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Myotonic dystrophy CTG expansion affects synaptic vesicle proteins, neurotransmission and mouse behavior

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Running title: *Synaptic dysfunction in myotonic dystrophy*

ABSTRACT

Myotonic dystrophy type 1 is a complex multisystemic inherited disorder, which displays multiple debilitating neurological manifestations. Despite recent progress in the understanding of the molecular pathogenesis of DM1 in skeletal muscle and heart, the pathways affected in the central nervous system are largely unknown. To address this question, we studied the only transgenic mouse line expressing CTG trinucleotide repeats in the central nervous system. These mice recreate molecular features of RNA toxicity, such as RNA foci accumulation and missplicing. They exhibit relevant behavioral and cognitive phenotypes, deficits in short-term synaptic plasticity, as well as changes in neurochemical levels. In the search for disease intermediates affected by disease mutation, a global proteomics approach revealed RAB3A upregulation and Synapsin I hyperphosphorylation in the central nervous system of transgenic mice, transfected cells and *post-mortem* brains of myotonic dystrophy type 1 patients. These protein defects were associated with electrophysiological and behavioral deficits in mice, and altered spontaneous neurosecretion in cell culture. Taking advantage of a relevant transgenic mouse of a complex human disease, we found a novel connection between physiological phenotypes and synaptic protein dysregulation, indicative of synaptic dysfunction in myotonic dystrophy type 1 brain pathology.

Key words: myotonic dystrophy, transgenic mice, synaptic transmission, RAB3A, synapsin I

Abbreviations: CELF = CUGBP/Elav-like factor; CNS = central nervous system; DM = myotonic dystrophy; DM1 myotonic dystrophy type 1; DMPK = myotonic dystrophy protein kinase; MBNL = muscleblind-like; PPF = paired-pulse facilitation; SYN1 = synapsin I.

INTRODUCTION

Myotonic dystrophy (DM) is the most common form of adult muscular dystrophy (Harper, 2001). DM1 is caused by the expansion of a CTG trinucleotide repeat in 3'UTR of the DM protein kinase (*DMPK*) gene (Brook *et al.*, 1992, Fu *et al.*, 1992, Mahadevan *et al.*, 1992). Repeat number correlates directly with disease severity and inversely with age of onset. The DM1 repeat shows a marked tendency towards further expansion in intergenerational transmissions and somatic tissues (Gomes-Pereira and Monckton, 2006). The prevailing model of disease pathogenesis points to a *trans*-dominant effect of expanded *DMPK* transcripts, which accumulate in nuclear foci, interfering with at least two families of alternative splicing regulators: the muscleblind-like (MBNL) and CUGBP/Elav-like (CELF) proteins. MBNL1 loss of function, through sequestration into ribonuclear foci (Miller *et al.*, 2000), and CELF1 upregulation (Timchenko *et al.*, 2001) disturb a developmentally regulated splicing program, resulting in aberrant expression of embryonic isoforms in adult skeletal muscle and heart (Ranum and Cooper, 2006). Missplicing explains important DM1 symptoms, such as myotonia (Charlet *et al.*, 2002, Lueck *et al.*, 2007, Mankodi *et al.*, 2002) and insulin resistance (Savkur *et al.*, 2001).

Although traditionally considered a muscle disease DM type 1 (DM1) presents many debilitating neurological manifestations. Adult-onset DM1 patients show prevalent hypersomnia and fatigue, as well as visuoconstructive impairment, attention deficits, reduced initiative and apathy (Harper, 2001, Meola and Sansone, 2007), suggesting executive dysfunction and the involvement of frontal lobes (Gaul *et al.*, 2006, Meola and Sansone, 2007, Meola *et al.*, 2003, Sistiaga *et al.*, 2010). Reduced intelligence quotients (IQ) were measured in one third of adult and in the majority of childhood-onset patients (Angeard *et al.*, 2007, Meola and Sansone, 2007), while

moderate to severe mental retardation is a feature of congenital DM1 (Harper, 2001). Additional signs of personality disorder, such as increased anxiety, depression and anhedonia, were reported in a proportion of DM1 patients (Bungener *et al.*, 1998, Delaporte, 1998, Meola *et al.*, 2003, Winblad *et al.*, 2005). Region-specific structural abnormalities (including both white and grey matter affection) and metabolic changes, revealed by imaging studies, may contribute to DM1 neuropsychological manifestations (Minnerop *et al.*, 2011, Romeo *et al.*, 2010, Weber *et al.*, 2010).

RNA foci accumulate in *post-mortem* DM1 brains and co-localize with MBNL1 and MBNL2 (Jiang *et al.*, 2004). Nevertheless, the mechanistic links between the genetic mutation and neuropsychological abnormalities remain elusive (Meola and Sansone, 2007). We previously generated transgenic mice expressing *DMPK* transcripts in multiple tissues under the control of the human gene promoter, within the environment of the human DM1 locus. In contrast to control DM20 lines (overexpressing short 20-CTG tracts), homozygous mice from two independent DM300 expansion lines (carrying 500-600 CTG), expressing enough toxic *DMPK* transcripts, showed wide RNA foci accumulation in a variety of tissues and developed a multisystemic phenotype (Guiraud-Dogan *et al.*, 2007, Panaite *et al.*, 2008, Seznec *et al.*, 2001). Dramatic intergenerational instability in DM300 mice generated DMSXL animals carrying >1000 CTG, which develop a more severe phenotype (Gomes-Pereira *et al.*, 2007, Huguet *et al.*, 2012). We now used DMSXL mice to characterize RNA toxicity in the brain and to identify disease intermediates and pathways affected by DM1 in the central nervous system (CNS). We gathered electrophysiological, neurochemical and molecular evidence of synaptic protein dysregulation and synaptic dysfunction, which translates into DM1-associated behavior deficits and most certainly mediates neurological symptoms.

MATERIALS AND METHODS

Transgenic mice. The DM transgenic mice used in this study carried 45 kb of human genomic DNA cloned from a DM1 patient as described (Gomes-Pereira *et al.*, 2007, Seznec *et al.*, 2000). Transgenic mice were raised and kept at Centre d'Exploration et de Recherche Fonctionnelle Expérimentale (CERFE, Genopole, Evry, France). Animal housing, care and handling were performed according to the French and European legislations, and the ethical guidelines of the host institution. Genotyping procedures are described in the Supplementary Materials and Methods online. The generation, characterization and genotyping of *Mbn11-* and *Mbn12* knock-out mice is described elsewhere (Charizanis *et al.*, 2012, Kanadia *et al.*, 2003).

Quantitative real-time RT-PCR quantification of *DMPK* and *Rab3A* transcripts.

Total RNA extraction and cDNA synthesis were performed as previously described (Gomes-Pereira *et al.*, 2007). *DMPK*, *Dmpk* and *Rab3A* transcripts were amplified in a 7300 Real Time PCR System (Applied Biosystems) using SybrGreen detection and oligonucleotide primer sequences described in Supplementary Table 1. *DMPK* and *Rab3A* mRNA levels were expressed relative to *18S* transcripts. The quantification of *DMPK* transcript levels was performed on a cDNA sample synthesized from equal RNA quantities extracted from three individual DMSXL homozygous mice. *Rab3A* transcript levels were quantified in six individual DMSXL homozygotes and wild-type controls. Two independent replicate experiments were performed.

Fluorescent in situ hybridization (FISH). Ribonuclear inclusions were detected with a 5'-FITC-labelled 2'-O-methyl-(CAG)₁₀ oligonucleotide probe, or a 5'-Cy3-labelled (CAG)₅ PNA probe, as previously described (Seznec *et al.*, 2001). RNA foci

quantification was performed using Discovery Automaton (Roche) (Bassez *et al.*, 2008). Mouse brain regions of interest were anatomically identified and the percentage of cells containing nuclear foci was calculated based on the observation of >100 cells in each brain region, in each mouse.

Immunofluorescence (IF) combined with FISH. Following the 1X SSC post-hybridization wash of the FISH procedure, sections were incubated in primary antibodies overnight at 4°C (antibody references and working dilutions are listed in Supplementary Table 2), washed five times with PBS for 2 min, and then incubated in secondary antibody and 0.001% (m/v) diamidino-2-phenylindole (DAPI) for 15 min at room temperature. Sections were washed five times in PBS prior to mounting.

RT-PCR analysis of alternative splicing. Total RNA extraction, cDNA synthesis and semi-quantitative RT-PCR analysis were performed as previously described (Gomes-Pereira *et al.*, 2007), using oligonucleotide primers described in Supplementary Table 1. The inclusion ratio of alternative exons in each individual animal was determined in two replicate PCR amplifications to minimize experimental variation. The percentage of exon inclusion was calculated as $[\text{exon inclusion band}/(\text{exon inclusion band} + \text{exon exclusion band})] \times 100$.

Behavioral analysis. Since DMSXL mice display muscle deficits (Huguet *et al.*, 2012) behavioral tests were carefully selected, designed and interpreted to minimize the confounding effects of muscular impairment on brain functional deficits. All the experiments were performed according to the guidelines of the French Ministry of Agriculture for laboratory animal experimentation (law 87-848; Agreement N°: A-45-234-8) and the mouse genotype was unknown to the researcher. Mouse behavioral

testing was conducted in order to reduce animal stress to the minimum. Three groups of animals were studied, in order to limit the number of tests performed on each animal. Group A: Open-field, Y maze, passive avoidance and sucrose intake. Group B: Marble-burying and Morris water maze. Group C: Saccharine intake. Further details on behavioral phenotyping are described in the Supplementary Materials and Methods online.

Electrophysiological profiling. Input/output (I/O) properties, Paired-Pulse Facilitation (PPF), Long Term Depression (LTD) and Long Term Potentiation (LTP) were assessed on male DMSXL homozygotes and wild-type controls, aged seven months (I/O, PPF and LTD) or four months (LTP). Mice were sacrificed by fast decapitation, without previous anesthesia. Brains were processed in oxygenated buffer and artificial cerebro-spinal fluid as previously described (Steidl *et al.*, 2006). Extracellular field excitatory post-synaptic potentials (fEPSP) were measured with Multi-Electrode Arrays (MEA) technology (100 μm distant electrodes) on 350 μm thick hippocampal slices. One of the electrodes stimulated Schaeffer collaterals at the CA3/CA1 interface. The stimulus, consisting of a monopolar biphasic current pulse (negative for 60 μs and then positive for 60 μs), evoked responses (field potentials: fEPSP) in the CA1 region. *I/O properties:* I/O curves were plotted by measuring the response evoked by stimulation intensities varying between 100 μA and 800 μA . Details on the electrophysiological procedures are included in the Supplementary Materials and Methods online.

Two-dimensional protein electrophoresis and mass spectrometry. Ten-month-old female mice were used to compare the global proteomic profile in frontal cortex and brainstem between six homozygotes carrying expanded repeats (~500-600 CTG) and six females carrying short 20-CTG control tracts. Isoelectric focusing and mass

spectrometry was performed as previously described (Sergeant *et al.*, 2003). CELF1 and CELF2 phosphorylation was assessed by two-dimensional gel electrophoresis and immunodetection, using antibodies in Supplementary Table 2.

Western blot analysis. Primary antibody references and working dilutions are shown in Supplementary Table 2.

Neurosecretion assays. Neurosecretion assays in cell culture were performed as previously described (Lee *et al.*, 2007).

Human tissue samples. Autopsy materials were obtained from nine DM1 patients (mean age 60.9 years, range 32–73 years) and six non-DM1 controls (three with no neurologic disease, one with Charcot-Marie-Tooth disease, one with a brain tumor and one with Limb-girdle muscular dystrophy; mean age 68.2 years, range 53-79 years). Six DM1 patients (i, j, m, n, o) had signs of classical adult-onset DM1, and they all died of complications related to the disease (respiratory failure or heart disease). Patient “DM1 g” developed late onset DM1. Patient “DM1 h” carried a large CTG repeat expansion in blood and brain tissue; her clinical form of DM1 was not fully described by the clinicians, but she presented gait problems and general brain atrophy evidenced by neuroimaging. The clinical form of patient “DM1 l” was not fully described either; she carried large CTG repeats, presented gait problems and mild cognitive impairment, and imaging assessment revealed general brain atrophy. Patient “DM1 k” exhibited mental retardation and was diagnosed with congenital DM1. He died at the age of 32, of pneumonia. Neuropsychological profiling and neuroimaging was not systematically conducted on the majority of these patients. Further patient details are given in Supplementary Table 3. All experiments using human samples were approved by the

Ethics Committee of Asahikawa Medical Center and Okayama University. Written informed consent specimen use for research was obtained from all patients.

Statistical analysis. Statistical analyses were performed with JMP 5, Prism 5 and Excel software. When two groups were compared, a two-tailed Student's *t*-test for statistical significance was performed, unless otherwise stated. The significance level was set at *P* values less than 0.05 for all statistical analyses. All data are expressed as mean \pm SEM, unless otherwise stated.

RESULTS

Expression of DM1 CTG expansions results in RNA foci accumulation and missplicing in the CNS of DMSXL mice

The toxic RNA hypothesis proposes that accumulation of CUG-containing transcripts is the initiating pathogenic event in DM1. To assess to what extent DMSXL mice recreate central molecular aspects of DM1, we measured *DMPK* transgene expression in CNS regions dissected from one-month-old DMSXL homozygous mice and found expanded transcripts in all CNS regions investigated, with some regional differences (Supplementary Fig. 1A): *DMPK* transcript levels were higher in the hippocampus, thalamus/hypothalamus, cerebellum and brainstem, than in frontal and temporal cortex and in striatum. Overall, the expression levels of the *DMPK* transgene in frontal cortex and cerebellum were approximately three times higher than those of the endogenous *Dmpk* gene (Huguet *et al.*, 2012). Expanded *DMPK* transcripts accumulated in one to ~20 ribonuclear inclusions per cell nucleus in both neurons and astrocytes (Fig. 1A) and co-localized with MBNL1 and MBNL2 (Fig. 1B). Astrocytes showed a significantly higher frequency of foci than neurons overall (62% versus 44% foci-containing nuclei) (Fig. 1C). Interestingly, foci distribution was not homogeneous throughout DMSXL brains: they were present in 20% nuclei of striatum, up to 70% in brain cortex and ~80% in the dorsal raphe and raphe magnus nuclei of brainstem (Supplementary Fig. 1B). In addition to the accumulation of RNA foci in neurons previously reported in DM1 human brains (Jiang *et al.*, 2004), we found nuclear foci in human astrocytes (Supplementary Fig. 1C).

To investigate the *trans*-dominant effect of *DMPK* transcripts in the CNS, we studied the alternative splicing of candidate genes. We focused primarily on foci-rich brain regions relevant to disease manifestations: while frontal cortex is involved in

executive tasks (Robbins and Arnsten, 2009), brainstem is involved in the control of sleep cycles, respiratory and cardiac function (Izac and Eeg, 2006). Mild region-specific missplicing events were detected in frontal cortex and brainstem of one-month-old DMSXL homozygotes (Fig. 1D, Supplementary Fig. 2A).

The missplicing of *Mbnl1* exon 7 and *Ldb3* exon 11, two alternative exons specifically regulated by MBNL1 activity (Kalsotra *et al.*, 2008), confirmed the impact of the sequestration of MBNL proteins (Fig. 1B) on alternative splicing deregulation. Similarly, mild missplicing of CELF1-dependent exons 15 and 16 of *Fxr1* predicted a role of CELF1 in CNS spliceopathy. To confirm this hypothesis we quantified the steady-state levels of CELF proteins and found significant CELF1 and CELF2 upregulation (of about 30%) in DMSXL frontal cortex (Fig. 1E; Supplementary Fig. 1D). In DMSXL brainstem, only CELF1 was significantly upregulated. CELF1 upregulation was associated with mild protein hyperphosphorylation in DMSXL frontal cortex and brainstem (Supplementary Fig. 1E). CELF2 phosphorylation levels did not differ between genotypes in frontal cortex and appeared to decrease in DMSXL brainstem. The upregulation of CELF proteins was more pronounced in human DM1 frontal cortex: CELF1 levels were 70% higher and CELF2 showed a four-fold increase relative to non-DM individuals (Fig. 1F; Supplementary Fig. 1F).

Interestingly, wild-type splicing profiles were region-specific (e.g. *Grin1/Nmdar1*, Fig. 1D, Supplementary Fig. 2A). Regional differences were associated with varying steady-state levels of MBNL and CELF proteins between frontal cortex and brainstem in adult mice (Supplementary Fig. 1G), suggesting a determining role of the ratio between antagonistic splicing regulators in the establishment of adult splicing profiles.

We studied the alternative splicing of alternative genes throughout wild-type mouse brain development (Supplementary Fig. 2B), and found that the DMSXL

spliceopathy in the CNS modified certain transcripts towards embryonic/newborn splicing profiles. For instance, the splicing patterns of *Grin1* exon 21 and *Mbnl2* exon 7 in DMSXL frontal cortex, as well as *App* exon 8 and *Frax1* exons 15/16 in DMSXL brainstem resembled, to a limited extent, the splicing patterns of newborn wild-type mice. The effect was less pronounced in other alternative exons, which displayed intermediate inclusion ratios in DMSXL mice, between those characteristic of the embryonic and adult developmental stages (e.g. *Ldb3* exon 11 in frontal cortex and brainstem; *Grin1* exon 5 in brainstem).

CTG repeat expansions induce mouse behavioral abnormalities

Following the validation of toxic RNA expression, foci accumulation and missplicing in DMSXL brains, we then investigated the impact of expanded *DMPK* transcripts on mouse behavior and cognition through blinded phenotyping of adult DMSXL homozygotes. We first assessed mouse activity in the open-field test. DMSXL mice displayed overall levels of horizontal ($P=0.402$, Student's *t*-test) and vertical activity ($P=0.355$, Student's *t*-test) similar to those of wild-type controls, excluding a major effect of muscular deficits on mouse performance in this test (Supplementary Fig. 3A). However, DMSXL mice showed a significant decrease in exploratory activity, shortly after transfer into the open-field arena. While the total number of rearings did not differ between the two genotypes, the percentage of rearings over the first minute out of the first five minutes spent in the arena was significantly lower in DMSXL mice (Fig. 2A). This result reveals freezing behavior in response to a new and unfamiliar environment and indicates novelty-induced inhibition, suggestive of increased anxiety. This was confirmed by the assessment of obsessive-compulsive behavior using a marble-burying test. DMSXL mice displayed a statistically significant shift towards a higher number of fully buried marbles (Fig. 2B), indicative of increased anxiety.

We then examined if the expression of expanded *DMPK* transcripts resulted in memory impairment as observed in DM1 patients. Mouse spatial memory was assessed by the Morris water maze. The platform was located in the same target quadrant during the training period, and removed during the probe trial. DMSXL mice showed impaired spatial memory, illustrated by a significantly lower number of entries in the target quadrant during the probe trial (Fig. 2C). DMSXL swimming speed was not significantly different between genotypes (Supplementary Fig. 3B), excluding biases introduced by impaired motor performance. In the assessment of working memory, a component of the executive function (Robbins and Arnsten, 2009), both genotypes were capable of finding the platform in the acquisition trial. The apparent lower latency ($P=0.3066$, Student's *t*-test) and shorter distance ($P=0.4490$, Student's *t*-test) travelled by DMSXL mice were not statistically different from those of wild-type controls. Both genotypes showed similar performances in the acquisition trial. As expected, wild-type mice performed significantly better in the retention than in acquisition trial (latency: $P=0.0019$; distance travelled: $P=0.0148$; Student's *t*-test), indicating that working memory was present. In contrast, DMSXL mouse performance did not improve significantly between the two trials (latency: $P=0.1916$; distance travelled: $P=0.2726$; Student's *t*-test), suggesting possible mild working memory impairment (Fig. 2D). Non-spatial long-term memory, assessed by the passive avoidance conditioning test, was not affected in DMSXL mice (Supplementary Fig. 3C).

Anhedonia, previously reported in DM1 patients, was assessed by the consumption of a highly palatable solution of saccharine (Schweizer *et al.*, 2009). The similar volume of water drunk during the habituation period by DMSXL and wild-type mice (day 1: $P=0.136$; day 2: $P=0.538$; Student's *t*-test) shows similar satiety between the two genotypes (Supplementary Fig. 3D). Once saccharine became available, both animal groups showed a pronounced initial interest for the saccharine

solution, showing again similar satiety over the first three days of testing (test 1: $P=0.980$; test 2: $P=0.450$; test 3: $P=0.843$; Student's *t*-test). However, as the test progressed DMSXL interest for the saccharine solution was significantly lower, as compared to wild-type controls (test 4: $P=0.043$; test 5: $P=0.037$; test 6: $P=0.003$; test 7: $P=0.043$; Student's *t*-test), suggesting an anhedonic-like behaviour (Fig. 2E).

DMSXL mice exhibit deficits in short-term synaptic plasticity

In parallel to the behavioral and cognitive phenotyping of DMSXL mice, we have assessed the physiological impact of toxic *DMPK* transcripts on synaptic function. To this end we performed the electrophysiological profiling of DMSXL hippocampus, a brain region showing foci accumulation and missplicing (Supplementary Fig. 4). We first examined basal synaptic transmission by stimulating hippocampal Schaffer collaterals at increasing intensities and generating input/output (I/O) curves from measures of field excitatory post-synaptic potentials (fEPSP). DMSXL homozygotes exhibited slightly higher fEPSP relative to wild-type controls, but the overall I/O curves were similar between both genotypes (Fig. 3A), indicating no major deficits in basal transmission. We next investigated some short-term plasticity properties through the quantification of paired-pulse facilitation (PPF). DMSXL slices displayed significantly reduced PPF ratios, indicative of presynaptic dysfunction (Fig. 3B). Finally, we measured long-term depression (LTD) and potentiation (LTP), which support some forms of learning and memory. Standard LTD and LTP protocols did not reveal overt abnormalities in DMSXL mice (Figs. 3C and 3D). However, early after low frequency stimulation, LTD amplitude was slightly lower in DMSXL than in wild-type slices ($36\pm 7\%$ vs. $47\pm 3\%$), which might suggest mild impairment of the post-synaptic response. The difference between genotypes, however, did not reach statistical significance ($P=0.142$, repeated measures two-way ANOVA). Depression stabilized

after 20 minutes, being similar for both genotypes at the endpoint of the experiment. In summary, the number of animals studied did not show marked abnormalities in long-term plasticity, but revealed significant deficits in short-term synaptic plasticity.

DMSXL brains show abnormal levels of dopamine and serotonin metabolites

Neurotransmitter dysregulation can cause behavioral and electrophysiological dysfunction. Little is known about neurochemical signaling and metabolism in DM1 brains. To further dissect the neurological phenotype of DMSXL mice and assess whether behavioral and electrophysiological phenotypes were associated with changes in neurotransmitter levels, we measured key neurosignaling molecules in DMSXL brains. Adult DMSXL mice revealed a significant reduction of dopamine (DA) in the frontal cortex, as well as a tendency to decreased levels of its precursor (L-DOPA) and metabolites (DOPA, HVA). A significant decrease of 5-hydroxyindoleacetic acid (5-HIAA, the main serotonin metabolite) was detected in the brainstem of DMSXL mice (Fig. 3E).

Molecular abnormalities in synaptic proteins in the CNS of DMSXL mice and DM1 patients

To identify pathways affected in the CNS that might contribute to the behavioral and electrophysiological phenotypes, we compared the proteomic profiles of adult homozygous mice carrying CTG expansions, with those of DM20 control mice overexpressing short *DMPK* transcripts. The use of DM20 controls excluded the identification of disease intermediates possibly affected by overexpression of DMPK protein. The proteomics analysis suggested altered expression of RAB3A and post-translational modifications of Synapsin I (SYN1). Western blot quantification confirmed a statistically significant upregulation of RAB3A and hyperphosphorylation of serine

residues of SYN1 in DMSXL frontal cortex and hippocampus at four months of age (Fig. 4A, Supplementary Figs. 5A and 5B). To further test if synaptic protein dysregulation could be mediated by overexpression of short *DMPK* transcripts and protein, we analysed DM20 mice carrying 20 CTG repeats by western blot. This *DMPK* overexpressing control line did not show RAB3A upregulation or SYN1 hyperphosphorylation (Fig. 4B), indicating that the defects observed are mediated by the expression of expanded CUG RNA repeats. We extended the analysis to other synaptic proteins to assess the extent of synaptic dysfunction, but found no additional abnormalities (Supplementary Figs. 5C and 5D). Our data suggest that, rather than interfering with synaptic proteins in general, the repeat expansion may affect (directly or indirectly) a limited number of synaptic targets.

To address the mechanisms of synaptic protein dysregulation, we first tested if these abnormalities were associated with missplicing of known alternative exons of *Rab3A* and *Syn1* transcripts. RT-PCR analysis revealed that *Rab3A* and *Syn1* alternative splicing was not affected in DMSXL mice at four months (Supplementary Fig. 5E), an age when synaptic protein abnormalities are detected. RAB3A and SYN1 protein changes are not mediated by spliceopathy of the candidate alternative exons studied. Interestingly, RAB3A upregulation was associated with a significant increase of mRNA transcript levels in DMSXL frontal cortex and hippocampus (Fig. 4C).

We then tested if *Mbn1* or *Mbn2* inactivation was sufficient to dysregulate synaptic proteins, through the analysis of knock-out mice (Charizanis *et al.*, 2012, Kanadia *et al.*, 2003). Western blot analysis revealed that *Mbn1* inactivation (but not *Mbn2*) resulted in a significant increase of RAB3A protein levels in mouse frontal cortex (Fig. 4D). Neither *Mbn1* nor *Mbn2* loss of function affected SYN1 phosphorylation levels. To test whether SYN1 hyperphosphorylation is mediated by upregulation of CELF proteins, we transfected PC12 cells with expressing vectors

encoding CELF1 or CELF2. We studied SYN1 phosphorylation by western blot and found protein hyperphosphorylation in PC12 cells overexpressing CELF1 or CELF2 (Fig. 4E). This analysis indicated that upregulation of CELF proteins is sufficient to dysregulate SYN1 phosphorylation levels.

To ascertain whether RAB3A and SYN1 dysregulation in mice reflected a pathophysiological event of the human condition, we investigated *post-mortem* DM1 frontal cortex (Fig. 4F). Western blot quantification confirmed statistically significant RAB3A upregulation in DM1 patients, when compared to non-DM1 subjects ($P=0.0054$, Student's *t*-test). SYN1 was hyperphosphorylated in Ser9 ($P=0.0282$, Student's *t*-test) and Ser553 ($P=0.0032$, Student's *t*-test) amino acid residues in DM1 individuals. Abnormal phosphorylation was not accompanied by changes in protein steady-state levels ($P=0.7658$, Student's *t*-test). These results show that proteins playing important roles in synaptic function are dysregulated in DM1.

Expanded CUG-containing *DMPK* transcripts affect neuronal exocytosis in culture.

To investigate the functional consequences of DM1 repeat expansions, and in particular altered expression of synaptic proteins on vesicle trafficking, we studied exocytosis in an established transfected cell culture model of neurosecretion. Regulated exocytosis is triggered by extracellular stimulus and exhibits low basal spontaneous secretion in the absence of stimulation (Sudhof, 2004). Basal and regulated neurosecretion can be measured in cultured PC12 cells transiently transfected with human growth hormone (hGH) (Lee *et al.*, 2007, Sugita, 2004). The percentage of hGH secreted prior and following osmotic stimulation serves as an estimate of basal and regulated neurosecretion, respectively. To assess the effect of CTG repeats on vesicle trafficking, PC12 cells were co-transfected with an hGH-producing plasmid and with *DMPK*

constructs carrying a CTG expansion or no CTG repeats. Expanded CUG-containing RNA accumulated in the nucleus of PC12 cells, co-localized with MBNL1 and MBNL2, and induced mild missplicing. More importantly, transfected PC12 cells exhibited RAB3A upregulation and SYN1 hyperphosphorylation (Supplementary Fig. 6). The neurosecretion assay demonstrated that the expression of toxic RNA repeats enhanced basal neurosecretion in the absence of stimulation, relative to no-repeat control constructs (Fig. 5). The effect was observed in four independent experiments with a significant average basal secretion enhancement of $60 \pm 16\%$, during 15 minutes of incubation. Interestingly, CUG-containing transcripts did not disturb regulated neurosecretion after osmotic stimulation.

DISCUSSION

To explore DM1 neuropathology, we studied DM1 transgenic mice that reproduce key molecular aspects of RNA toxicity and exhibit relevant behavioral phenotypes. DMSXL mice display neurochemical and electrophysiological signs of synaptic dysfunction, which are associated with molecular abnormalities in synaptic proteins, observed not only in mice but also in transfected cells and *post-mortem* DM1 brain samples. The association between physiological and molecular phenotypes of the synapse indicates synaptic dysfunction in DM1 neuropathophysiology.

The expression of expanded *DMPK* transcripts in the CNS of transgenic mice results in the accumulation of RNA foci in neurons and astrocytes. RNA toxicity in both cell types indicates that DM1 may be associated not only with neuronal dysfunction, but also with glial abnormalities. Interestingly, brain areas showing the highest foci content (e.g. frontal cortex) did not necessarily express the highest *DMPK* levels, suggesting that RNA aggregation into foci may depend on region- and/or cell type-specific factors. Like in skeletal (Lin *et al.*, 2006, Orengo *et al.*, 2008) and cardiac (Wang *et al.*, 2007) muscles, CUG toxicity interferes with developmental alternative splicing in the CNS, increasing to a limited extent the abnormal expression of embryonic isoforms of some transcripts in adult DMSXL brains. Splicing abnormalities in the CNS may contribute to DMSXL phenotype. In particular, *Grin1/Nmdar1* and *Mapt/Tau* splicing defects likely participate in synaptic dysfunction detected in DMSXL mice. Further experiments are required to investigate how *Grin1/Nmdar1* missplicing influences intracellular localization and function of this receptor, as well as the impact of *Mapt/Tau* missplicing on DM1 neurofibrillary degeneration.

The missplicing of alternative exons specifically regulated by MBNL or CELF proteins (Kalsotra *et al.*, 2008) indicated that MBNL loss of function by protein

sequestration, and CELF upregulation induce missplicing in the CNS. In support of this view, *Mbnl1*- or *Mbnl2*-deficient mice show abnormal splicing in brain (Charizanis *et al.*, 2012, Suenaga *et al.*, 2012).

CELF1 upregulation in CNS appears to be mediated by mild protein hyperphosphorylation, as in DM1 heart (Kuyumcu-Martinez *et al.*, 2007). It remains to be investigated whether PKC activity is increased in the DM1 brains. In contrast, CELF2 upregulation in DMSXL frontal cortex was not associated with protein hyperphosphorylation, suggesting alternative mechanisms of protein regulation. The marked upregulation of CELF2 in human DM1 frontal cortex predicts a pathogenic role for this protein in DM1 neuropathogenesis. The modulation of CELF2 expression by miRNA species, recently reported in a transgenic mouse model of spinal-bulbar muscular atrophy (SBMA) suggests new possibilities for miRNA-mediated therapies in DM1 (Miyazaki *et al.*, 2012)

The behavioral phenotyping of DMSXL mice revealed reduced exploratory activity, increased anxiety, spatial memory impairment and anhedonia, which resemble DM1 neurological manifestations. Previous neuropsychological assessment of DM1 patients revealed low scores in the exploratory scale (Winblad *et al.*, 2005), a higher prevalence of anxiety-related behaviors (Delaporte, 1998, Meola *et al.*, 2003), visual-spatial impairment (Modoni *et al.*, 2004) and anhedonia associated with emotional blunting and depressive symptomatology (Bungener *et al.*, 1998). The phenotypic parallel between patients and transgenic mice illustrates the impact of toxic *DMPK* transcripts on CNS physiology, and corroborates the use of the DMSXL line to recreate DM1 brain pathology. Mild deficits in working memory were also found in DMSXL mice. Although not statistically significant, the initial difference between the two genotypes in the acquisition trial, during the working memory assessment, might confound the analysis, and the results should be interpreted with some caution. Alternative

behavioral tests (*e.g.* elevated or multiple arm radial mazes) might prove useful to provide definitive evidence of working memory deficits in DMSXL mice.

The behavioral abnormalities of DMSXL mice are associated with deficits in short-term plasticity, as well as changes in neurochemicals, suggesting altered synaptic function and neurotransmission in response to the CTG repeat expansion. The neurochemical data, in particular, provide insight into the neuronal circuits affected by DM1. Decreased dopamine in frontal cortex may account for motivation and reward deficits (Arias-Carrion and Poppel, 2007), while the reduced serotonin metabolism in brainstem may increase susceptibility to depressive-like behaviors (Werner and Covenas, 2010), thereby contributing to anhedonia. The high foci content in dopaminergic (substantia nigra) and serotonergic (raphe nucleus) brain centers may contribute, at least partially, to the neurochemical deficits of DMSXL mice. In humans, loss of catecholaminergic neurons (dopamine is an abundant catecholamine) and serotonin-containing neurons was previously reported (Ono *et al.*, 1998, Ono *et al.*, 1998). The involvement of dopaminergic and serotonergic pathways in DM1 neuropathology requires further investigation, and may provide insight into future means of therapeutic intervention, such as the modulation of the dopaminergic and serotonergic circuits.

In the search for pathways affected by the DM1 repeat mutation in the CNS, we found RAB3A upregulation and SYN1 hyperphosphorylation, not only in transgenic mice expressing large CTG expansions, but also in *post-mortem* DM1 brains. Transgenic mice over-expressing short *DMPK* transcripts did not show abnormal synaptic protein dysregulation, indicating that RAB3A and SYN1 misregulation is specifically associated with expanded transcripts.

Decreased glucose metabolism (Fiorelli *et al.*, 1992) and blood perfusion (Meola *et al.*, 1999) have been previously described in the frontal lobe of DM1 patients. Since

abnormal glucose metabolism can alter the protein content of synaptic vesicles (Gaspar *et al.*, 2010), it is reasonable to speculate that changes in the exocytotic machinery in frontal cortex might result (at least partially) from brain hypoperfusion and/or hypometabolism. Despite the possible contribution of altered glucose metabolism to synaptic dysfunction, cells transfected with DM1 repeat expansions displayed RAB3A upregulation and SYN1 hyperphosphorylation in culture, supporting the view that synaptic protein dysregulation is also a direct consequence of CUG RNA toxicity, rather than a simple indirect effect, secondary to general brain dysfunction. To further support this hypothesis, the analysis of complementary animal and cell models of DM1 revealed that RAB3A upregulation is mediated by MBNL1 inactivation, and SYN1 hyperphosphorylation is mediated by upregulation of CELF proteins. We propose that transcriptional dysregulation (Osborne *et al.*, 2009) or altered post-transcriptional regulation of mRNA decay (Masuda *et al.*, 2012) through loss of function of MBNL proteins could mediate RAB3A upregulation. Hyperphosphorylation of SYN1 may result from dysregulated kinase and/or phosphatase activities, as a result of altered CELF levels. Dysregulation of micro-RNAs, also reported in DM1 skeletal muscle and heart (Gambardella *et al.*, 2010, Perbellini *et al.*, 2011, Rau *et al.*, 2011), may extend to the CNS, opening new avenues for future research.

RAB3A is an abundant synaptic vesicle protein that regulates neurotransmission (Sudhof, 2004). Mouse *Rab3a* inactivation results in increased hippocampal PPF (Geppert *et al.*, 1997), while RAB3A overexpression in cell culture activates spontaneous exocytosis (Schluter *et al.*, 2002), similar to PC12 cells transfected with expanded *DMPK* constructs. Given the involvement of RAB3A in short-term synaptic plasticity and neurotransmitter release, RAB3A upregulation in DMSXL mice likely contributes to altered PPF and mediates the increase in basal neurosecretion in transfected PC12 cells. In addition RAB3A has been implicated in visual-spatial learning

(D'Adamo *et al.*, 2004). Therefore, altered RAB3A expression may contribute to the cognitive deficits of DMSXL mice and DM1 patients. Synapsins comprise the most abundant proteins in synaptic vesicles. SYN1 serves as a phosphorylation-dependent regulator of neurotransmitter release (Rosahl *et al.*, 1993): while non-phosphorylated SYN1 attaches synaptic vesicles to the actin cytoskeleton, stimulation-dependent phosphorylation decreases the affinity for synaptic vesicles and potentiates exocytosis (Fdez and Hilfiker, 2006). Consistent with a role of SYN1 in short-term plasticity (Fiumara *et al.*, 2007), chemically-induced hyperphosphorylation of SYN1 *in vivo* was previously associated with decreased PPF (Tallent *et al.*, 2009), like in DMSXL mice. The marked RAB3A upregulation and abnormal SYN1 hyperphosphorylation in *post-mortem* DM1 brains corroborate their role in DM1 neuropathology. RAB3A and SYN1 variability between patients may be associated with different degrees of disease severity, as previously reported for splicing abnormalities in skeletal muscle and heart (Orengo *et al.*, 2008, Wang *et al.*, 2007). In summary, we found RAB3A upregulation and SYN1 hyperphosphorylation in DM1 transgenic mice, transfected cells and in human DM1 brain samples. These protein defects were associated with electrophysiological and behavioral abnormalities in mice, as well as altered spontaneous neurosecretion in cell culture.

If changes in synaptic proteins reflect greater abnormalities in the dynamics and microstructure of the brain cell membrane, the molecular abnormalities identified might have wider implications and may correlate with the prevalent white matter lesions reported in DM1 brains (Minnerop *et al.*, 2011). Future MRI (magnetic resonance imaging) of DMSXL brain integrity will address this possibility, through the characterization of tissue changes in response to the DM1 mutation. Additional imaging measurements of regional brain blood flow and/or glucose metabolism in transgenic mice by PET (positron emission tomography) and/or SPECT (single-photon emission

computed tomography) must be performed to evaluate and localize the functional impact of DM1 in the CNS, and provide insight into the underlying mechanisms.

There is currently debate whether CNS dysfunction in DM1 is neurodegenerative, neurodevelopmental or neurofunctional. Although clinical data are not enough to answer this difficult question, transgenic mice may provide significant insight. The molecular and electrophysiological abnormalities detected in DMSXL mice are consistent with functional deficits in adult brain. Future analyses of cell loss and the CNS investigation throughout mouse development will be required to address the contribution of neurodegeneration and impaired neurodevelopment, respectively, towards DM1 CNS dysfunction. It remains possible that these processes are not mutually exclusive and that they all participate in DM1 brain pathophysiology."

In the context of the pre-clinical assessment of future therapies, it will be interesting to investigate whether therapeutic schemes targeting the CNS (and in particular RAB3 and SYN1) will be able to reverse the behavioral phenotypes (*e.g.* visual-spatial memory impairment, increased anxiety, anhedonia) and/or electrophysiological profiles (*e.g.* PPF deficits) of DMSXL mice. In cell culture, RAB3A and SYN1 can modulate miniature endplate currents of neurons (Chiappalone *et al.*, 2009, Wang *et al.*, 2011). It is tempting to investigate whether RAB3A knocking-down and/or SYN1 dephosphorylation in primary cultures can rescue the electrophysiological phenotype of DMSXL neurons.

Using molecular and physiological approaches to explore the molecular mechanisms of a complex human disease, we generated evidence of the impact of DM1 on synaptic proteins, vesicle secretion, neurotransmission and synaptic plasticity. The validation of our molecular findings in *post-mortem* DM1 brains substantiate a role of synaptic dysfunction in DM1, through changes in proteins involved in the regulation of synaptic vesicle release.

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SUPPLEMENTARY MATERIAL

Supplementary material includes five figures and two tables.

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FIGURE TITLES AND LEGENDS

Figure 1. Expression of an expanded *DMPK* transgene induces foci accumulation and splicing dysregulation in the CNS. **(A)** Fluorescent *in situ* hybridization (FISH) and immunofluorescence (IF) revealed nuclear foci of transgenic *DMPK* mRNA in both NeuN-positive neurons and GFAP-positive astrocytes, in multiple regions of the brain of one-month-old DMSXL homozygotes. **(B)** MBNL1 and MBNL2 colocalized with nuclear RNA foci. No RNA foci were observed in DM20 and wild-type control animals (data not shown). Punctuated MBNL2 staining was occasionally detected and did not correspond to RNA aggregates. DAPI was used for nuclear staining. Scale bar represents 5 μ m. **(C)** Quantification of the percentage of astrocytes and neurons exhibiting nuclear RNA aggregates (\pm SEM) throughout the brain of one-month-old DMSXL homozygotes (n=2) (**, $P < 0.01$, Chi-square test). **(D)** Percentage of inclusion of alternative exons in mRNA transcripts encoding: GRIN1/NMDAR1 glutamate receptor, ATP2A1/SERCA1 endoplasmic calcium ATPase, microtubule-associated protein tau (MAPT/TAU), amyloid beta precursor protein (APP), insulin receptor (INSR), MBNL1 and MBNL2 splicing regulators, LDB3/CYPHER cytoskeleton-interacting protein and FXR1 RNA binding protein. The analysis was performed in frontal cortex and brainstem of homozygous DMSXL mice (n=9) and wild-type controls (n=9) at one month of age. Splicing profiles were compared with those of wild-type newborns collected as postnatal day P1 (n=3). The graphs show the average fractional inclusion of the specified exon in triplicate assays (\pm SEM). **(E)** Quantification of CELF proteins in homozygous DMSXL (n=3) and wild-type mice (n=3) at one month. **(F)** Quantification of CELF proteins in frontal cortex of adult DM1 individuals (n=9) and non-DM controls (n=3). The graphs in **(E)** and **(F)** show the average steady-state levels relative to normalized controls. *,

$P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 2. DMSXL exhibit novelty-induced inhibition, anxiety, spatial and working memory impairment and anhedonia. **(A)** Assessment of novelty-induced inhibition in DMSXL homozygotes ($n=16$) and age-matched controls ($n=16$). The graph represents the percentage of the number of rearings (\pm SEM) in the first minute out of the first five minutes spent in the first open-field session. **(B)** Assessment of mouse anxiety levels by the marble-burying test. The graph represents the average number of marbles (\pm SEM) unburied and buried by DMSXL ($n=15$) and wild-type mice ($n=15$). The DMSXL line shows a significant shift towards a higher number of buried marbles ($P=0.0087$, Fisher's exact test). **(C)** Spatial memory assessment by the Morris water maze test. The graph represents the number of entries in the target quadrant (\pm SEM) during the probe trial, for DMSXL ($n=15$) and wild-type ($n=15$) mice. **(D)** Working memory assessment by the Morris water maze test. The graphs represent the average time and distance travelled (\pm SEM) to reach the platform in acquisition and retention trials for both genotypes ($n=15$ per genotype). **(E)** Saccharine consumption test for anhedonia. The graph on the left represents the average volume of saccharine solution (\pm SEM) drank by DMSXL homozygotes ($n=12$) and wild-type controls ($n=12$). The volume of water consumed once saccharine became available did not differ between the two genotypes (graph on the right). *, $P < 0.05$; **, $P < 0.01$.

Figure 3. DMSXL mice exhibit deficits in short-term synaptic plasticity and changes in neurochemical levels. **(A)** Electrophysiological profiling of DMSXL mice and age-matched controls. I/O characteristics in the CA1 region. Mean value of fEPSP (\pm SEM) is expressed as a function of stimulation intensity. **(B)** PPF in the CA1 region. The mean ratio of the second peak compared to the first one ($fEPSP_2/fEPSP_1$, \pm SEM) is expressed

as a function of the inter-stimulus interval. PPF values were significantly lower in DMSXL hippocampal slices (repeated measures two-way ANOVA). The difference between genotypes was more pronounced for inter-stimulus intervals of 100, 200 and 300 ms. **(C)** LTD in the CA1 region. Mean value of normalized fEPSP amplitude (\pm SEM) is expressed as a function of time. LTD amplitude was slightly lower in DMSXL slices, shortly after low frequency stimulation (grey box), but overall LTD amplitude was not significantly different between DMSXL and wild-type mice. **(D)** LTP in the CA1 region. Mean value of normalized fEPSP amplitude (\pm SEM) is expressed as a function of time. LTP did not significantly differ between DMSXL and wild-type mice. I/O, PPF and LTD data correspond to values averaged from 16 independent slices prepared from DMSXL (n=5) and wild-type mice (n=5). LTP data correspond to values averaged from 10 independent slices prepared from four DMSXL (n=4) and five independent slices from three wild-type mice (n=3). **(E)** Quantification of neurochemicals in the brain of DMSXL (n=5) and wild-type controls (n=5) at four months of age. Average concentration (\pm SEM) of L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovalinic acid (HVA), noradrenaline (NA), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) are plotted for frontal cortex and brainstem. *, $P < 0.05$; **, $P < 0.01$.

Figure 4. Abnormal metabolism of RAB3A and SYN1 in the CNS of DMSXL mice and DM1 patients. **(A)** Average steady-state levels (\pm SEM) of RAB3A, and phosphorylated SYN1 in the frontal cortex, brainstem and hippocampus of homozygous DMSXL mice (n=9), relative to normalized age-matched wild-type controls (n=9) at four months of age. **(B)** Western blot analysis of RAB3A protein levels and SYN1 phosphorylation in 4-month-old DM20 and wild-type mice (n=2 per genotype). **(C)** RAB3A protein upregulation in DMSXL brains is associated with increased transcript levels. Real-time

quantitative PCR of *Rab3A* mRNA in frontal cortex and hippocampus of DMSXL (n=6) and wild-type mice (n=6). The graph shows the average *Rab3A* relative expression (\pm SEM) at four months of age. *, $P < 0.05$. **(D)** Western blot analysis of RAB3A protein levels and SYN1 phosphorylation in frontal cortex of knock-out mice inactivated for *Mbn1*- and *Mbn2*-deficient mice. The graph on the right represents the average RAB3A steady state-levels (\pm SEM) in knock-out mice (n=3), relative to normalized age-matched wild-type controls (n=3). **(E)** Western blot analysis of RAB3A protein levels and SYN1 phosphorylation in PC12 cells overexpressing CELF1 or CELF2. NT, non-transfected cells. **(F)** RAB3A upregulation and SYN1 hyperphosphorylation in frontal cortex of adult DM1 patients (n=9), relative to non-DM controls (n=6). One non-DM control ("b") did not show a suitable SYN1 signal (possibly due to protein degradation) and was excluded from the analysis. *, $P < 0.05$; **, $P < 0.01$.

Figure 5. Toxic CUG repeats dysregulate neurosecretion in culture. Quantification of the effect of CTG repeat expansion on neurosecretion. The average secretion from hGH-expressing PC12 cells co-transfected with expanded DT960 or no-expansion DMPKS plasmids is plotted as a percent of total hGH content (\pm SEM). Basal secretion was measured in control medium containing 5.6 mM KCl. Stimulus-dependent secretion was measured in media containing 56 mM or 100 mM KCl.