Synaptic protein dysregulation in myotonic dystrophy type 1
Disease neuropathogenesis beyond missplicing

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The toxicity of expanded transcripts in myotonic dystrophy type 1 (DM1) is mainly mediated by the disruption of alternative splicing. However, the detailed disease mechanisms in the central nervous system (CNS) have not been fully elucidated. In our recent study, we demonstrated that the accumulation of mutant transcripts in the CNS of a mouse model of DM1 disturbs splicing in a region-specific manner. We now discuss that the spatial- and temporal-regulated expression of splicing factors may contribute to the region-specific spliceopathy in DM1 brains. In the search for disease mechanisms operating in the CNS, we found that the expression of expanded CUG-containing RNA affects the expression and phosphorylation of synaptic vesicle proteins, possibly contributing to DM1 neurological phenotypes. Although mediated by splicing regulators with a described role in DM1, the misregulation of synaptic proteins was not associated with missplicing of their coding transcripts, supporting the view that DM1 mechanisms in the CNS have also far-reaching implications beyond the disruption of a splicing program.

Myotonic Dystrophy and the Central Nervous System

Myotonic dystrophy type 1 (DM1) is the most common form of inherited muscular dystrophy in adults, with a worldwide incidence of 1 in 8,000 individuals. DM1 is a typical multisystemic disease, affecting a large number of tissues and organs in the human body.1 The central nervous system (CNS) is compromised to different extents in adult, juvenile and congenital forms of the disease. Several neuropsychological symptoms have been reported in adult-onset DM1 patients, such as excessive daytime sleepiness and fatigue, visuoconstructive impairment, attention deficits, reduced initiative and apathy, increased anxiety and anhedonia, as well as reduced intelligence quotients. Marked mental retardation and delayed psychomotor development are found in the congenital cases.2 In addition to the clinical evidence, imaging and histopathological techniques have also illustrated brain dysfunction in DM1. MRI scans have revealed that white and gray matter are affected in DM1 brains, while PET-SPECT imaging techniques revealed deficits in brain glucose metabolism and hypoperfusion.3,4 The histopathological distribution of tau protein in the brain revealed the accumulation of pathogenic protein isoforms in DM1 individuals,5 in association with changes in the alternative splicing of tau transcripts,6 resulting in the classification of DM1 as a tauopathy.

The neurological manifestations of the disease are highly debilitating and have a tremendous impact on the quality of life of DM1 patients and their families. As a result of their intellectual impairment and
behavioral deficits, DM1 patients experience low education achievements, low employment, poor familial environment, as well as social, economic and material deprivation.⁷

### Unraveling the Molecular Mechanisms of DM1 in the CNS

DM1 is caused by the abnormal expansion of a non-coding CTG trinucleotide repeat in the 3'UTR of the DMPK gene.⁸ Experimental evidence supports a prevailing model of disease pathogenesis, in which the DM1 phenotype is mainly mediated by a deleterious gain-of-function of expanded DMPK transcripts.⁹,¹⁰ CUG repeat-containing expanded transcripts form secondary RNA structures that bind to and sequester muscleblind-like proteins (MBNL) into ribonuclear inclusions or nuclear RNA foci,¹¹ and upregulate the CUG/Elav-like family (CELF) proteins.¹² Given the antagonistic role of MBNL and CELF proteins in the control of a developmentally regulated splicing program, CUG-associated RNA toxicity results in the aberrant expression of embryonic isoforms in adult skeletal muscle and heart.¹³,¹⁴ Typical DM1 symptoms, such as myotonia, muscle weakness and insulin resistance, are explained by abnormal splicing of the CLCN1 chloride channel,¹⁴,¹⁵ BIN1 bridging integrator protein¹⁶ and the insulin receptor,¹⁷ respectively. Although splicing defects have been described in human DM1 brains,¹⁸ we do not understand the functional impact of MAPT/TAU, GRIN1/NMDAR1 and APP RNA missplicing in DM1 neuropathophysiology. Nor do we know the cell populations, neuronal circuits, molecular pathways and neurological functions that are primarily disturbed in DM1 brains.

In addition to spliceopathy, evidence has shown that additional elements may contribute to (or at least modify) disease pathogenesis, such as chromatin rearrangements within the DM1 locus, leaking of transcription factors away from active chromatin, dysregulated miRNA metabolism, altered protein translation, and accumulation of toxic peptides resulting from non-conventional repeat-associated RNA translation.⁹,¹⁰

### Recreating RNA Toxicity in the CNS of DM1 Transgenic Mice

Given the compromised function of the CNS in DM1 patients and the impact of the neurological symptoms on their daily life, a growing effort has been made to unravel the mechanisms of DM1 neuropathogenesis over the last few years. We have been tackling this question by using DMSXL transgenic mice, previously generated in our laboratory.¹⁹,²⁰ These animals carry a large fragment of the human DM1 locus containing more than 1000 CTG repeats in the 3'UTR of the DMPK gene. Homozygous DMSXL mice produce sufficient toxic RNA transcripts to reproduce some critical and highly relevant molecular features of DM1, such as RNA foci accumulation and missplicing, as well as muscle phenotypes.²¹ In order to investigate whether RNA toxicity extended to the CNS, we have studied nuclear RNA foci accumulation and found CUG-containing ribonuclear inclusions in DMSXL mice, not only in the brain, but also in the spinal cord.²² RNA foci were particularly abundant in the frontal cortex and in some neuronal nuclei of the brainstem—two brain regions that are considered to play critical roles in the development of some of the most characteristic neurological symptoms of DM1. The nuclear accumulation of toxic RNA foci was associated with sequestration of MBNL proteins, upregulation of CELF proteins and resulted in the disruption of alternative splicing in DMSXL frontal cortex and brainstem.²²

While most of the exons misspliced in DMSXL brains were previously reported as misregulated in post-mortem DM1 brains,⁶,¹⁸ the missplicing of GRIN1/NMDAR1 exon 21, MBNL1 and MBNL2 exon 7 in human patients was poorly documented. To confirm that the splicing changes of these exons in DMSXL mice recreate relevant molecular events characteristic of DM1, we have now validated these abnormalities in human DM1 frontal cortex and brainstem. The RT-PCR analysis confirmed increased inclusion of GRIN1/NMDAR1 exon 21, MBNL1 exon 7 and MBNL2 exon 7 in human DM1 frontal cortex and brainstem, relative to non-DM1 control individuals (Fig. 1A), suggesting that the DMSXL splicing changes detected mimic true DM1 molecular features. Our RT-PCR analysis also revealed that missplicing was more pronounced in human frontal cortex than in brainstem, particularly for MBNL1 and MBNL2 transcripts. Coincidently, the missplicing of Mbnl1 and Mbnl2 are among the most noticeable and reproducible defects detected in the brain of DMSXL mice, and may provide useful molecular biomarkers for the pre-clinical assessment of therapies aiming to correct DM1spliceopathy in the CNS.

The extent and nature of the spliceopathy in DMSXL brains is region-specific, as illustrated by Grin1/Nmdar1 transcripts. Grin1/Nmdar1 exon 5 is abnormally excluded in DMSXL brainstem (but not in frontal cortex), while exon 21 is abnormally excluded in DMSXL frontal cortex (while it remains unaffected in brainstem).²³ The regional splicing defects of this gene (and others) might be the consequence of a spatially regulated splicing program. In support of physiological region-specific mechanisms of splicing regulation, we have found that the distribution of Grin1/Nmdar1 isoforms varied between frontal cortex and brainstem in wild-type animals: while exon 5 is preferentially included in brainstem, exon 21 shows a much higher inclusion ratio in frontal cortex.²² These differences suggest that the levels and/or activity of the key splicing regulators that control these events vary between different brain territories. We have addressed this hypothesis and found higher levels of CELF1 and CELF2 proteins in adult frontal cortex than in brainstem of wild-type mice. In contrast, the levels of MBNL proteins were higher in brainstem than in frontal cortex.²² Additionally, the distribution of MBNL2 protein isoforms differed significantly between frontal cortex and brainstem: western blot immunodetection following long electrophoresis migration revealed high molecular weight isoforms that were specifically present in frontal cortex, as well as a higher expression of low molecular weight isoforms in brainstem (Fig. 1B). Given the role of CELF and MBNL proteins in the control of alternative splicing in the CNS,²⁵,²⁶ these
differences may contribute to the regional splicing profiles in wild-type brains, and to the region-specific susceptibility of alternative exons to the accumulation of toxic RNA transcripts in DMSXL mice. Consistent with this view, a role of CELF2 in the regional regulation of alternative splicing in the mouse brain has been previously reported.26 Our data suggest that the regional distribution of different splicing isoforms in the brain is regulated by the interplay between multiple RNA-binding proteins, which include not only CELF family members, but also MBNL proteins.

Missplicing in DMSXL CNS was not confined to frontal cortex and brainstem areas of the brain, and it was found throughout the CNS to a variable extent, including the hippocampus22 and spinal cord (Fig. 1C), among other regions. These results demonstrate that CUG toxicity operates throughout the entire CNS. In agreement with this view, signs of neuronopathy, the expression of pathological forms of MAPT tau protein and a reduced number of lumbar motor neurons were reported in the spinal cord of DMSXL mice.27 However, the correlation between histopathology and splicing dysregulation in spinal cord remains to be further explored.

Expression of Splicing Regulators is Regulated during Mouse CNS Development

The missplicing events detected in DMSXL brains increased the expression of embryonic splicing profiles in adult animals, to a limited extent,12,13 as previously reported in skeletal muscle and heart.12,13 To gain insight into the mechanisms underlying the developmental splicing program in the CNS, we have studied the steady-state levels of MBNL and CELF proteins throughout brain development and aging in wild-type mice. We found that CELF1 and CELF2 protein levels experienced a pronounced decrease in brainstem. In the frontal cortex, however, only CELF1 displayed a very mild decrease from embryonic day 12.5 (E12.5) onwards. In contrast, MBNL1 and MBNL2 levels increased significantly in adult frontal cortex and brainstem, particularly between post-natal day eight (P8) and the first month of age (M1). MBNL1 and MBNL2 were hardly detected in embryonic stages and at post-natal day 1 (P1) even following long exposures (Fig. 1D). The dramatic change in MBNL1 levels at one month of age coincided with a striking switch in the splicing patterns of Mbnl1 and Ldb3/Cypher transcripts previously detected.22 This is not surprising, since the alternative splicing of the alternative exons in these genes is specifically regulated by MBNL1.28 In contrast, the splicing profile of Fxr1 (a gene specifically regulated by CELF1) showed a less pronounced developmental splicing transition,21 possibly due to the mild changes in the levels of CELF1 throughout brain development.

More importantly, the analysis of CELF and MBNL protein levels throughout wild-type brain development and aging revealed that DM1-associated CELF upregulation (particularly in the brainstem), and the functional inactivation of MBNL proteins by sequestration into ribonuclear foci recreated an embryonic scenario, thereby contributing to the abnormal expression of embryonic isoforms in adult DMSXL brain tissues.

We have extended our analysis to the hnRNP H ribonucleoprotein, an alternative splicing regulator with a described role in DM1 spliceopathy.29 hnRNP H steady-state levels exhibited a marked decrease from wild-type embryonic stages to adult ages, in both frontal cortex and brainstem to similar extent (Fig. 1D). In contrast to the upregulation of hnRNP H reported in DM1 myoblasts,29 the steady-state levels of hnRNP H did not differ significantly between DMSXL and wild-type brain regions at one month of age (Fig. 1E).

Changes in Synaptic Vesicle Proteins are Not Associated with Missplicing

To identify dysfunctional disease intermediates and pathways behind CUG-associated brain dysfunction, we investigated the proteomic profile of DM1 transgenic mice and found abnormal RAB3A upregulation and synapsin I (SYN1) hyperphosphorylation relative to control animals. We extended our findings from mouse to human brains, and confirmed a statistically significant RAB3A upregulation and SYN1 hyperphosphorylation in post-mortem DM1 frontal cortex.22 Furthermore, higher RAB3A levels and SYN1 phosphorylation were also detected in neuronal-like PC12 cells expressing expanded CUG-containing transcripts. In these cells, the abnormal metabolism of synaptic vesicle proteins was associated with aberrant exocytosis, indicating a physiologically relevant consequence of the expression of toxic DMPK transcripts in neuronal cell lineages. Overall, we gathered electro-physiological, neurochemical, cellular and molecular data suggesting that the DM1 neuropsychological manifestations are mediated by the dysregulation of synaptic vesicle proteins, which likely affects neuronal vesicle release and exocytosis, and disrupts synaptic function in the CNS.

Since it has been suggested that DM1 molecular features, particularly splicing dysregulation, recreate embryonic events,19 we asked whether synaptic protein expression and/or phosphorylation were also developmentally regulated. More importantly, we were interested in investigating whether RAB3A upregulation and SYN1 hyperphosphorylation in adult DMSXL mice recreated embryonic events, and supported a contribution of neurodevelopmental deficits behind DM1 neuropathology. To answer these questions, we studied protein expression and/or phosphorylation levels throughout wild-type brain development and aging (Fig. 2A). The analysis revealed that RAB3A levels increased at one month of age (M1), and indicated that RAB3A upregulation detected at four months of age did not mimic an embryonic expression profile, and it was unlikely to be a direct consequence of defective developmental regulation of this protein. Similarly, the total levels of SYN1 also showed a marked post-natal increase, particularly after one month of age. However, SYN1 phosphorylation at residues serine-9 and serine-553 preceded the pronounced increase in the steady-state levels of this protein. As a result, the phosphorylation of SYN1 appears to peak at post-natal day eight (P8), but mainly as a result of the low steady-state of SYN1 at this stage (Fig. 2A). This situation differs from the SYN1
profiles in adult DMSXL mice, which show increased SYN1 phosphorylation but unchanged steady-state levels relative to wild-type control mice.22 Therefore, we conclude that the hyperphosphorylation of SYN1 in adult DMSXL brains does not
truly mimic the early post-natal metabolism of this protein. In summary, our data are consistent with the view that DM1-associated RAB3A upregulation and SYN1 phosphorylation is more likely mediated by neurofunctional abnormalities, rather than CUG-associated neurodevelopmental deficits.

To gain insight into the mechanisms of synaptic protein dysregulation, we studied additional mouse and cell models of DM1. We found that RAB3A was upregulated in mouse brain in response to the inactivation of MBNL1, while SYN1 was hyperphosphorylated in PC12 cells over-expressing CELF1 or CELF2. Given the role of MBNL and CELF protein families in the regulation of alternative splicing,
RAB3A and SYN1 events in the analysis confirmed additional splicing mouse and human genes. Our RT-PCR to the remaining internal exons of the SYN1 of exon 13 in both human and mouse to the use of an alternative 5' splice site inclusion of a short 38-bp sequence due site in mouse RAB3A through the use of an alternative 5' splice site in mouse transcripts; the RAB3A the alternative inclusion of exon 2 in both previously described in the literature, such as human brains (CUG-containing transcripts in mouse or changes induced by the expression of toxic we did not detect significant splicing dependent of missplicing events. Similarly, even if SYN1 is hyperphosphorylated as a result of the upregulation of CELF1 and/ or CELF2 splicing regulators, the splicing of SYN1 transcripts remains unaltered. In summary, although involving the dysfunction of known splicing factors (such as MBNL and CELF family members), DM1 neuropathogenesis goes beyond the abnormal expression of embryonic splicing isoforms, and implicates other molecular mechanisms.

**Conclusion**

DMSXL mice recreate relevant signs of RNA toxicity in the CNS, associated with behavioral, electrophysiological and neurochemical changes. In the search for the mechanisms and dysfunctional pathways behind these phenotypes, we found altered expression and phosphorylation of synaptic proteins, which are independent of splicing dysregulation. RAB3A is an abundant synaptic vesicle protein that regulates neurotransmission, through the interaction with other synaptic proteins that control vesicle fusion to the cell membrane. SYN1 regulates neuronal vesicle release in a phosphorylation-dependent manner (Fig. 3). As a result, RAB3A upregulation and SYN1 hyperphosphorylation disrupts synaptic function and neurotransmitter release, likely contributing to the cognitive and behavioral deficits of DMSXL mice and the neuropsychological manifestations of DM1 patients.

**Disclosure of Potential Conflicts of Interest**

No potential conflict of interest was disclosed.

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