approaches revealed a higher number of dysregulated transcripts in DMSXL OL *versus* OPC. GO analysis of abnormal transcripts pointed to an enrichment for clusters of genes participating in cytoskeleton rearrangements, motility and cell adhesion. In agreement with the low signs of RNA toxicity and transcriptome abnormalities, cell functional studies demonstrated that DMSXL OPC show only subnormal, substrate-dependent, migration defects, while DMSXL OL display significant impairment of differentiation-associated process outgrowth and branching. Defective process complexity in response to the CTG repeat expansion was confirmed in independent cell models of rat oligodendroglia and human iPSC-derived oligodendrocytes, strongly corroborating the view that the differentiation delay of oligodendroglia is a direct consequence of toxic CUG RNA.

Molecular validation approaches showed that the expression and splicing abnormalities of DMSXL OL recreate to a certain extent the transcriptome profile of OPC, thereby reproducing the molecular signature of undifferentiated cells. These findings provide mechanistic support for the differentiation defects of DMSXL oligodendroglia. In particular the OPC-like profiles of transcripts involved in cytoskeleton remodeling (such as *Itga6* and *Arhgef12*) found in mature OL may explain the defective process extension of DMSXL OL (Michalski et al., 2016, Zuchero and Barres, 2013). In particular, ARHGEF12 has been implicated in OL differentiation from neuronal stem cells, which demonstrate the importance of this factor in lineage specification (Suzuki et al., 2007). Furthermore, the downregulation of other integrins, such as *Itga3* reported here, may further aggravate the dysregulation of signaling pathways that connect the extracellular matrix to the cytoskeleton.

The different degrees of splicing dysregulation found in individual transcripts, and the extent to which they recreate truly OPC splicing profiles may be accounted for different mechanisms: 1) a dose-dependent compensation by MBNL members, in exons that are permissive to both MBNL1 and MBNL2 splicing regulation, can explain the partial retaining of OPC-like profiles; 2) reduced levels of functional MBNL1 or MBNL2 proteins in DMSXL OL are not sufficient to trigger the splicing switch of MBNL1- or MBNL2-exclusive exons, hence resulting in full retention of OPC splicing profiles; or 3) the competition/interaction of MBNL proteins with other splicing factors is altered, leading to “exacerbated” OPC splicing profiles or different splicing defects. All these mechanisms are well described in DM1, in response to MBNL protein inactivation by toxic RNA foci (Goodwin et al., 2015, Batra et al., 2014, Mohan et al., 2014, Kalsotra et al., 2008). Additionally to MBNL sequestration, the missplicing of *Mbnl1* and *Mbnl2* transcripts can directly affect total protein levels (Konieczny et al., 2018, Konieczny et al., 2014), potentially contributing towards MBNL protein downregulation and further exacerbating missplicing of target transcripts.

The partial inactivation and downregulation of MBNL proteins in DMSXL oligodendroglia may therefore have a significant contribution towards the differentiation defects reported. Previously these proteins were shown to be involved in the molecular regulation of pluripotency during cell reprogramming (Han et al., 2013). It

is therefore conceivable that the decreased activity of MBNL1 and MBNL2 in DMSXL OL impairs the progression of cell differentiation.

Oligodendroglia dysfunction: the combined effect of intrinsic and extracellular factors

The morphological defects of DMSXL OL were associated with mild MBP downregulation at early stages of differentiation, which was later resolved in mature cells. MBP expression is also delayed in the brain of young DMSXL mice and human patients, in association with a reduced number of myelinated axons in mice (Chapter III). Interestingly the defect in MBP was abolished when cells grew on laminin-containing cell substrates. These results are in agreement with the reported benefits of LN2 to myelin production (Hu et al., 2009). More importantly, these findings suggest a critical role played by external cues on the phenotypes of DMSXL mice.

Additionally to the role in MBP synthesis, LN2 is also a key element in the regulation of cytoskeleton dynamics and differentiation of oligodendrocytes through its interaction with ITGA6/beta 1 receptor, providing a signaling pathway for cell communication with the extracellular matrix (ECM). ECM-Integrin signaling activates RhoGTPases effectors and promotes actin filament polymerization and/or depolymerization, a process that is crucial for OL process outgrowth and branching (O'Meara et al., 2013, Warner et al., 2019, Seixas et al., 2019). Therefore, the rescuing of MBP expression in DMSXL OL may be accounted by a positive effect of LN2 on the integrin signaling pathway, that is otherwise more sensitive to its absence as a result of *Itga6* missplicing and *Itga3* downregulation, among other possible molecular defects. LN2 may compensate for these defects and drive the cells towards molecular differentiation, but is not sufficient to overcome process extension phenotypes. Thus, potential effects of other LN2 receptors in OL physiology, such as dystroglycan, must also to be considered.

It will be worth testing if process extension and branching defects seen in DMSXL OL (observed in cells cultured in PDL/LN2-coated plates) would be further impaired in the absence of LN2. Similarly, it would be interesting to evaluate the proliferation of OPC grown in absence of LN2, to check whether LN2 and downstream signaling cascades influence the proliferative capacity of progenitor oligodendroglia.

VI. Conclusion

Taken together, our findings may point to both cell-autonomous and non-autonomous mechanisms to explain the impact of CTG repeats on the DMSXL OL differentiation. The phenotypes observed in DM1-derived OL corroborate this view, revealing this mixed cell culture system as an asset to study the interaction between different brain cell types and their contribution to general DM1-associated brain disease.

Further studies on the implication of molecular events dysregulated in OL phenotypes will provide insight into cell-specific pathways dysregulated by the repeat mutations, and how they contribute to DM1 white matter abnormalities and overall brain pathology. Hence opening new routes for the identification of pharmacological targets for future rational therapies, notably through the modulation of signaling pathways triggered by the interaction with the extracellular matrix.

VII. Supplementary information

A. Supplementary tables

Supplementary table 1 - Primary antibodies for IF and WB (related to figures 1, 2, 8 and 9).

| Antigene | Supplier; catalogue number | IF blocking and incubation conditions | WB blocking and incubation conditions | Dilution (IF; WB) |
|----------|-------------------------------|------------------------------------------|------------------------------------------|----------------------|
| OLIG2 | Millipore; AB9610 | 0.2% BSA, 10% NGS, 0.3% TX-100; 1h, RT | 5% blotto, 1h, RT | 1/250; 1/1000 |
| MBNL1 | MB1A from Glen Morris (gift) | 0.2% BSA, 10% NGS; 1h, RT | 5% blotto, 1h, RT | 1/10; 1/200 |
| MBNL2 | MB2A from Glen Morris (gift) | 0.2% BSA, 10% NGS; 1h, RT | 5% blotto, 1h, RT | 1/10; 1/200 |
| ITGA6 | GeneTex; GTX100565 | - | 2% BSA, 1h, RT | 1/1000 |
| MBP | Abcam; ab40390 | - | 5% blotto, 1h, RT | 1/500 |
| MBP | Abcam; ab7349 | 10% NGS; 1h at RT | - | 1/500 |

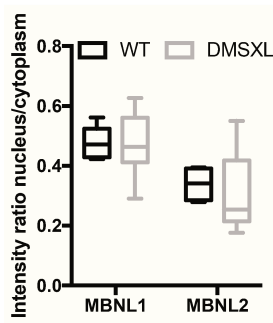
| | | | | |
|----------------|----------------------|-------------------------------|-------------------|--------------|
| PDGFR α | Santa Cruz; sc-338 | 0.5% BSA, 0.2% TX-100; 1h, RT | 5% blotto, 1h, RT | 1/200; 1/100 |
| O4 | R&D systems; MAB1326 | 10% NGS; 1h at RT | - | 1/100 |
| beta-Actin | Genetex; GTX109639 | 10% NGS; 1h at RT | - | 1/400 |

NGS, normal-goat serum; h, hour; RT, room temperature; BSA, bovine serum albumin

Supplementary table 2 - Oligonucleotide primer sequences used in RT-PCR analysis in mouse oligodendrocytes (related to Figure 1, 2, 4, 5 and 8)

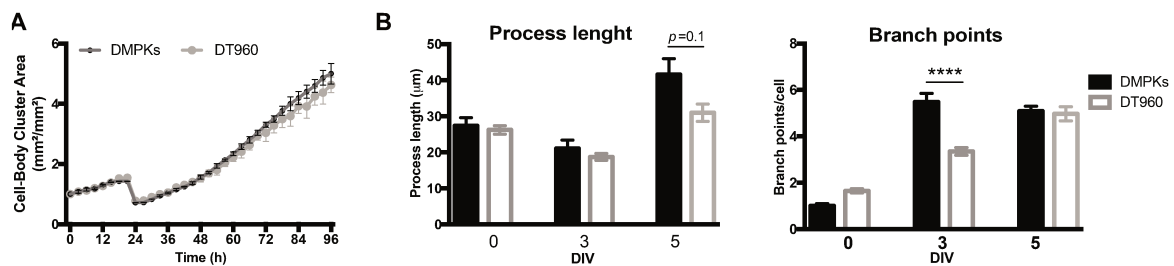
| Gene | Spliced exon | Forward primer | Reverse primer | Product size (bp) |
|------------------|--------------|------------------------------|------------------------------|-------------------|
| <i>Anxa V</i> | - | ACTCCTGACCGACAGCATCAT | GCATCAGCCCTGCCATCAAAT | 75 |
| <i>Anxa 6</i> | - | GAAGCCCATCGAAGCCAGTA | TTCGGATGCACTTCACCACG | 83 |
| <i>Serpina3n</i> | - | AGGAAACAGACCCAGGGGAT | GGCACCTTCCATTTGGCTTTA | 107 |
| <i>Mbnl1</i> | 7 | TGGTGGGAGAAATGCTGTATGC | GCTGCCAATACCAGGTCAAC | 270/216 |
| <i>Mbnl2</i> | 5 | CTTTGGTAAGGGATGAAGAGCAC | ACCGTAACCGTTTGTATGGATTAC | 255/201 |
| <i>Cacna1a</i> | 39 | TGCTGAATCTCTTTGTCGCTGTC | GAAGTGTGTTGTCATCTGCTACCG | 173/270 |
| <i>Inf2</i> | 21 | CTGAAGATACCCCGGATGCC | CCGACGAGAGCACTCACTT | 471/528 |
| <i>Capzb</i> | 8 | GCACGCTGAATGAGATCTACTTTG | CCGTTAGCGTGAAGCAGAG | 280/192 |
| <i>Sorbs1</i> | 27 | CCAGCTGATTACTTGGAGTCCACAGAAG | GTTACCTTCATACCAGTTCTGGTCAATC | 349/181 |
| <i>St3gal3</i> | 4 | CCTCTTCTGGTCTGGGAT | GGAGGAAGCCCAGCCTATCA | 95/143 |
| <i>Mpp6</i> | 8 | TCGACAAGGTCTGCTTCACG | TAGTCTGCTTCTTTGCAGGG | 232/274 |
| <i>Magi1</i> | 27 | GAAGTATCAAGAATGGCGGC | CTGGACTCCAAGGCTGGATG | 176/265 |
| <i>Dgki</i> | 22 | CAGTGTCGGGAAGTGATGCT | AAGCTTCCCGGAGTTTGTCC | 262/325 |
| <i>Arhgef12</i> | 5 | TTGAACCGAGAGTCACCAACA | GATCACACAGCGCTGAACAA | 96/153 |
| <i>Mpdz</i> | 28 | TGAACAAGCTGTGGAAGCCA | GGAGGGAGGAACATTGCACA | 139/238 |
| <i>Numa1</i> | 16 | GACCCACTTGGCTGAAATGC | GTCAGCTTCTTACTTAGTTCTTCC | 171/213 |
| <i>Abi2</i> | 8 | GAGAACAGCGGAAGTGGTAGTGTG | TTGTAGGTGGTGTATGGCGAGAG | 133/316 |
| <i>Myo9a</i> | 27 | GCCCACAAACAAGATGAATCAGC | CAGAAAATGGATGAGCAGGTGTC | 313/526 |
| <i>Socs7</i> | 4 | CGTGGACATTTCTCAGCGGG | ATAGAGGGGACACTGGAGGTGC | 166/268 |
| <i>Add1</i> | 16 | CAGAAAGAGAAGAGTCTCCAGACC | TTGAAGGTGGTGGCATCACTG | 129/166 |
| <i>Myo5a</i> | 31 | GAGCCTAAAGGAAGAAAACAACCG | TCCATCTGCCCCACTTCTAGTTC | 231/306 |
| <i>Itga6</i> | 27 | GGGATTCTGATGCTGGCTCTATTAG | GGCTTTGGGTAGTGTGAGGTGTTT | 246/376 |
| <i>Pol2ra</i> | - | GGCTGTGCGGAAGGCTCTG | TGTCCTGGCGGTTGACCC | 155 |

B. Supplementary figures



Supplementary figure 1 - MBNL protein intracellular distribution in OL.

Quantification of nucleo-cytoplasmic localization of MBNL1 and MBNL2 in primary OL (MBNL1 n>400 cells per genotype; MBNL2 n>250 cells per genotype).



Supplementary figure 2 - Toxic CUG repeats dysregulate oligodendrocyte branching in culture.

(A) Quantification of the effect of CTG repeat expansion on CG4 proliferation by videomicroscopy. The area occupied by the cell bodies per mm² of plate well is plotted through time, in cells transfected with expanded DT960 or no-expansion DMPKS plasmids (B) Measurement of cell process length and branching, by immunolabeling of the actin cytoskeleton on transfected CG4 cells with DT960, compared with DMPKS-expressing cells, at 0, 3 and 5 div time points. Data represent means \pm SEM. **** $p<0.0001$

VIII. Author contribution

Sandra O. Braz: mouse breeding and genotyping; oligodendrocyte cell cultures setup, analysis of RNA toxicity in culture (foci accumulation, MBNL sequestration and missplicing); sample preparation for RNA sequencing; RNA sequencing validation analysis; analysis of OPC and OL phenotypes; iPSC-derived OL cultures setup, data collection and post-acquisition analysis; study design; data collection and analysis; preparation of figures; manuscript writing.

Aurélien Cordier: : analysis of alternative splicing in primary cultures.

Raphäel Blain: analysis of alternative splicing in primary cultures.

Olivier Alibeu: RNA sequencing processing and data analysis.

Cyril F. Bourgeois: Bioinformatics analysis of alternative splicing.

Aline Huguet: mouse colony maintenance and genotyping.

Christine Bole-Feysot: RNA sequencing design and data analysis.

Cecile Martinat: Provided iPSC samples and scientific discussion.

Didier Auboeuf: Bioinformatics analysis of alternative splicing.

Geneviève Gourdon: study design; data analysis.

Mário Gomes-Pereira: study design; data analysis; manuscript revision.

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Chapter V

Conclusions and general discussion

I. Discussion

My PhD work has contributed to expand the knowledge on DM1 brain disease by investigating the unexplored implication of oligodendrocytes and myelin in CNS pathophysiology, and the characterization of the molecular and functional consequences of the CTG repeat expansion on this cell type. Using the relevant DMSXL mouse, mouse and human cell culture models of the disease, as well as human DM1 tissue samples, I have shown that oligodendroglia lineage is affected by the expanded CTG repeats, with a subsequent impact on myelin biology. *In vivo*, oligodendrocytes from DMSXL brains present molecular hallmarks of CUG RNA toxicity that are more pronounced in early developmental ages. Interestingly, RNA toxicity in oligodendrocytes was associated with transient CC phenotypes, including reduced size (hypoplasia) and hypomyelination with reduced number of myelinated fibers and preferential myelination of larger axons. These phenotypes are likely accounted for, at least in part, by inefficient differentiation of the oligodendroglia cell lineage. Myelination deficits were extended to other DMSXL brain regions, which are relevant for DM1 CNS dysfunction. In particular, the frontal cortex shows dysregulation of important myelin proteins, such as MBP, CNP or MOG in developing young mice, further supporting delayed myelination process, which is later recovered in older animals. DM1-associated defects in myelin defects were further confirmed in independent mouse models, characterized by the dysfunction of canonical mediators of the disease: *Mbnl* knock-out mice also exhibited significant downregulation of MBP, corroborating the critical role of MBNL proteins in the etiology of myelin disease in DM1. Furthermore, human validation studies revealed that the toxic RNA foci detected in oligodendrocytes of DM1 *post mortem* brain tissue appear to impact oligodendrocyte and myelin proteins in a similar way, i.e. impairing MBP expression characteristic of fully matured oligodendroglia cells.

In the search for the mechanisms behind these phenotypes, the transcriptomics of primary DMSXL cell cultures showed dysregulated expression and alternative splicing of genes implicated on oligodendrocyte differentiation. To study the functional consequences of such abnormalities with single cell resolution, I took advantage of sophisticated videomicroscopy approaches, which I have set up and optimized during

my PhD. Using live cell imaging, I visualized and quantified the morphological differentiation of DMSXL oligodendrocytes, and found differentiation deficits, which are likely to contribute to myelination impairment in DMSXL mice. Importantly, independent cell culture models of transfected cells and DM1-derived oligodendrocytes showed similar differentiation phenotypes, illustrating the impact of the CTG repeat expansion on oligodendrocyte physiology and the involvement of this cell type on DM1 pathology.

In addition to the intrinsic oligodendrocyte contribution, non-cell autonomous mechanisms might be envisaged. It is conceivable that axon-myelin miscommunication, as well as astrocyte deficient support may influence oligodendrocyte physiology and contribute towards myelination deficits, since neuronal and astrocytic physiology are also both affected in DMSXL CNS (Hernandez-Hernandez et al., 2013a, Hernandez-Hernandez et al., 2013b, Sicot et al., 2017). Therefore, combined cell cultures experiments must be conducted to explore the cross talk between cell populations and the contribution of other cell types to the myelination phenotypes in DM1.

Pathomechanisms behind oligodendrocyte and myelin dysfunction

Differentiation-regulated splicing transition and beyond

The perturbation of gene expression and alternative splicing appears to be involved in the impairment of oligodendrocyte differentiation associated with DM1. In particular, RNA sequencing of DMSXL oligodendrocytes revealed the abnormal alternative splicing of a significant number of transcripts, which are usually developmentally dysregulated during cell differentiation. Such molecular defects affect the expression of fully mature splicing profiles in cultured DMSXL oligodendrocytes, providing strong molecular evidence of impaired cell differentiation.

Previously, DM1 molecular pathogenesis was previously characterized by the failure in the post-natal transition of developmental alternative splicing in muscle, heart and brain. DM1 missplicing in these tissues results in the abnormal expression of embryonic splicing isoforms in adult tissues, which is not compatible with proper tissue function (Kalsotra and Cooper, 2011, Kalsotra et al., 2008, Lin et al., 2006, Hernandez-Hernandez et al., 2013a). Here, we have demonstrated for the first time that similar mechanisms operate in the oligodendroglia cell lineage, and that such molecular

abnormalities might translate into delayed cell maturation of and defective tissue function in adults. Together with the transient myelination defects, these data support the view that DM1 oligodendroglia and myelin dysfunction might have a developmental origin.

In contrast to *Mbnl1/Mbnl2* double knockout (KO) mice, in which developmental regulated MBP proteins isoforms, generated by alternative splicing, are altered and possibly contribute to MBP downregulation, the low MBP expression in DMSXL, *Mbnl2* single KO and human patients is not associated RNA missplicing. Interestingly, MBP

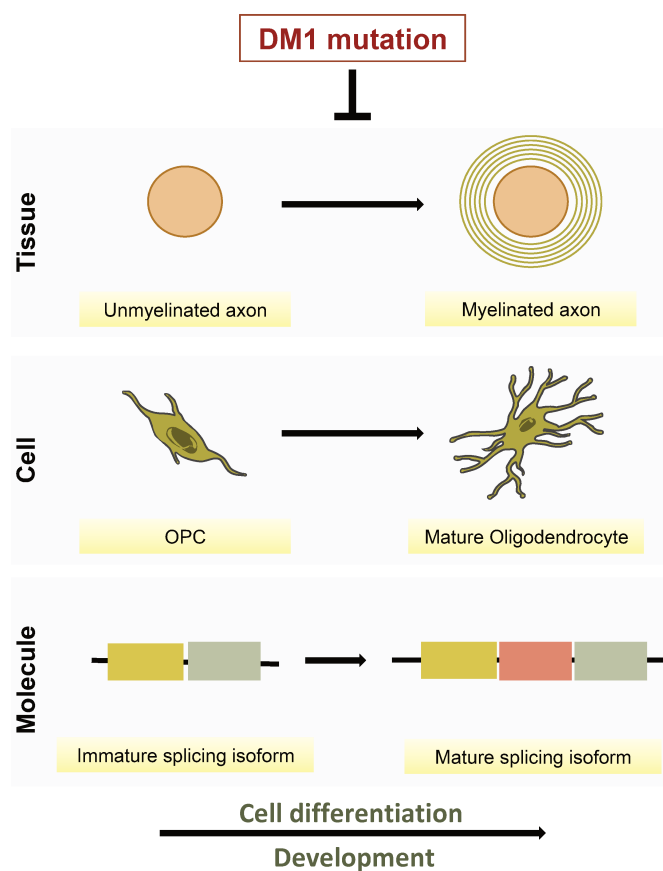


Figure 1 – Summary of processes impaired by the DM1 mutation at tissue, cellular and molecular levels.

protein levels remain unchanged in *Mbnl1* KO mice, possibly as a result of an MBNL2-dependent compensatory mechanisms. These findings indicate that the pathomechanisms implicated in myelin dysfunction are not entirely explained by the dysregulation of alternative splicing, and point to an additional role of MBNL2 protein on the regulation of MBP expression by other means. Other alternative routes for the action of MBNL2 on MBP expression are the regulation of alternative polyadenylation and/or *Mbp* transcripts localization within the cell; both mechanisms that can regulate the levels of locally translated MBP protein (Batra et al., 2014, Wang et al., 2012).

Extracellular cues, cell adhesion and oligodendroglia differentiation

Under physiological conditions oligodendrocyte differentiation is regulated by cell intrinsic and extrinsic cues, and the imbalance of either signaling paths might have

long-term pathological implications (van Tilborg et al., 2018). Gene ontology enrichment analyses of DMSXL transcriptomics data have pointed to altered biological processes related with cytoskeleton dynamics and global cell adhesion. Extracellular signaling has a great influence on these processes. In support of the role of extracellular factors, we found that the extracellular matrix molecule laminin improved DMSXL OPC performance and enhanced of MBP expression by DMSXL oligodendrocytes. However, process outgrowth and branching defects were unaffected by the presence of laminin on the culture dish. The contribution of laminin and its receptors to the DM1 related phenotypes (as well as other components of the extracellular matrix and their interacting proteins) should be further investigated by assessing their expression levels and activity. Interestingly, similar to DM1, in the congenital muscular dystrophy, caused by laminin $\alpha 2$ deficiency, patients show abnormalities in the (WM) matter tracts and disease mouse models present reduced myelinated axons, among other myelin defects (Cognato et al., 2005).

Recently, reports on depressed individuals indicate affected oligodendrocyte-astrocyte intercellular communication through the abnormal distribution of gap junction proteins within oligodendrocytes (Tanti et al., 2019). The depressive-like behaviors of DM1 patients (Sistiaga et al., 2010, Serra et al., 2014) could be mediated, at least partly, by similar defects in cell adhesion, a hypothesis that is supported by the astrocyte dysfunction reported in DM1 (Hernandez-Hernandez et al., 2013a, Sicot et al., 2017) and the transcriptomics alteration of DMSXL OL (Chapter IV). Defective adhesion between OL and other cell types may lead to oligodendrocyte dysfunction and myelin abnormalities in DM1.

The role of neurotransmitters

DM1 mouse models have provided evidence of both gamma-aminobutyric acid (GABA) and glutamate signaling defects associated with the CUG RNA toxicity. Neuronal hyperexcitability has been reported in *Mbnl2* KO and DMSXL mice, suggestive of GABA and glutamatergic altered neurotransmission (Braz et al., 2018). Among the potentially contributing factors are the missplicing of glutamate ionotropic receptor NMDA type subunit 1 (*Grin1*) and associated genes (such as *Tanc2* and *Cacna1d*), which likely perturb N-methyl-D-aspartate (NMDA) receptor functioning (Charizanis et al., 2012), as

well as the reported downregulation of glutamate transporter 1 (GLT1) levels (Sicot et al., 2017).

In oligodendroglia, the expression of both GABA and glutamate receptors is implicated in lineage maintenance and progression (v. Blankenfeld and Kettenmann, 1991, Gallo and Ghiani, 2000). Signaling through GABA_B receptors promote oligodendrocyte differentiation by enhancing processes branching and MBP expression (Serrano-Regal et al., 2019). It is therefore conceivable that altered GABA metabolism or release in response to CTG expansion may affect oligodendrocyte and myelin physiology.

The function of glutamate signaling on the oligodendroglia cell lineage is more diverse and complex, and involves different receptors. NMDA receptors are the main type of glutamate receptors involved in the modulation of oligodendrocyte differentiation (Tauheed et al., 2016), however AMPA receptor are also crucial for OL survival and subcortical myelination (Kougioumtzidou et al., 2017). Interestingly, mice with disrupted AMPA receptor signaling in OPC, show reduced number of mature oligodendrocytes in developing CC without major myelin ultrastructure deficits (Kougioumtzidou et al., 2017), like the DMSXL mice described here. Regardless the receptor involved, oligodendroglia cells, especially in the mature stage, are sensitive to glutamate excitotoxicity (McDonald et al., 1998, Deng et al., 2004, Matute et al., 1997), which can contribute to WM lesions in human disorders, such as ischemia, stroke or multiple sclerosis (Káradóttir et al., 2005). Thus, the potential effects of glutamate signaling on DMSXL oligodendrocyte lineage may be two-fold: 1) it is conceivable that the splicing defects of glutamate receptors impair oligodendroglia differentiation and/or function; and 2) it is possible that DMSXL oligodendrocytes are affected by excessive extracellular glutamate concentration, due to the reduced levels of GLT1. Microdialysis quantification of extracellular levels of glutamate and GABA in DMSXL brains, as well as the investigation of the alternative splicing of their receptor subunits in primary cell cultures should be conducted to uncover the implications of neurotransmission signaling on the abnormalities of OL and myelin in DM1.

Neuronal activity-dependent vesicular release of neurotransmitters serve as a communication route between neurons and oligodendrocytes to fine tune the onset and extent of myelination (Mensch et al., 2015, Wake et al., 2011, Bergles et al., 2000, Wake

et al., 2015). Since the expression and phosphorylation of some neuronal synaptic proteins are altered in the brains of DMSXL mice and human patients (Hernandez-Hernandez et al., 2013a, Hernandez-Hernandez et al., 2013b), changes in vesicle release and dynamics may represent an alternative or additional mechanism to explain DMSXL myelination delay. Stimulating primary DMSXL oligodendrocyte cultures with conditioned medium from wild-type neurons, to evaluate the differentiation progress, might elucidate the impact of vesicular release on DMSXL myelin deficits.

Metabolism and insulin signaling

Insulin metabolism has been classically associated with oligodendrocyte physiology during development. The insulin growth factor 1 (IGF1) affects oligodendrocyte proliferation, maturation and myelination (McMorris et al., 1993, Saneto et al., 1988, Ye et al., 2002), through IGF1 receptor that is expressed in developing mouse brains (Zeger et al., 2007). In adult DMSXL mice though, IGF levels in serum are slightly decreased (Huguet et al., 2012). Moreover splicing abnormalities of the insulin receptor were found in the skeletal muscle and liver of transgenic DM300 mice in association with transient insulin resistance, while abnormal expression was reported in the hypothalamus of the same mice (Guiraud-Dogan et al., 2007). Together these findings point to the dysregulation of insulin signaling and metabolism, which may contribute to oligodendrocyte differentiation and myelination delays. Recently, an imaging study on adults at risk for Alzheimer disease, has correlated WM fraction changes with insulin levels and resistance. In DM1 patients, the typical insulin resistance is associated with insulin receptor splicing defects in skeletal muscles (Moxley et al., 1984, Savkur et al., 2001). However, the consequences of deficient insulin metabolism to DM1 brain disease, notably WM alterations, have not been explored. Combined imaging and metabolic studies are needed in DM1 mouse models and human patients to clarify this question.

Neuroinflammation and microglia signaling

Imflammation has been associated with DM1 (Olejniczak et al., 2015), notably the connection between the CTG repeat expansion and inflammation in congenital DM1

(CDM) muscle has been recently established (Nakamori et al., 2017). The activation of the nuclear factor κ B (NF- κ B) in response to the expanded CUG expression upregulates the muscle interleukin 6 (IL-6) pathway, which is associated with muscle immaturity in CDM patients (Nakamori et al., 2017). In the brain, the first evidence for neuroinflammation comes from *Mbnl2* KO mice, which display transient increase of IL-1 β -expressing, proinflammatory microglial cells (Ramon-Duaso et al., 2018). Microglia activation has severe consequences for myelin in demyelinating disorders, such as multiple sclerosis. Both steady-state and activated microglia have dual effects on oligodendrocyte differentiation and myelination (Domingues et al., 2016). In physiological conditions IL-6 and IL-1 β , among other microglia-secreted factors, promote OPC survival, proliferation and differentiation into mature oligodendrocytes, likely through the activation of NF- κ B and PDGFA signaling (Nicholas et al., 2001). However, the positive effect of certain cytokines is dose-dependent: low levels are pro-differentiation, while higher levels are deleterious. Is it conceivable that a similar damaging effect of the cytokines overexpressed in DM1 contributes to the impairment of oligodendrocyte maturation and myelination. To further understand the interplay between neuroinflammation a myelin deficits, additional studies must be performed, to better characterize cytokine and chemokine profiles in DMSXL mouse brains. Furthermore, mouse and human mixed cultures provide useful tools to investigate the impact of selected signaling molecules and cytokines on oligodendrocyte development.

Implications of DMSXL myelin dysfunction to the etiology of DM1 disease and CNS symptoms

The origin and nature of the WM lesions in DM1 brains are not known, the jury is still out on whether they are due to a dysregulated developmental process, or to the accumulation of defects during disease progression. The actual view proposes different origins of WM lesions depending on the clinical form of DM1. Although a degenerative nature of WM injuries fits better to the adult onset cases, growing evidence supports that changes occurring during oligodendrocyte and myelin development can permanently alter WM structures and translate into neurological manifestations. It is possible that the transient myelin phenotypes in young mice described in chapter III, may induce WM changes that are possibly related with the DM1-related cognitive and

behavioral deficits showed by adult DMSXL mice (Hernandez-Hernandez et al., 2013a). In humans, the lack of imaging data on developing pre-symptomatic adult onset patients does not allow the definite establishment of such cause-consequence relationships in DM1.

To shed some light on the impact of WM abnormalities on DM1 clinical phenotypes, imaging analysis must be performed in DMSXL mice, throughout development, at different ages. If WM lesions are confirmed in this transgenic line, we could then investigate if the removal of the expanded CTG repeat specifically from the oligodendroglia lineage rescues, at least to some extent, some of the mouse neurological phenotypes.

Implications on learning and cognition

In mice, the learning and consolidation of new motor tasks relies both on the formation of new oligodendrocytes and in the maturation of pre-existent ones in the CC region (McKenzie et al., 2014). This is consistent with studies in humans showing increasing WM volume when learning a new skill (Bengtsson et al., 2005, Scholz et al., 2009). Similarly, complex cognitive exercises, such as learning a new language, can induce rapid WM changes (Schlegel et al., 2012), in a process that requires myelin plasticity for both creation of new myelinating cells or remodeling the existent myelin. Moreover, dyslexic children show abnormalities in CC WM microstructure correlated with word reading and comprehension (Hasan et al., 2012).

In line with these reports, the lack of mature oligodendrocytes and myelin in important brain regions, such as CC and frontal cortex, may impair the cognition of DMSXL mice. Similar defects may affect learning and cognition of DM1 patients, particularly those with earlier onset, in which WM abnormalities are more pronounced (Wozniak et al., 2011, Minnerop et al., 2011, Cabada et al., 2017). Interestingly, the acquisition of working memory and executive functions during development and adulthood is based on myelin-associated synchronicity of neuronal networks (James et al., 2008). Given the prevalent impairment of these cognitive functions in DM1, it is possible that these neurological manifestations are an indirect consequence of myelin defects.

Effects of motor activity and social interaction

Recent results from a clinical trial aiming to improve the quality of life of DM1 patients, by promoting regular activity and social interaction, revealed beneficial effects on CNS-related symptoms (Okkersen et al., 2018). Growing evidences on mouse models demonstrate that experience and neuronal activity are at the basis of adaptive myelination through adult life, by shaping the cellular and ultrastructural properties of myelin, which in turn modifies network dynamics resulting in animal behavior changes (Mount and Monje, 2017). In line with these results, one could hypothesize that the positive effects of social interaction on DM1 neurological symptoms can be mediated, at least in part, by adaptive myelination improvements. For instance, adult adaptive myelination in response to neuronal activity stimulation of motor areas has been reported in mice, revealing that activity enhance motor behavior (Gibson et al., 2014). The adaptive response was dependent on OPC proliferation and oligodendrocyte differentiation and the effect on cell activation was relatively permanent. On the other hand, social isolation paradigms with young mice, but not with adults, induced drastic alterations in WM, mediated through permanent deficits on oligodendrocyte maturation and myelination capacity in medial prefrontal cortex, leading to behavior deficits (Makinodan et al., 2012, Liu et al., 2012). This effect was specifically mediated by oligodendrocytes, since the treatment with an enhancer of oligodendrocyte differentiation rescued oligodendrocyte and myelin phenotypes provoked by social interaction deprivation, along with the recovery of social abilities (Liu et al., 2016), revealing myelin as key player on adaptation to social experiments.

Lack of motivation and social avoidance are common features of DM1 patients (Yu et al., 2011, Winblad et al., 2006, Jean et al., 2014) that often results in inactivity and isolation. In light of the mouse findings, adaptive myelination may be related to and/or impaired by these symptoms. Moreover, DM1 patients have problems in recognizing other's emotional states (Kobayakawa et al., 2012, Serra et al., 2016), while exhibiting social anxiety, attention deficits and some autistics traits (Douniol et al., 2012, Ekstrom et al., 2008). Similar difficulties are experienced by children with sensory processing disorders and autism spectrum disorders (ASD) (Owen et al., 2013, Hughes, 2007). Interestingly, a transversal feature to all these conditions is the WM abnormalities. In

particular, an ADS mouse model shows hypomyelination and reduced total number of cells of the oligodendroglia lineage, and mature oligodendrocytes in particular, with unaltered OPC population (Graciarena et al., 2019), similar to the findings reported in DMSXL mice (chapter III).

It is therefore possible, that transient oligodendrocytes and myelin alterations in DM1 are closely associated with the impaired social cognition of patients.

White matter abnormalities and sleep disturbances

The DM1 most frequent non-muscular complaint is the excessive day-type sleepiness. The cause of such symptom is poorly known, but it is thought to have a central origin (Laberge et al., 2009, Yu et al., 2011, Romigi et al., 2013), possibly even related with mild neuronal loss in areas of the brain that control sleep cycles (Ono et al., 1996, Mizukami et al., 1999). A recent work has shown that narcoleptic patients (that share similar sleep symptoms with DM1) present complex widespread WM microstructural disruption, indicating a combination of decreased myelination, lower axonal density and/or larger axons (Gool et al., 2019). Thus, changes in WM in sleep-related brain areas and connective tracts may offer a structural explanation for DM1 sleep disturbance.

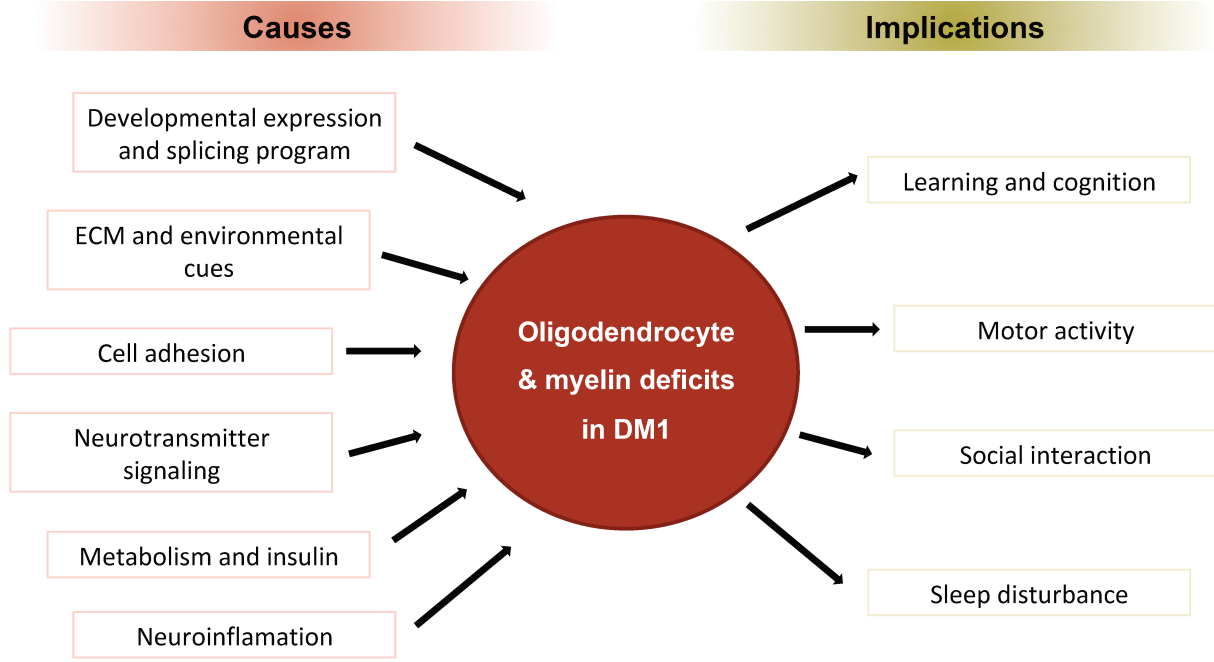


Figure 2 – Contributing factors for myelination defects, and possible consequences related to DM1 CNS symptoms.

It remains however, to be determined whether myelin defects drive the DM1 clinical manifestations discussed above, or if they are exacerbated by the disease symptoms. The two hypothesis are not mutually exclusive and can co-operate to aggravate myelin phenotypes and symptoms severity over time.

II. General conclusions

My work has suggested for the first time a cellular mechanism to explain, at least partially, the typical and frequent imaging alterations observed in DM1 patients. The myelin defect found in the DMSXL may as well contribute to DM1 CNS manifestations. In spite of the remaining gaps in our knowledge, these findings have opened new avenues for in-depth investigation of the mechanisms behind myelin and WM defects in DM1 and their functional consequences to neuropathology and disease manifestations.

The cellular models established, as well the videomicroscopy analysis developed during this work will accelerate the identification of critical individual cellular events, and their functional validation, in the onset of disease phenotypes. Together my findings offer also the opportunity to conceive and imagine pre-clinical and proof-of-principle experiments to test pharmacological therapeutics targeting the correction of key molecular events and associated phenotypes.

All together my research have contributed to an integrative view of the DM1 brain disease, using a multiple technical approaches and a small collection of various disease models. This research line fits well in the long-lasting DM1 framework, which over the years has followed the rational and sequential path, starting from the discovery of the disease mutation, moving to the identification of disease intermediates and dysregulated pathways, to finally create opportunities to translate our biomedical knowledge into the first clinical trials.

I hope my work has pushed DM1 field one step forward, getting us closer to the development of efficient therapies that patients have waited for so long.

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