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(54) Title: METHODS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT OF THE NEUROPATHOLOGY OF PATIENTS SUFFERING FROM MYOTONIC DYSTROPHY TYPE 1 (DM1)

(57) Abstract: The present invention relates to methods and pharmaceutical compositions for the treatment of the neuropathology of patients suffering from myotonic dystrophy type 1 (DM1). In particular, the present invention relates to a method of treating of the neuropathology of a patient suffering from myotonic dystrophy type 1 (DM1) comprising administering to the patient a therapeutically effective amount of an agent that normalizes, enhances, or potentiates glutamate uptake by glia.



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METHODS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT OF THE NEUROPATHOLOGY OF PATIENTS SUFFERING FROM MYOTONIC DYSTROPHY TYPE 1 (DM1)

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FIELD OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for the treatment of the neuropathology of patients suffering from myotonic dystrophy type 1 (DM1).

10 BACKGROUND OF THE INVENTION:

Expanded non-coding RNAs can exhibit a *trans*-dominant deleterious gain-of-function causing disease through abnormal interactions with RNA-binding proteins (Sicot & Gomes-Pereira, 2013). Myotonic dystrophy type 1 (DM1) is the most common inherited neuromuscular disease and the prototypical example of RNA toxicity (Sicot *et al.*, 2011). It is
15 a highly multisystemic disorder characterised by a wide range of clinical manifestations, such as myotonia, muscle weakness and wasting, cardiac arrhythmia, and other variable symptoms (Harper, 2001; Ashizawa & Sarkar, 2011). Given the variability in age of onset and clinical symptoms, four clinical forms of the disease have been described: late, adult, childhood and congenital onset (Harper, 2001). Clinical evidence and neuropsychological assessment
20 revealed the involvement of the central nervous system (CNS), especially in the most severe forms of the disease. Indeed, congenital DM1 is characterised by intellectual disability, whereas the childhood-onset form exhibits reduced IQ, low processing speed, as well as attention and executive deficits (Angeard *et al.*, 2007). Juvenile DM1 patients show some features of the autism spectrum, such as abnormal social interaction and communication, and
25 perform poorly at school, with a slow processing speed and visuo-spatial impairment (Angeard *et al.*, 2011). They are frequently diagnosed with attention deficit hyperactivity disorder and have emotional and behavioural problems, even prior to the onset of muscle symptoms (Steyaert *et al.*, 1997; Steyaert *et al.*, 2000). The neurological manifestations of the adult form show some overlapping with the juvenile cases, but they are usually milder with a
30 higher degree of inter-individual variability (Harper, 2001; Meola *et al.*, 2003; Winblad *et al.*, 2006; Meola & Sansone, 2007; Sansone *et al.*, 2007; Sistiaga *et al.*, 2009). DM1 neuropsychological manifestations of the disease are highly debilitating and have a tremendous impact on the quality of life of DM1 patients and their families, as illustrated by the prevalent hypersomnia in all clinical forms (Meola & Sansone, 2007; de Leon & Cisneros,

2008). As a result of their intellectual impairment, DM1 patients experience low education achievements, low employment, poor familial environment, as well as social, economic and material deprivation (Laberge *et al.*, 2007; Gagnon *et al.*, 2010). The involvement of the brain in DM1 is further supported by histopathological changes such as neurofibrillary tangles, cell loss, cytoplasmic neuronal inclusions, ubiquitin positive aggregates and disordered neuronal migration (Meola & Sansone, 2007; de Leon & Cisneros, 2008). White matter lesions, changes in brain connectivity and hypometabolism have also been detected by imaging techniques (Minnerop *et al.*, 2011; Caliandro *et al.*, 2013; Serra *et al.*, 2014; Wozniak *et al.*, 2014), and may contribute to the characteristic DM1 neuropsychological executive dysfunction. Most of the CNS research has concentrated on brain cortex, brainstem and hippocampus, based on the nature of DM1 neuropsychological symptoms.

SUMMARY OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for the treatment of the neuropathology of patients suffering from myotonic dystrophy type 1 (DM1). In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

Brain function is compromised in myotonic dystrophy type 1 (DM1), however we do not know the cell populations, neuronal circuits or pathways primarily affected in the CNS. In a DM1 mouse model the inventors found fine motor discoordination and abnormal Purkinje neuron firing, suggestive of cerebellum dysfunction. Purkinje abnormalities are associated with extensive disease-associated RNA aggregates and missplicing in the surrounding Bergmann cells, suggestive of glial dysfunction. A proteomics approach revealed downregulation of the glia-specific GLT1 glutamate transporter in the cerebellum of DM1 mice and patients, as a result of partial MBNL1 inactivation. Upregulation of GLT1 corrected Purkinje electrophysiology and motor deficits of DM1 mice, indicating that cerebellum dysfunction is mediated by defective glutamate uptake by Bergmann glia. The results demonstrate the critical role of neuroglial communication in DM1 neuropathology, not only in the cerebellum but also in other brain regions where GLT1 is also downregulated. Importantly, the data open new perspectives towards therapeutic pharmacological manipulation of glutamate metabolism in the brain of DM1 patients.

Accordingly, one aspect of the present invention relates to a method of treating of the neuropathology of a patient suffering from myotonic dystrophy type 1 (DM1) comprising administering to the patient a therapeutically effective amount of an agent that normalizes, enhances, or potentiates glutamate uptake by glia.

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As used herein, the term "treatment" or "treat" refer to both prophylactic or preventive treatment as well as curative or disease modifying treatment, including treatment of subjects at risk of contracting the disease or suspected to have contracted the disease as well as subjects who are ill or have been diagnosed as suffering from a disease or medical condition, and includes suppression of clinical relapse. The treatment may be administered to a subject having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay the onset of, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment. By "therapeutic regimen" is meant the pattern of treatment of an illness, e.g., the pattern of dosing used during therapy. A therapeutic regimen may include an induction regimen and a maintenance regimen. The phrase "induction regimen" or "induction period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the initial treatment of a disease. The general goal of an induction regimen is to provide a high level of drug to a subject during the initial period of a treatment regimen. An induction regimen may employ (in part or in whole) a "loading regimen", which may include administering a greater dose of the drug than a physician would employ during a maintenance regimen, administering a drug more frequently than a physician would administer the drug during a maintenance regimen, or both. The phrase "maintenance regimen" or "maintenance period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the maintenance of a subject during treatment of an illness, e.g., to keep the subject in remission for long periods of time (months or years). A maintenance regimen may employ continuous therapy (e.g., administering a drug at a regular intervals, e.g., weekly, monthly, yearly, etc.) or intermittent therapy (e.g., interrupted treatment, intermittent treatment, treatment at relapse, or treatment upon achievement of a particular predetermined criteria [e.g., disease manifestation, etc.]).

In some embodiments, the method of the present invention is particularly suitable for the treatment of glial dysfunction in a patient suffering from DM1.

In some embodiments, the agent that normalizes, enhances, or potentiates glutamate uptake by glia is an agent that raises the expression of *Glt1*.

As used herein, the term "*Glt1*" has its general meaning in the art and refers to the gene encoding for the high affinity glutamate transporter (GLT1) also named excitatory amino acid transporter 2 (EAAT2) or solute carrier family 1 member 2 (SLC1A2). GLT1 is a glial-specific glutamate transporter that ensures glutamate re-uptake from the intersynaptic space to avoid neuroexcitotoxicity of postsynaptic neurons (O'Shea, 2002; Kanai & Hediger, 2004).

Accordingly, the expression "agent that raises the expression of *Glt1* expression" refers to any compound natural or not (i.e. synthetic) that is able to raise the expression of *Glt1* in cells, in particular Bergmann cells. Typically, the agent stimulates *Glt1* promoter activity and transcription, or induces protein upregulation through increased translation (Karki *et al.*, 2014; Kong *et al.*, 2014).

In some embodiments, the agent that raises the expression of *Glt1* is raloxifene.

In some embodiments, the agent that raises the expression of *Glt1* is riluzole.

In some embodiments, the agent that raises the expression of *Glt1* is LDN/OSU-0212320.

In some embodiments, the agent that raises the expression of *Glt1* is a beta-lactam compound.

As used herein, the term "beta-lactam compound" refers to compounds that contain a beta-lactam nucleus in their molecular structure. The beta-lactam compounds of the invention include those beta-lactam compounds that are known in the art. See, e.g., U.S. Pat. Nos. 5,310,897 and 6,031,094. These and other beta-lactam compounds of the invention may be synthesized by standard chemical techniques as is well known in the art.

In some embodiments, the beta-lactam compound of the present invention is a beta-lactam antibiotic selected from the group consisting of benzylpenicillin, procaine

benzylpenicillin, penicillin V, penicillin V potassium, benzathine penicillin, hetacillin, cloxacillin, carbenicillin, flucloxacillin, ampicillin, ampicillin sodium, amoxicillin, co-amoxiclav, carboxypenicillin, ticarcillin, timentin, tazocin, piperacillin, pivmecillinam, amoxicillin-clavulanate, oxacillin, bacampicillin HCl, nafcillin sodium, cefaclor, cefadroxil, cefadyl, cefalexin, cefamandole, cefazolin, cefditoren, cefepime, cefetamet, cefdinir, cefixime, cefizox, cefotaxime, cefmetazole, cefobid, cefonicid, cefoperazone, cefoperazone sodium, cefotan, cefotetan, cefoxitin, cefpirome, cefpodoxime, cefpodoxime proxetil, cefprozil, cefradine, ceftazidime, ceftibuten, ceftidoren, ceftin, ceftizoxime, ceftriaxone, ceftriaxone sodium, cefuroxime, cefuroxime axetil, cephalixin, cefzil, cephalothin, cephalothin sodium, cephalirin sodium, aztreonam, imipenem, meropenem, ertapenem and FK-037.

In some embodiments, the beta-lactam compound of the present invention is selected from the compounds disclosed in the international patent application WO2014197536. In particular, the beta-lactam compound of the present invention is selected from the group consisting of:

- (3S, 4R)-3-((R)- (1-hydroxy-ethyl)-4-((R)-[1-methyl-2-(4-methyl-piperazin-1-yl)-2-oxo-ethyl]-azetidin-2-one;
- tert-butyl 4-((R)-2-((2R,3S)-3-((R)- 1 -hydroxyethyl)-4-oxoazetidin-2-yl)propanoyl)piperazine- 1 -carboxylate;
- (3S, 4R)-3-((R)-(1-Hydroxy-ethyl)-4-((R)-(1-methyl-2-oxo-2-piperazin-1-yl-ethyl)-azetidin-2-one;
- (3S, 4R)-4-((R)-(1 -(4-acetylpiperazin- 1 -yl)- 1 -oxopropan-2-yl)-3-((R) (1 -hydroxyethyl)azetidin-2-one;
- (3 S,4R)-4-((R)- 1 -(4-ethylpiperazin- 1 -yl)- 1 -oxopropan-2-yl)-3-((R)- 1 -hydroxyethyl)azetidin-2-one;
- (3 S,4R)-3-((R)- 1 -hydroxyethyl)-4-((R)- 1 -(4-(methylsulfonyl) piperazin- 1 -yl)- 1 -oxopropan- 2-yl)azetidin-2-one;
- (3 S,4R)-4-((R)- 1 -(4-cyclohexylpiperazin- 1 -yl)- 1 -oxopropan-2-yl)-3-((R)- 1 -hydroxyethyl)azetidin-2-one;
- (3 S,4R)-4-((R)- 1 -(4-benzoylpiperazin- 1 -yl)- 1 -oxopropan-2-yl)-3-((R)- 1 -hydroxyethyl)azetidin-2-one;
- (3 S,4R)-3-((R)- 1 -hydroxyethyl)-4-((R)- 1 -oxo- 1 -(4-phenyl piperazin- 1 -yl)propan-2-yl)azetidin-2-one;

(3 S,4R)-3-((R)- 1 -hydroxyethyl)-4-((R)- 1 -oxo- 1 -(4-propyl piperazin- 1 -yl)propan-2- yl)azetidin-2-one;

(3 S,4R)-3-((R)- 1 -hydroxyethyl)-4-((R)- 1 -(4-(4-methoxyphenyl) piperazin- 1 -yl)- 1 - oxopropan-2-yl)azetidin-2-one;

5 (3 S,4R)-4-((R)- 1 -(4-(tert-butyl)piperazin- 1 -yl)- 1 -oxopropan-2-yl)-3-((R)- 1 -hydroxyethyl)azetidin-2-one;

4-((R)-2-((2R,3 S)-3-((R)- 1 -hydroxy ethyl)-4-oxoazetidin-2-yl)propanoyl)piperazine- 1 - carboxamide;

10 (3 S,4R)-3-((R)- 1 -hydroxyethyl)-4-((R)- 1 -(4-methyl-3,4-dihydro quinoxalin- 1 (2H)-yl)- 1 - oxopropan-2-yl)azetidin-2-one;

(R)- 1 -((2R,3 S)-2-((R)- 1 -(4-methylpiperazin- 1 -yl)- 1 -oxopropan-2-yl)-4-oxoazetidin-3- yl)ethyl acetate;

(R)- 1 -((2R,3 S)-2-((R)- 1 -(4-methylpiperazin- 1 -yl)- 1 -oxopropan-2-yl)-4-oxoazetidin-3- yl)ethyl butyrate;

15 (R)- 1 -((2R,3 S)-2-((R)- 1 -(4-methylpiperazin- 1 -yl)- 1 -oxopropan-2-yl)-4-oxoazetidin-3- yl)ethyl isobutyrate;

(R)- 1 -((2R,3 S)-2-((R)- 1 -(4-methylpiperazin- 1 -yl)- 1 -oxopropan-2-yl)-4-oxoazetidin-3- yl)ethyl pivalate;

or a pharmaceutically acceptable form thereof.

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As used herein, the term "effective amount" refers to a quantity sufficient to achieve a therapeutic effect (e.g. treating cerebellum dysfunction). In the context of therapeutic or prophylactic applications, the amount of a composition administered to the subject will depend on the type and severity of the disease and on the characteristics of the individual, such as general health, age, sex, body weight and tolerance to drugs. It will also depend on the degree, severity and type of disease. The skilled artisan will be able to determine appropriate dosages depending on these and other factors. In some embodiments, an effective amount of the agent for achieving a therapeutic or prophylactic effect, range from about 0.000001 mg per kilogram body weight per day to about 10,000 mg per kilogram body weight per day. Typically, the dosage ranges are from about 0.0001 mg per kilogram body weight per day to about 100 mg per kilogram body weight per day. For example dosages can be 1 mg/kg body weight or 10 mg/kg body weight every day, every two days or every three days or within the range of 1-10 mg/kg every week, every two weeks or every three weeks. In some embodiments, a single dosage of peptide ranges from 0.1-10,000 micrograms per kg body

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weight. In some embodiments, aromatic- cationic peptide concentrations in a carrier range from 0.2 to 2000 micrograms per delivered millilitre.

Typically, the agent of the present invention is combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form pharmaceutical compositions. "Pharmaceutically" or "pharmaceutically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. Typically, the pharmaceutical compositions contain vehicles, which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. Sterile injectable solutions are prepared by incorporating the agent of the present invention in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:**Figure 1. Behavioural and electrophysiological phenotyping revealed cerebellum**

dysfunction in DMSXL mice. (A) Assessment of cerebellum-dependent motor-coordination of DMSXL homozygotes (n=19) and wild-type controls (n=21) in the runway test over five consecutive days. The graph represents the average number of hind leg slips per trial (four trials per day). The DMSXL line shows a significant increased number of hind leg slips, during five consecutive days, as well as three weeks later, following a test-free period (* $p < 0.05$, *** $p < 0.001$). Learning was assessed by number of slips at day five, relative to day, and retention of the task was assessed at day 26 following a three-week period without testing (###, $p < 0.001$). **(B)** *In vivo* electrophysiological analysis of mouse cerebellum activity in alert DMSXL homozygous (n=4) and wild-type (n=3) mice at two months of age. DMSXL mice showed higher single spike (SS) frequency rate and higher rhythmicity (RI), compared to the wild-type controls (* $p < 0.05$, *** $p < 0.001$). **(C)** Patch clamp electrophysiological recordings of Purkinje cells activity in DMSXL and wild-type cerebellum slices. A schematic representation of the typical shape and phases of action potentials (AP) in DMSXL and wild-type Purkinje cells is shown on the left. The graphs show the mean threshold current required to initiate an AP, the frequency of the spontaneous activity and the action potential afterhyperpolarization (AHP) recovery time constant (τ_{AHP}). DMSXL Purkinje cells exhibited statistically significant differences in all electrophysiological parameters studied, indicating dysfunctional Purkinje cells (* $p < 0.05$).

Figure 2. Distribution of nuclear RNA foci and missplicing in DMSXL cerebellum.

(A) *DMPK* RNA foci (red) were detected by FISH associated with fluorescent immunodetection of MBNL1 or MBNL2 (green) in DMSXL mice. Foci were very abundant in the cell population surrounding Purkinje cells, but rarely detected in the nucleus of Purkinje neurons. RNA foci co-localise with MBNL proteins, except in Purkinje cells. No RNA foci were observed in DM20 and wild-type control animals (*data not shown*). The scale bar represents 10 μm . **(B)** Real-time quantitative PCR of *DMPK* transgene expression in Purkinje neurons and Bergmann astrocytes, microdissected from DMSXL cerebellum (n=3). An average of 100 cells of each type were analysed per animal. **(C)** Splicing profiles of different mRNA transcripts were compared in the whole cerebellum of DMSXL and wild-type mice

(n=4, each genotype) by RT-PCR analysis. Ratio of inclusion of alternative exons was determined in mRNA transcripts encoding: MBNL1 and MBNL2 splicing regulators, microtubule-associated protein tau (MAPT/TAU), GRIN1/NMDAR1 glutamate receptor, amyloid beta precursor protein (APP), FXR1 RNA binding protein and LDB3 cytoskeleton-interacting protein. Mild missplicing of alternative exons was detected in DMSXL cerebellum. **(D)** Representative nested RT-PCR products and quantification of *Mbnl1* and *Mbnl2* alternative exon inclusion in Purkinje cells and in neighbouring Bergmann glia collected by laser microdissection DMSXL and wild-type mice (n=3, each genotype) at one month of age. Three independent replicates were performed for each mouse (* p <0.05, *** p <0.001).

Figure 3. GLT1 downregulation in DMSXL cerebellum. **(A)** Quantification of GLT1 expression levels in cerebellum DMSXL revealed significant reduction of protein levels in DMSXL mice, relative to wild-type controls (n=4, each genotype). **(B)** Quantification of *Glt1* mRNA levels by quantitative real-time PCR did not show transcript level differences between DMSXL homozygotes and wild-type controls (n=4, each genotype).

Figure 4. Transcriptional GLT1 downregulation is mediated by partial MBNL1 inactivation. **(A)** Quantification of *GLT1* transcripts by quantitative RT-PCR revealed a significant downregulation in the cerebellum and frontal cortex of the DM1 patients showing the most pronounced protein decrease (n=5), relative to non-DM controls (n=4), and **(B)** in human T98G glial cells transfected with expanded *DMPK* constructs containing 960 interrupted CTG repeats (DT960), relative to no-repeat (DMPKS) and mock transfected controls. **(C)** Western blot detection of stably transfected GLT1 in C6 glioma cells revealed significant downregulation following DT960 transfection, relative to DMPKS and mock controls. β -Actin was used as loading control. **(D)** Quantification of *GLT1* mRNA by quantitative RT-PCR, following MBNL1 and/or MBNL2 knocking down in T98G cells, showed significant GLT1 downregulation in cells depleted of MBNL1 alone. **(E)** Western blot detection and quantification of MBNL1 and MBNL2 protein levels in primary neurons and astrocytes. Decreasing amounts of a protein pool of whole cell lysate from three wild-type cultures were electrophoresed and immunodetected. The graph represents the MBNL1/MBNL2 expression ratio in each cell type, and shows that MBNL1 relative expression is twofold higher in mouse primary astrocytes than in neurons. β -Actin was used as loading control. **(F)** Quantification of *GLT1* mRNA levels by quantitative RT-PCR

following CELF1 or CELF2 overexpression in T98G cultured cells. CELF protein overexpression did not alter GLT1 expression (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s. not statistically significant).

5 **Figure 5. Rescuing of DMSXL cerebellum phenotype following GLT1 upregulation by ceftriaxone.** Profiles of LFP oscillations recorded over 690 minutes in the Purkinje cell layer of (A) *Glut1*^{+/-} mice (n=4) and wild-type controls (n=2) and in (B) DMSXL mice (n=5) prior and following ceftriaxone i.p. injection. (C) The power peak of the LFP oscillation was calculated by FFT analysis. *Glut1*^{+/-} mice (n=4) showed fast LFP oscillations, with a power peak significantly higher than wild-type controls (n=2) ($F(1,46)=35,899$, $P < 0,000001$). Following ceftriaxone injection, the fast LFP power of *Glut1*^{+/-} mice was significantly reduced ($F(1,38)=20,341$; $p < 0.0006$), back to wild-type levels. (D) Power peak of the LFP oscillation in DMSXL mice (n=5) was significantly higher than in wild-type controls (n=2), and dramatically reduced by ceftriaxone ($F(1,76)=21,276$, $p < 0,00002$). In contrast, PBS injection did not change the amplitude of the fast oscillation in DMSXL cerebellum (n=4). (E) Quantification of GLT1 protein expression levels in the cerebellum of DMSXL mice, following i.p. injection of ceftriaxone for five days revealed a significant increase up to wild-type levels, relative to PBS-treated mice (n=2, each group) (* $p < 0.05$, *** $p < 0.001$). (F) Motor coordination improved in DMSXL mice injected with ceftriaxone (n=9), relative to control animals injected with PBS (n=9), as illustrated by a significant reduction in the number of hind leg slips over the first three days of the runway test (#, $p < 0.05$). Ceftriaxone reduced hind leg slips to values that were indistinguishable from those detected in wild-type controls (n=5) in 4 out of the 5 days of testing (§, $p < 0.05$). DMSXL mice injected with PBS performed consistently worse than wild-type controls throughout the entire test (#, $p < 0.05$).

30 **Figure 6. Analysis of RNA foci accumulation and GLT1 downregulation in human DM1 cerebellum.** (A) Immunofluorescence combined with FISH revealed abundant nuclear RNA foci in cerebellar Bergmann astrocytes of DM1 patients, which co-localise with MBNL1 and MBNL2 proteins. (B) The RT-PCR analysis of candidate alternative exons in human cerebellum tissue samples revealed mild missplicing events in adult DM1 patients (n=7), relative to non-DM controls (n=6). (C) Western blot quantification of GLT1 protein confirmed showing GLT1 downregulation in DM1 cerebellum (n=7), relative to non DM1 controls (n=6) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

EXAMPLE:**Materials and Methods**

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Transgenic mice and genotyping. DMSXL transgenic mice were generated and genotyped as previously described (Hernandez-Hernandez *et al.*, 2013a). All DMSXL mice used for this work were adult (2-4 months) homozygotes, unless stated otherwise. *Glt1* knock-out mice on C57Black/6 background (Tanaka *et al.*, 1997), were provided by Prof. Niels Christian Danbolt (University of Oslo, Norway). The *Glt1* transgenic status was determined by multiplex PCR of tail DNA. *Glt1* wild-type alleles generate a 469-bp product, while the disrupted allele generated a 210-bp allele. All experiments were produces with wild-type controls of the same litter to reduce the effect of variability between different litters. Animal care and handling was performed according to the French and European legislations, and the ethical guidelines of the host institution.

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Tissue samples. Mouse cerebellum tissues were microdissected at different ages and stored at -80°C. Human cerebellum samples were collected from different laboratories and shipped to us by Dr. Yasuhiro Suzuki (Asahikawa Medical Center, Japan), Dr. Tohru Matsuura (Okayama University, Japan), Dr. Adolfo Lopez-de-Munain (Navarra University, Spain), Dr. Christopher Pearson (The Hospital for Sick Children, Toronto, Canada). All experiments using human samples were approved by the Ethics Committees of the host institutions. Written informed consent specimen use for research was obtained from all patients.

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Behavioural and electrophysiological assessment.

The runway test: Motor coordination was examined by the runway test as previously described (Servais & Cheron, 2005). In this test, DMSXL (n=20) and control (n=19) mice, male and females included, ran along an elevated runway with low obstacles intended to impede progress. The runway was 100 cm long and 0,7 cm width. Obstacles being of 1 cm diameter wood rod and 0.7 cm width were placed every 10 cm along the runway. Mice were placed on one extremity of the runway and had to move along the runway to reach the other end. The number of slips of the right hind leg was counted. Each mouse underwent four trials

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per day during 5 consecutive days. The test was repeated following a test-free period of three weeks, over one day (four consecutive trials), to assess the learning capacity of mice.

In vivo electrophysiological study in alert mice. DMSXL and control mice, aged from one to two months, were surgically prepared for chronic recording of neuronal activity in the cerebellum. The experimental session for extracellular recording of Purkinje Cells (PCs) activity and local field potential (LFP) analysis in the cerebellar cortex was performed as previously described (Cheron *et al.*, 2004). The strength of the rhythmicity was quantified with a rhythm index (Sugihara & Furukawa, 1995).

Fluorescent in situ hybridization (FISH). Ribonuclear inclusions were detected with a 5'-Cy3-labelled (CAG)₅ PNA probe, as previously described (Huguet *et al.*, 2012). Immunofluorescence (IF) combined with FISH. Immunofluorescence combined with FISH was performed as previously described (Hernandez-Hernandez *et al.*, 2013a).

RT-PCR analysis of alternative splicing. Total RNA was extracted from half mouse cerebellum using a TRIZOL extraction protocol combined with a commercially available RNA Purification Kit, as previously described (Huguet *et al.*, 2012). cDNA synthesis and semi-quantitative RT-PCR analysis were performed as described elsewhere (Gomes-Pereira *et al.*, 2007; Hernandez-Hernandez *et al.*, 2013a). All samples were normalized to TATA-binding protein (Tbp).

Western blot analysis. Total protein was extracted from 20-30 mg brain tissue sample using RIPA buffer (ThermoFisher Scientific; 89901), supplemented with 0.05% CHAPS (Sigma; C3023), 1x complete protease inhibitors (Roche; 04693124001), 1x Phospho STOP phosphatase inhibitors (Roche; 04693124001). Protein concentrations in the supernatants were determined using a Bio-Rad DCTM protein assay (Bio-Rad; 500-0114). Protein integrity was checked by Coomassie stain of a 10% SDS-polyacrylamide gel. Volumes corresponding to 30-60 µg of protein were mixed with Laemmli sample buffer and boiled for 5 min. Proteins were resolved in 10% or 12% SDS-polyacrylamide gels and transferred onto PVDF membranes. Following Ponceau red staining to verify the efficiency of protein transfer, membranes were blocked in 1X TBS-T (10 mM Tris-HCl, 0.15 M NaCl, 0.05% Tween 20) containing blotto (Santa Cruz Biotech; sc2325) and incubated overnight at 4°C with the corresponding primary antibody. After three washes with 1X TBS-T, membranes were incubated at room temperature during 1 hr with the appropriated HRP-secondary antibody.

After washing with 1X TBS-T, antibody binding was visualized by chemiluminiscence (PerkinElmer). Densitometric analysis with Quantity One® 1D Analysis Software (Bio-Rad) has been performed to quantify signal intensity.

5 **Laser Capture Microdissection (LCM).** Purkinje cells and surrounding cells were individually microdissected from one to two-month-old DMSXL and wild-type control mouse cerebellum, using a Palm Micro Beam (Carl Zeiss). RNA extraction, cDNA synthesis and RT-PCR analysis of candidate genes performed as previously described (Peixoto *et al.*, 2004). Average of 100 Purkinje cells and 300 surrounding cells were separately collected in triplicate
10 from three DMSXL mice and from three wild-type control mice. For each cDNA sample, three replicates of the RT-PCR reactions were performed.

2 Dimensions Gel Electrophoresis. Cerebellum tissues from one to two-month-old DMSXL and wild-type control mouse were extracted as previously described above. Seven
15 centimeter linear ReadyStrips IPG strips (Bio-Rad; 163-2001) were loaded with 150 µg of proteins by passive rehydration overnight. Isoelectric focusing was performed on an IPGphor device (Pharmacia Biotech) and was carried out up to a total of 10 kVh. The IPG strips were rinsed thoroughly with distilled water, quickly dried on filter paper and focused proteins were reduced (50 mM Tris/HCl, pH 6.8, 6 M urea, 2% w/v SDS, 30% v/v glycerol,
20 2% w/v DTT) and alkylated (50 mM Tris/HCl, pH 6.8, 6 M urea, 2% w/v SDS, 30% v/v glycerol, 4.5% w/v iododacetamide) for 20 min each. Strips were then placed on top of 12% SDS polyacrylamide gel. The process of the second dimension has been performed as previously described.

25 **iTraq analysis.** The analysis of mouse brain proteome by isobaric tagging for relative and absolute quantifications (iTRAQ) mass spectrometry was performed on individual DMSXL and wild-type mice (n=2 per genotype). Detailed protocol is described in Supplementary Material and Methods.

30 **Microscope and images processing.** Images were taken with a fluorescent microscope Zeiss ApoTome 2, using Zeiss 2011 software and were treated with ImageJ 1.45s software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA).

Statistically analysis. Statistical analyses were performed with Prism (GraphPad Software, Inc), SPSS (v14.0, SPSS Inc©), Statistica (v6.0, StaatSoft®) and/or Excel software. When two groups were compared, a two-tailed Student's t-test with equal or unequal variance was performed as appropriate. For ANOVA, if statistical significance was achieved, we performed post-test analysis to account for multiple comparisons. Statistical significance was set at $p < 0.05$. Statistical analyses of the differences between electrophysiological profiles of DMSXL and wild-type mice were performed using the Student's t-test and one-way analysis of variance (ANOVA) for repeated measures. Unless indicated otherwise, the results are presented as means $\pm$ standard error of the mean ($\pm$ SEM) per genotype group.

Ceftriaxone treatment. Mouse intraperitoneal injections of ceftriaxone (Sigma; C5793) in PBS (20 $\mu\text{g}/\mu\text{l}$) were performed through a 27G needle to a final dose of 200 mg/kg.

Results:

Motor-coordination abnormalities of DMSXL mice suggest cerebellar dysfunction

To investigate DM1 neuropathogenesis, we have been taking advantage of the DMSXL transgenic mouse model, which carries more than 1000 CTG in 3'UTR of the *DMPK* gene, compared to the control DM20 line that carries non-pathogenic 20-CTG tracts. DMSXL derived from the DM300-328 line after successive breeding (Seznec *et al.*, 2000; Gomes-Pereira *et al.*, 2007). Homozygous DMSXL mice express enough toxic CUG-containing RNA under the control of the human *DMPK* promoter to develop important features of the disease, including nuclear RNA foci accumulation and missplicing in multiple tissues (Huguet *et al.*, 2012; Hernandez-Hernandez *et al.*, 2013a), which are associated with a multi-systemic phenotype, which includes muscle and respiratory deficits (Huguet *et al.*, 2012; Panaite *et al.*, 2013), as well as behavioural and electrophysiological abnormalities (Hernandez-Hernandez *et al.*, 2013a). The design of the initial phenotyping of DMSXL mice was grounded on clinical data, thus concentrating primarily on aspects of behavioural and cognition that depend on frontal cortex and hippocampus, in order to assess the involvement of these brain regions in DM1 neuropathology (Hernandez-Hernandez *et al.*, 2013b). However, since the detailed neuropathology of DM1 is not fully known, and given the wide expression of the transgene in DMSXL brains (Hernandez-Hernandez *et al.*, 2013a), we did

not limit our study to these brain areas and explored the susceptibility of other regions and neuronal circuits to the expression of toxic CUG RNA repeats.

The extended behavioural analysis of DMSXL mice included the assessment of cerebellum-dependent motor coordination, through the runway test. In this test, mice must run along an elevated runway with low obstacles intended to impede their progress. The test assesses motor coordination by the cerebellum, while limiting the influence of muscle performance on the final outcome. The number of slips of the right hind leg was counted and taken as a direct assessment of motor discoordination. DMSXL mice consistently showed a significantly higher number of hind leg slips over five consecutive days, relative to wild-type controls (Figure 1.A). The poor performance of DMSXL was noticeable from the first day of testing (4.7 versus 3.0 slips, $p=0.029$), and the difference between genotypes accentuated as the tested progressed (2.2 slips versus 0.8 slips at day five, $p<0.001$). These results indicate that DMSXL show motor impaired coordination and deficits in the fine-tuning of movements, suggestive of cerebellum dysfunction.

The mean number of hind leg slips of wild-type mice decreased significantly from day one to day five (3.0 at day one, versus 0.8 at day five, $p<0.001$). Similarly, the performance of DMSXL mice improved over the same period of time (4.7 slips at day one, versus 2.2 at day 5, $p=0.004$), demonstrating that, although performing poorly, DMSXL mice are capable of learning new motor functions (Figure 1.A). Finally, we tested the retention of the cerebellum-dependent task, through the re-assessment of mice following a test-free period of three weeks. The number of hind leg slips of both wild-type and DMSXL mice was still significantly lower at day 26 relative to the initial score measured at day one (3 slips versus 0.78 slips for wild-type mice, $p=1.97 \times 10^{-5}$; 4.7 slips versus 1.94 slips for DMSXL; $p=0.01$), which demonstrates that the retention of the cerebellum-dependent motor task is not affected in DMSXL mice.

Electrophysiological abnormalities of Purkinje cells in DMSXL cerebellum

To confirm cerebellum dysfunction in response to the expression of toxic *DMPK* transcripts, we explored the electrophysiological profile of DMSXL cerebellum. *In vivo* cerebellum electrophysiological recordings (Figure 1.B) were performed on alert DMSXL mice and compared to wild-type controls. Electrodes were positioned in the Purkinje cell layer, and recorded a significant increased frequency of simple spike firing rate of Purkinje cells (86 Hz versus 50 Hz, $p=1.47 \times 10^{-4}$), as well as a significant higher rhythmicity (0.13 versus 0.07, $p=0.02$) in DMSXL mice. These results demonstrate a hyperactivity of the

DMSXL cerebellum. The assign the cerebellar abnormalities recorded to Purkinje cell deficits, additional electrophysiological analysis were performed using the whole cell configuration of the patch-clamp technique, on acute cerebellar slices prepared from DMSXL and wild-type mice (Figure 1.C). The threshold current was significantly lowered in DMSXL versus wild-type Purkinje cells (203 pA versus 275 pA, $p<0.05$); while the frequency of the spontaneous activity of Purkinje cells was significantly increased in DMSXL (11.42 Hz versus 4.78 Hz, $p<0.05$). Finally, the action potential afterhyperpolarisation recovery time constant (τ_{AHP}) was significantly decreased in the DMSXL versus wild-type Purkinje cells (1.25 ms versus 0.7 ms, $p<0.05$). Taken together, the combined *in vivo* and *in vitro* electrophysiological analysis of DMSXL cerebellum demonstrated significant abnormalities in the activity of Purkinje cells.

DMSXL mice show abundant RNA accumulation in Bergmann glia

Accumulation of nuclear RNA foci is one of the initiating events in the molecular pathogenesis of DM1 (Sicot *et al.*, 2011). We have then investigated whether DMSXL motor coordination and Purkinje cell abnormalities were associated with the RNA aggregates. Fluorescent *in situ* hybridisation (FISH) revealed the regional accumulation of toxic *DMPK* transcripts in the cerebellum of adult DMSXL mice. In spite of widespread toxic RNA aggregates in the cerebellum, the highest foci content was consistently observed in a specific calbindin-negative cell population, which did not correspond to the Purkinje neurons but was located in the vicinity of the Purkinje layer (Figure 2.A). Both MBNL1 and MBNL2 co-localised with the RNA foci in cells neighbouring Purkinje neurons. In contrast, MBNL1 and MBNL2 produced a diffuse staining in the nucleus and cytoplasm of Purkinje cells, even in those rare Purkinje neurons that displayed RNA foci.

Given the intriguing regional distribution of RNA foci in DMSXL brains, we sought to identify the foci-enriched cell population. To this end, we combined FISH with the immunofluorescence against cell type-specific markers. NeuN is a widely used marker of mature neurons, but in the cerebellum it stains almost exclusively the granular neurons. Fox1 and Fox2 stain Purkinje and Golgi cells. In addition, Fox2 stains the granular cells neurons (Kim *et al.*, 2011). The analysis revealed that the majority of cells with the highest foci content in DMSXL mice and DM1 patients were NeuN-, Fox1- and Fox2-negative, indicating that foci accumulate preferentially in non-neuronal cells of the cerebellum. The non-neuronal nature of the foci-enriched cell type was confirmed by the immunodetection of GFAP, a glial-specific cell marker. Together with their localization around the Purkinje layer, the

expression of GFAP and absence of neuronal protein markers identified the foci-rich cells as Bergmann astrocytes.

To explore the reasons behind cell type-specific RNA foci distribution, we quantified expanded *DMPK* transcripts in different cell types collected by laser capture microdissection.

5 Nested real-time PCR revealed levels of toxic CUG RNA in Bergmann cells that were nearly three times higher than in neighbouring Purkinje cells (Figure 2.B), consistent with the higher foci abundance in the glial cell lineage, relative to the neuronal cell layer.

Bergmann-specific RNA spliceopathy

10 Following the accumulation of RNA foci accumulation and sequestration of MBNL proteins in the cerebellum, we investigated splicing dysregulation in adult mice. Splicing analysis revealed missplicing of a variety of alternative exons, such as *Mapt* exon 10, *Grin1* exon 21 and *App* exon 8 (Figure 2.C). Overall we were surprised by the mild spliceopathy in DM1 cerebellum, given the severe foci accumulation in this region, particularly in certain cell
15 populations. We therefore hypothesised that the regional distribution of RNA foci dictates cell type-specific spliceopathy: it is possible that the cells expressing higher CUG RNA repeats and showing the highest foci content exhibit pronounced splicing abnormalities. To test this hypothesis, we compared the splicing profiles of candidate genes between foci-rich Bergmann astrocytes and Purkinje neurons, by nested RT-PCR. We focused on two robust splicing
20 defects consistently detected in DMSXL mouse cerebellum. In line with our hypothesis, *Mbnl1* and *Mbnl2* splicing was specifically deregulated only in foci-rich DMSXL Bergmann glia, relative to wild-type controls. The inclusion of *Mbnl1* and *Mbnl2* alternative exons remained unaltered in DMSXL Purkinje neurons (Figure 2.D).

Glutamate transporter downregulation in DMSXL cerebellum

25 The Bergmann cell-specific foci accumulation and splicing dysregulation suggests that Purkinje cell deficits result from neuroglial miscommunication. To investigate disease intermediates and pathways underlying cerebellum-dependent behavioural and electrophysiological abnormalities, we used isobaric tag for relative and absolute
30 quantification (iTRAQ) methods, to identify proteins abnormally expressed in DMSXL cerebellum. Among misregulated proteins, we found significant downregulation of glial high affinity glutamate transporter (GLT1) [also named excitatory amino acid transporter 2 (EAAT2) or solute carrier family 1 member 2 (SLC1A2)], a glial-specific glutamate transporter that ensures glutamate re-uptake from the intersynaptic space to avoid

neuroexcitotoxicity of postsynaptic neurons (O'Shea, 2002; Kanai & Hediger, 2004). A ~70% decrease in GLT1 expression in the cerebellum of DMSXL mice was confirmed by western blot (Figure 3.A). To assess the extent of GLT1 downregulation, we studied additional brain regions, and found significantly decreased protein expression in DMSXL frontal cortex and brainstem, demonstrating general downregulation of this glutamate transporter.

GLT1 downregulation is mediated by MBNL1 inactivation

To gain insight into the mechanisms of GLT1 downregulation we first tested if reduced protein was associated with abnormal transcript levels. Quantitative RT-PCR revealed significantly lower levels of *GLT1* mRNA in the cerebellum and frontal cortex of DM1 patients with pronounced protein downregulation (**Figure 4.A**). In amyotrophic lateral sclerosis (ALS), missplicing of GLT1 results in RNA degradation and loss of protein (Lin *et al.*, 1998 Dykes-Hoberg M, Crawford T, Clawson L, Rothstein JD (1998). Aberrant RNA processing in a neurodegenerative disease: the cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis. *Neuron*: 20: 589-602). To test if similar mechanisms operate in DM1, we studied GLT1 splicing but we did not find obvious abnormalities in the cerebellum of DM1 patients or DMSXL mice. We then tested whether GLT1 downregulation was the direct result of the expression of CUG-containing RNA, or a secondary consequence associated with DM1 brain disease progression. To this end, we quantified GLT1 in cultured glial cells transfected with expanded *DMPK* constructs. The expression of expanded CUG RNA reduced significantly *GLT1* transcripts in human T98G glioblastoma cells, relative to no-repeat control constructs (**Figure 4.B**). In addition, GLT1 protein was also reduced in rat C6 glial cells stably transfected with GLT1 cDNA (Kalandadze *et al.*, 2002) (**Figure 4.C**).

MBNL proteins regulate various aspects of RNA metabolism. Hence, we tested whether MBNL1 or MBNL2 inactivation was sufficient to lower GLT1 levels in the absence of missplicing. We used shRNA to knock down MBNL1 and/or MBNL2 in T98G cells. Quantitative RT-PCR revealed that the downregulation of MBNL1 decreased *GLT1* mRNA levels (**Figure 4.D**). Modest MBNL2 downregulation however, did not decrease GLT1 protein. Similarly, GLT1 remained unchanged in T98G treated simultaneously with MBNL1 and MBNL2 shRNA. The role of MBNL1 in GLT1 regulation in astrocytes was associated with higher expression of MBNL1 in this cell type relative to neurons (**Figure 4.E**).

Since CELF2 is significantly upregulated in mouse and human DM1 brains, we studied whether CELF proteins regulated GLT1 expression. Transient transfection and upregulation of CELF1 and CELF2 in T98G cells did not result in lower *GLT1* mRNA levels (Figure 4.F). In summary, these results demonstrate that GLT1 protein downregulation in DM1 is associated with lower transcript levels without evidence of missplicing, and is mediated by partial inactivation of MBNL1 independently of CELF proteins.

Upregulation of GLT1 corrects the cerebellum phenotype of DMSXL mice

To establish a causal relationship between GLT1 dysregulation and the cerebellar phenotype of DMSXL mice, we have recorded local field potential (LFP) oscillations in the Purkinje cell layer of the cerebellar vermis of DMSXL and *Glt1*-deficient mice (Tanaka *et al.*, 1997), as an indication of the extracellular electrical activity. Both DMSXL and heterozygous *Glt1*^{+/-} mice exhibited a frequency peak of oscillations around 200 Hz (200 ± 27 Hz in DMSXL mice and 221 ± 28 Hz in *Glt1*^{+/-} mice) (Figure 5.A and B). Importantly, the amplitude of the power peak of LFP oscillations was significantly higher in *Glt1*^{+/-} ($2.0 \times 10^{-7} \pm 2.3 \times 10^{-8} \mu V^2$) and in DMSXL mice ($1.0 \times 10^{-6} \pm 1.9 \times 10^{-7} \mu V^2$), relative to wild-type controls ($1.0 \times 10^{-7} \pm 5.0 \times 10^{-8} \mu V^2$) (Figure 5.C and D), indicating abnormal Purkinje electrical activity in both mouse lines as a result of GLT1 inactivation.

To further assess the contribution of GLT1 levels to the power peak of spontaneous LFP oscillations of Purkinje cells activity, we have taken advantage of ceftriaxone, a β -lactam antibiotic that stimulates *Glt1* promoter activity and induces protein upregulation (Rothstein *et al.*, 2005). Ceftriaxone intraperitoneal injection for five consecutive days (single dose of 200 mg/kg per day) significantly reduced the power peak of LFP oscillations in *Glt1*^{+/-} mice to wild-type levels (Figure 5.C), indicating that the deleterious impact of GLT1 insufficiency on Purkinje neuronal activity can be corrected by the administration of ceftriaxone.

To directly demonstrate the determinant role of GLT1 in the cerebellar phenotype of DMSXL mice, we treated DMSXL animals with ceftriaxone (i.p. injection of 200 mg/kg over five consecutive days). We first confirmed the increase of GLT1 protein in DMSXL cerebellum, to levels undistinguishable from those detected in wild-type controls (Figure 5.E). Like in *Glt1*^{+/-} mice, ceftriaxone did not change the frequency peak of LFP oscillations in DMSXL mice (Figure 5.B), but resulted in a remarkable reduction in the amplitude of the power peak of Purkinje LFP oscillations, down to wild-type values (Figure 5.C).

We next assessed whether GLT1 upregulation and correction of the Purkinje electrophysiological activity by ceftriaxone was sufficient to ameliorate the motor

coordination of DMSXL mice. DMSXL mice were injected with the β -lactam antibiotic for five consecutive days, prior to the runway test. Ceftriaxone injections continued during motor assessment. Ceftriaxone resulted in a significant reduction in the average number of hind leg slips per trial over the first three days of testing. The effect was so dramatic that DMSXL injected with ceftriaxone, performed as well as wild-type controls from day two (Figure 5.F). However, the improvement of wild-type mouse performance on day five was not accompanied by ceftriaxone-treated DMSXL animals, resulting in a mild but significant difference between both groups at this time point. Overall, these results demonstrate that GLT1 upregulation through ceftriaxone injection rescued the cerebellum phenotype of DMSXL mice.

Bergmann-specific RNA foci accumulation and GLT1 downregulation in human DM1 cerebellum

To assess the implications of our findings to the human condition we have first investigated the regional distribution of the molecular hallmark of signs of CUG RNA toxicity in human cerebellum. Like in DMSXL mice, RNA foci were particularly abundant in Bergmann cells, where they co-localised with MBNL1 and MBNL2 proteins (Figure 6.A). Rare RNA foci accumulated in Purkinje cells but were not associated with pronounced sequestration of MBNL proteins: diffuse MBNL1 and MBNL2 staining was detected in the nucleus and cytoplasm of those rare DMSXL and DM1 Purkinje cells exhibiting nuclear foci. The enrichment of RNA foci in a confined cell population was associated with splicing defects in human cerebellum tissue (Figure 6.B). We then investigated whether the abnormalities of GLT1 found in DMSXL mice were relevant for the human condition. To this end we quantified GLT1 protein in *post-mortem* human tissue by western blot. Despite some inter-individual variability, GLT1 was dramatically reduced in five out of seven adult DM1 patients. Overall, GLT1 steady-state levels were significantly reduced by ~50% in DM1 subjects, relative to non-DM controls (Figure 6.C). The effect was not limited to the cerebellum and GLT1 was also decreased in the frontal cortex and brainstem of adult DM1 individuals

Discussion

The molecular bases of the neurological manifestations of DM1 are not fully understood. It was our goal to investigate the involvement of different brain regions, cell types and molecular pathways in DM1 neuropathology. Through the analysis of a transgenic

mouse model of the disease we have collected compelling evidence of cerebellum dysfunction, characterised by deficits in fine motor coordination and Purkinje neuronal activity. The downregulation of a glial glutamate membrane transporter plays a determinant role in the mediation of these phenotypes.

5 DMSXL mice showed impaired cerebellum-dependent motor coordination, performing poorly in the runway test, which is frequently used to evaluate motor coordination in mouse models of cerebellar disease. Although DMSXL mice exhibit reduced body weight and muscular deficits (Huguet *et al.*, 2012), these features have minimal impact on their performance in the runway test. In fact, the smaller size and lighter body mass of DMSXL
10 mice may facilitate their progress along an elevated runway. We confirmed cerebellum dysfunction through electrophysiological recordings of DMSXL neuronal activity. *In vivo* analysis of the spontaneous firing rate and rhythmicity constitutes an efficient way of assessing the functional states of the cerebellar neuronal network. Spontaneous simple spike firing reflects the integrated intrinsic excitability of Purkinje cells, resulting from the
15 combined inhibition by the molecular interneurons, and excitation by the parallel and climbing fibres (Sato *et al.*, 1992; Schwarz & Welsh, 2001; Servais & Cheron, 2005; Cheron *et al.*, 2008). Together, the increased simple spike firing rate, rhythmicity and power peak of spontaneous LFP oscillations in DMSXL cerebellum demonstrates hyper-excitability in response to the DM1 repeat expansion. The complementary electrophysiological profile of
20 acute cerebellar slices confirmed increased spontaneous activity of DMSXL Purkinje cells.

Consistent with a *trans*-dominant effect of toxic CUG RNA repeats, we found disease-associated nuclear ribonuclear aggregates in both DMSXL and human DM1 cerebellum, in association with mild overall spliceopathy.

Interestingly RNA foci distribution was not homogenous throughout the cerebellum.
25 In spite of their functional abnormalities, DMSXL Purkinje cells did not display abundant RNA foci, suggesting that the abnormal neuronal hyperactivity is not directly mediated by the accumulation of RNA aggregates in this cell type. RNA foci were, however, particularly abundant in the neighbouring Bergmann cells, co-localising with MBNL1 and MBNL2. RNA foci accumulation does not seem to affect MBNL1 and MBNL2 localization in Purkinje cells.
30 Our data corroborate previous reports of RNA foci in human DM1 Purkinje cells without MBNL1 co-localisation (Daughters *et al.*, 2009). MBNL proteins likely remain functional in foci-positive Purkinje cells, hence explaining why MBNL1-dependent splicing events did not show dysregulation in laser microdissected DMSXL Purkinje neurons. It is conceivable that *DMPK* transcript levels in Purkinje cells are insufficient to trigger mechanisms of RNA

toxicity in this cell type. Alternatively, Purkinje cells may express MBNL protein isoforms with reduced affinity to CUG repeats. Finally, Purkinje cells may express other proteins that compete with MBNL to bind CUG RNA repeats, thereby maintaining MBNL proteins soluble and functional in the cell.

5 The higher levels of toxic CUG-containing *DMPK* transcript levels and nuclear RNA foci and the more severe splicing dysregulation in Bergmann glia, indicates greater susceptibility of this cell type to disease pathogenesis and hints at glial dysfunction in the cerebellum. Bergmann glia is a population of astrocytes that contribute to synaptogenesis, synaptic transmission, and plasticity as well as to metabolite supply to neurons,
10 osmoregulation and neuroprotection (Bellamy, 2006; Wang *et al.*, 2012). Bergmann cell dysfunction in DM1 may therefore impair Purkinje cell activity.

 GLT1 is a glial glutamate transporter, expressed in multiple regions of the CNS. This transporter is responsible for the removal of the excitatory neurotransmitter glutamate from the synaptic cleft, thus playing a crucial role in protecting post-synaptic neurons against
15 glutamate excitotoxicity, neurodysfunction and ultimately neurodegeneration (O'Shea, 2002; Kanai & Hediger, 2004; Herman & Jahr, 2007). Nevertheless, no signs of neurodegeneration were detected in DMSXL cerebellum, suggesting that, although insufficient to cause cell death, DM1-associated GLT1 downregulation may increase the levels of extracellular glutamate, possibly leading to neuronal dysfunction. This hypothesis is corroborated by the
20 increased susceptibility of DMSXL to seizures induced by PTZ, an epileptogenic drug (Charizanis *et al.*, 2012).

 GLT1 upregulation in DMSXL mice abolished the hyperactivity of Purkinje cells, and corrected motor discoordination in a cerebellum-dependent task. These results demonstrate that GLT1 downregulation plays a determinant role in DMSXL cerebellum dysfunction, and
25 that GLT1 manipulation may provide novel therapeutic strategies. A five-day injection scheme was sufficient to ameliorate both electrophysiological and behavioural phenotypes, anticipating the benefits of therapeutic strategies grounded on the administration of ceftriaxone or analogue compounds.

 In cultured cells, MBNL1 inactivation decreased *GLT1* transcripts, possibly through
30 altered mRNA stability or polyadenylation. Interestingly, MBNL2 did not affect *GLT1* mRNA levels, possibly due to the simultaneous and compensating increase of MBNL1 in culture. Similarly, the milder efficiency of the double knock down did not affect GLT1 levels. It is conceivable that MBNL1 (but not MBNL2) specifically regulates GLT1 expression in

glial cells. Chemicals that prevent the sequestration of MBNL1 proteins into RNA foci may be of therapeutic use to avoid GLT1 downregulation.

GLT1 misregulation and accumulation of extracellular glutamate has been implicated in the development of several neurodegenerative diseases including Alzheimer's disease, Huntington disease and amyotrophic lateral sclerosis (Kim *et al.*, 2011). Ceftriaxone is a well-tolerated β -lactam antibiotic used for the treatment of a number of bacterial infections. It ameliorates the phenotype of Huntington disease transgenic mice, through the upregulation of GLT1 promoter activity (Miller *et al.*, 2008). The antibiotic is currently in clinical trial for amyotrophic lateral sclerosis (Berry *et al.*, 2013). Since in DM1 GLT1 downregulation is not limited to the cerebellum, but it extends to other brain areas, therapeutic strategies to modulate GLT1 levels and glutamate uptake may provide general benefits to DM1 neuropathology, beyond cerebellar dysfunction.

Other FDA-approved compounds, such as other β -lactams and their derivatives (Rothstein *et al.*, 2005), raloxifene (Karki *et al.*, 2014), and riluzole (Carbone *et al.*, 2012) can activate GLT1 transcription to different extents and are suitable for chronic administration. There are already data on the pharmacokinetics, pharmacodynamics and toxicology of these compounds. More recently, LDN/OSU-0212320 was found to activate GLT1 with higher efficacy than ceftriaxone, through increased translation (Kong *et al.*, 2014). However, we do not know the efficiency of these compounds to achieve sustained GLT1 upregulation in cells expressing toxic CUG RNA, nor do we know the consequences of their chronic administration in DM1.

The cerebellum is a complex brain region, traditionally implicated in motor coordination and balance. It is primarily involved in the control of skilled voluntary movements, as well as in the control of motor tone, posture and gait. Cerebellum dysfunction is usually associated with ataxia, lack of coordination or tremor. Although gait and coordination problems are not typical DM1 features, lack of equilibrium and sudden falls are a frequent complain. It remains possible that other subtle sub-clinical motor manifestations, such as mild episodes of loss of balance are masked by more prominent symptoms (*e.g.* muscle weakness and myotonia).

The cerebellum may also be involved in DM1 neuropathology through its non-motor functions. Cerebellar lesions have often resulted in a pattern of cognitive and behaviour abnormalities, such as executive dysfunction, blunting or flattening of affect, constrictions in social interaction and impaired spatial cognition (Schmahmann, 1998; Schmahmann & Sherman, 1998), which resemble to a certain extent those described in DM1 patients. Our

results are in line with a mediating role of cerebellum dysfunction in the onset of some of these neuropsychological manifestations, mediated by impaired of Bergmann glia function, glutamate transport and defective Bergmann/Purkinje cell communication.

In summary, our data provide new insight into the DM1 mechanism in the brain, demonstrating how CTG-associated glial molecular abnormalities impact neuronal activity through neuroglial miscommunication. Our results strongly suggest an unexpected role of cerebellum in DM1 neurobiology, a brain region that has been overlooked in this condition.

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A method of treating of the neuropathology of a patient suffering from myotonic dystrophy type 1 (DM1) comprising administering to the patient a therapeutically effective amount of an agent that normalizes, enhances, or potentiates glutamate uptake by glia.
2. The method of claim 1 wherein the agent is a beta-lactam compound.
3. The method of claim 2 wherein the beta-lactam compound is selected from the group consisting of benzylpenicillin, procaine benzylpenicillin, penicillin V, penicillin V potassium, benzathine penicillin, hetacillin, cloxacillin, carbenicillin, flucloxacillin, ampicillin, ampicillin sodium, amoxicillin, co-amoxiclav, carboxypenicillin, ticarcillin, timentin, tazocin, piperacillin, pivmecillinam, amoxicillin-clavulanate, oxacillin, bacampicillin HCl, nafcillin sodium, cefaclor, cefadroxil, cefadyl, cefalexin, cefamandole, cefazolin, cefditoren, cefepime, cefetamet, cefdinir, cefixime, cefizox, cefotaxime, cefmetazole, cefobid, cefonicid, cefoperazone, cefoperazone sodium, cefotan, cefotetan, cefoxitin, cefpirome, cefpodoxime, cefpodoxime proxetil, cefprozil, cefradine, ceftazidime, ceftibuten, ceftidoren, ceftin, ceftizoxime, ceftriaxone, ceftriaxone sodium, cefuroxime, cefuroxime axetil, cephalixin, cefzil, cephalothin, cephalothin sodium, cephalirin sodium, aztreonam, imipenem, meropenem, ertapenem and FK-037.
4. The method of claim 2 wherein the beta lactam compound is selected from the group consisting of:

(3S, 4R)-3-((R)- (1-hydroxy-ethyl)-4-((R)-[1-methyl-2-(4-methyl-piperazin-1-yl)-2-oxo-ethyl]-azetidin-2-one;

tert-butyl 4-((R)-2-((2R,3S)-3-((R)- 1 -hydroxyethyl)-4-oxoazetidin-2-yl)propanoyl)piperazine- 1 -carboxylate;

(3S, 4R)-3-((R)- (1-Hydroxy-ethyl)-4-((R)- (1-methyl-2-oxo-2-piperazin-1-yl-ethyl)-azetidin-2-one;

(3S, 4R)-4-((R)- (1 -(4-acetylpiperazin- 1 -yl)- 1 -oxopropan-2-yl)-3-((R) (1 -hydroxyethyl)azetidin-2-one;

- (3 S,4R)-4-((R)- 1 -(4-ethylpiperazin- 1 -yl)- 1 -oxopropan-2-yl)-3-((R)- 1 -hydroxyethyl)azetidin-2-one;
- (3 S,4R)-3-((R)- 1 -hydroxyethyl)-4-((R)- 1 -(4-(methylsulfonyl) piperazin- 1 -yl)- 1 -oxopropan- 2-yl)azetidin-2-one;
- 5 (3 S,4R)-4-((R)- 1 -(4-cyclohexylpiperazin- 1 -yl)- 1 -oxopropan-2-yl)-3-((R)- 1 -hydroxyethyl)azetidin-2-one;
- (3 S,4R)-4-((R)- 1 -(4-benzoylpiperazin- 1 -yl)- 1 -oxopropan-2-yl)-3-((R)- 1 -hydroxyethyl)azetidin-2-one;
- (3 S,4R)-3-((R)- 1 -hydroxyethyl)-4-((R)- 1 -oxo- 1 -(4-phenyl piperazin- 1 -yl)propan-2- yl)azetidin-2-one;
- 10 (3 S,4R)-3-((R)- 1 -hydroxyethyl)-4-((R)- 1 -oxo- 1 -(4-propyl piperazin- 1 -yl)propan-2- yl)azetidin-2-one;
- (3 S,4R)-3-((R)- 1 -hydroxyethyl)-4-((R)- 1 -(4-(4-methoxyphenyl) piperazin- 1 -yl)- 1 -oxopropan-2-yl)azetidin-2-one;
- (3 S,4R)-4-((R)- 1 -(4-(tert-butyl)piperazin- 1 -yl)- 1 -oxopropan-2-yl)-3-((R)- 1 -hydroxyethyl)azetidin-2-one;
- 15 4-((R)-2-((2R,3 S)-3-((R)- 1 -hydroxy ethyl)-4-oxoazetidin-2-yl)propanoyl)piperazine- 1 - carboxamide;
- (3 S,4R)-3-((R)- 1 -hydroxyethyl)-4-((R)- 1 -(4-methyl-3,4-dihydro quinoxalin- 1 (2H)-yl)- 1 -oxopropan-2-yl)azetidin-2-one;
- 20 (R)- 1 -((2R,3 S)-2-((R)- 1 -(4-methylpiperazin- 1 -yl)- 1 -oxopropan-2-yl)-4-oxoazetidin-3- yl)ethyl acetate;
- (R)- 1 -((2R,3 S)-2-((R)- 1 -(4-methylpiperazin- 1 -yl)- 1 -oxopropan-2-yl)-4-oxoazetidin-3- yl)ethyl butyrate;
- 25 (R)- 1 -((2R,3 S)-2-((R)- 1 -(4-methylpiperazin- 1 -yl)- 1 -oxopropan-2-yl)-4-oxoazetidin-3- yl)ethyl isobutyrate;

(R)- 1 -((2R,3 S)-2-((R)- 1 -(4-methylpiperazin- 1 -yl)- 1 -oxopropan-2-yl)-4-oxoazetidin-3- yl)ethyl pivalate;

or a pharmaceutically acceptable form thereof.

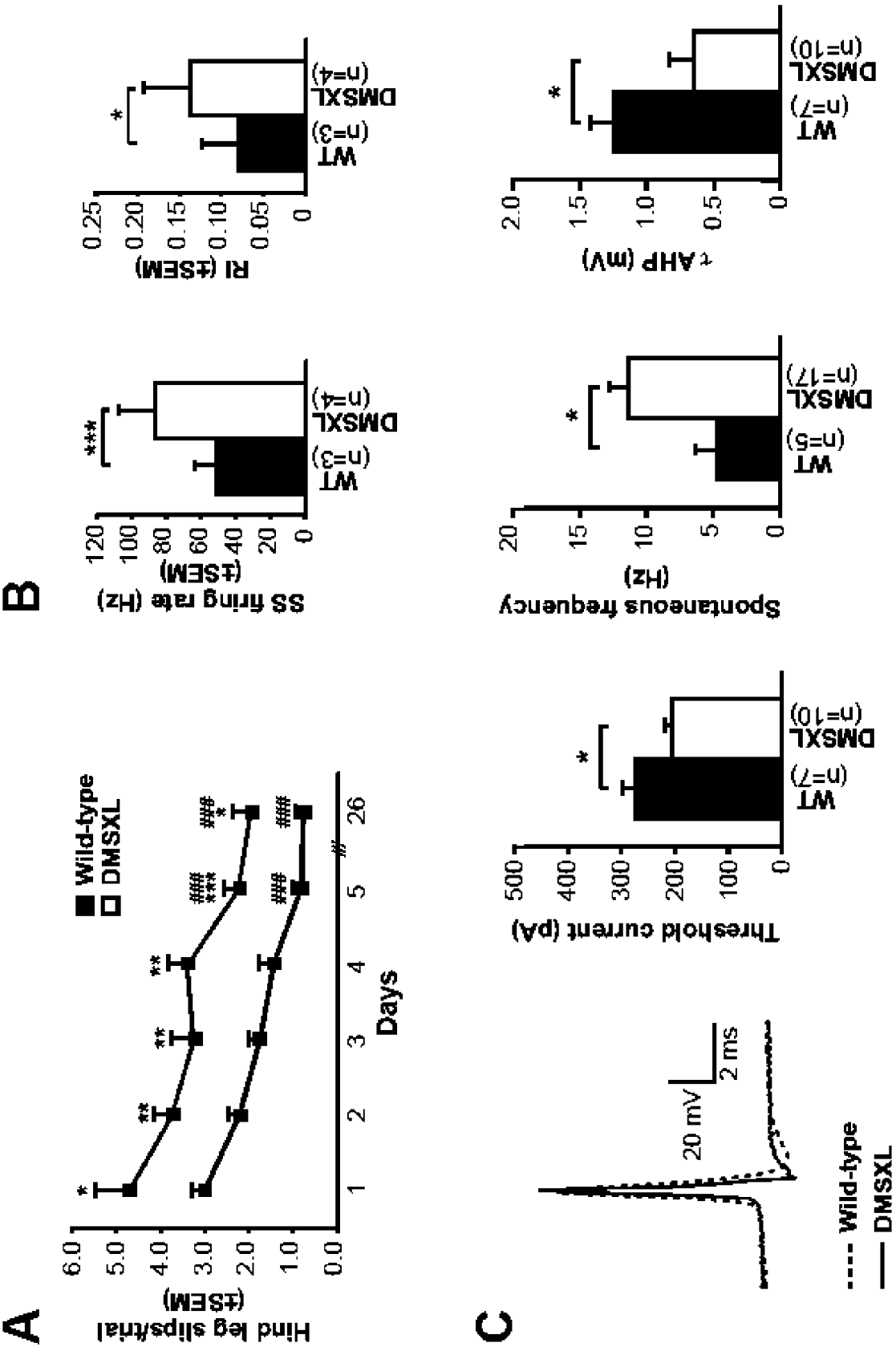


Figure 1

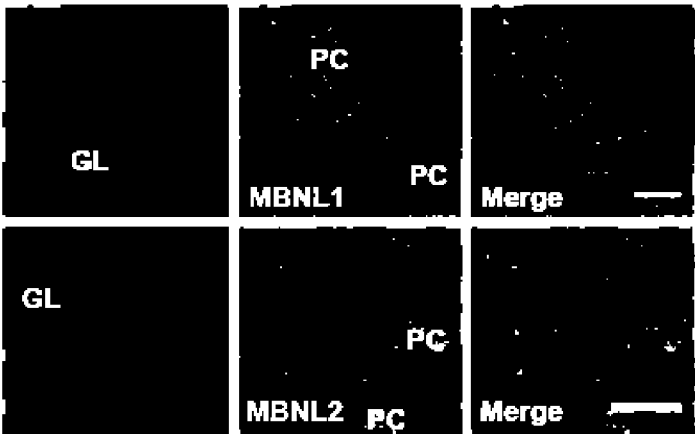


Figure 2A

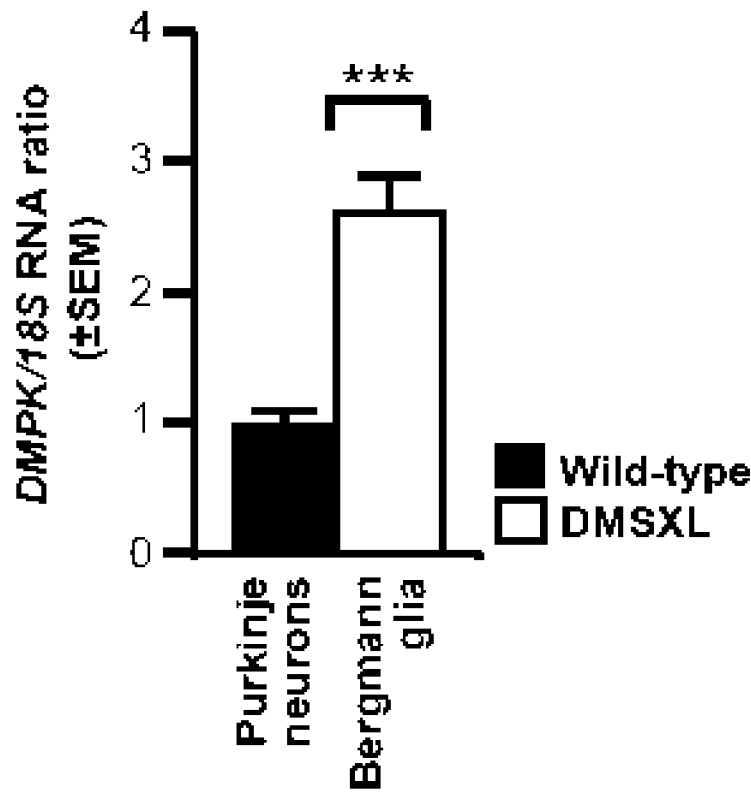


Figure 2B

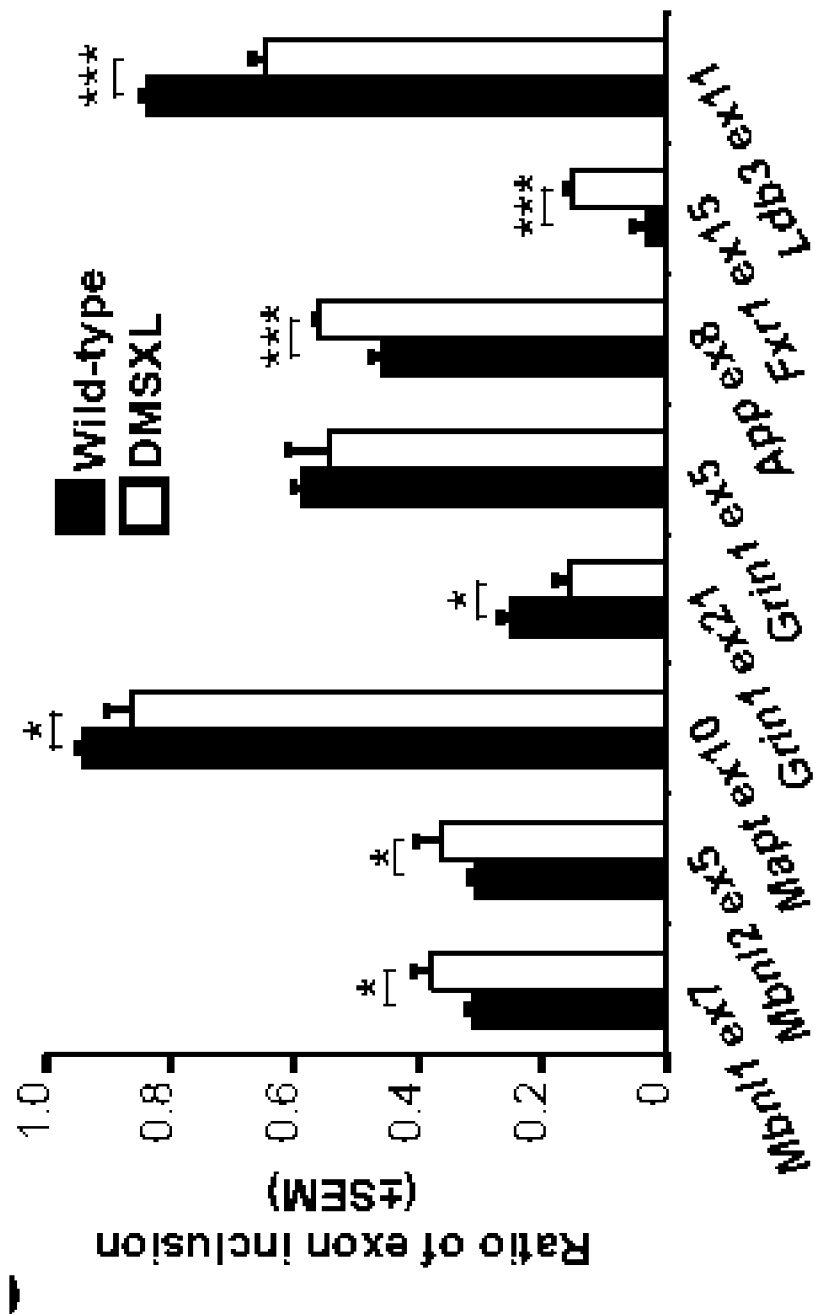


Figure 2C

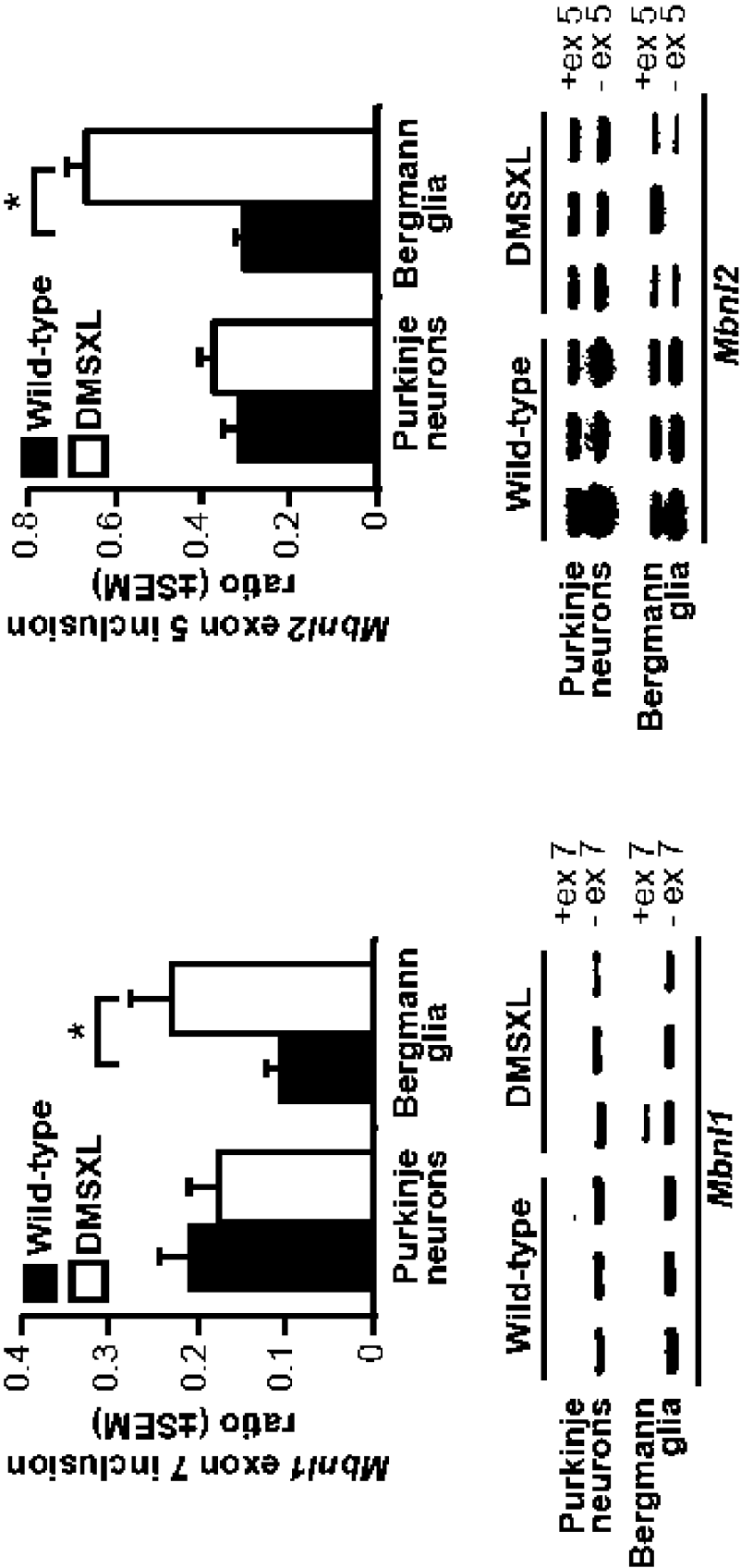


Figure 2D

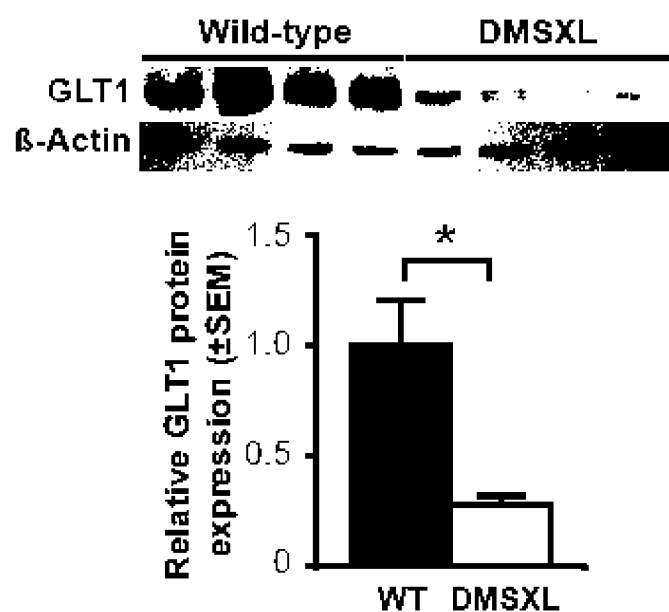
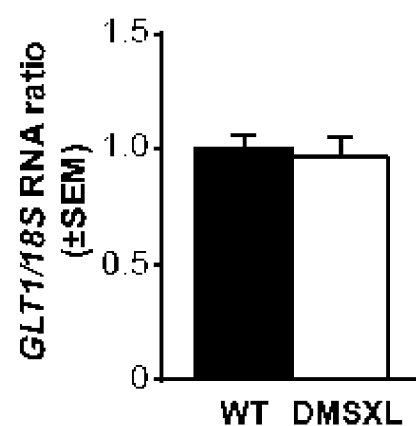
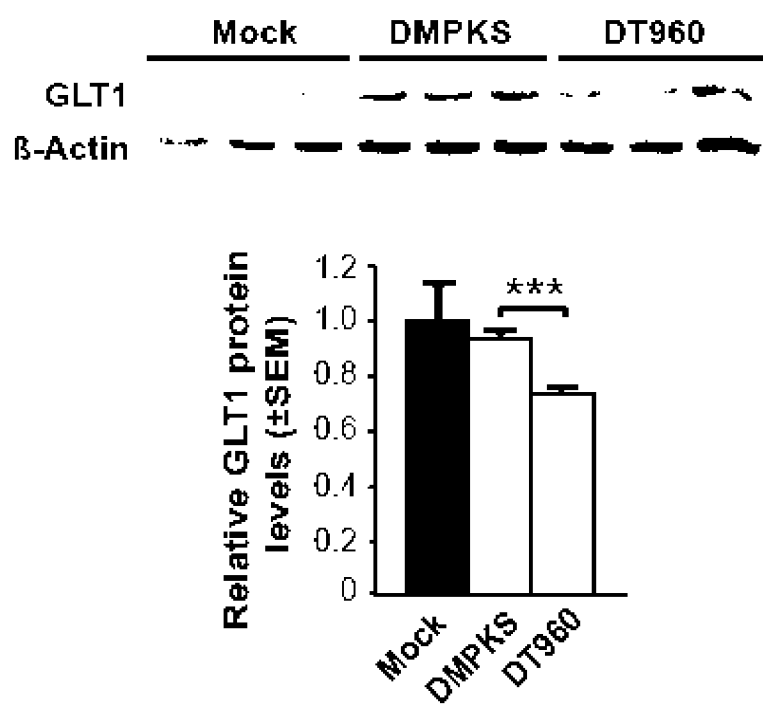
A**B****C**

Figure 3

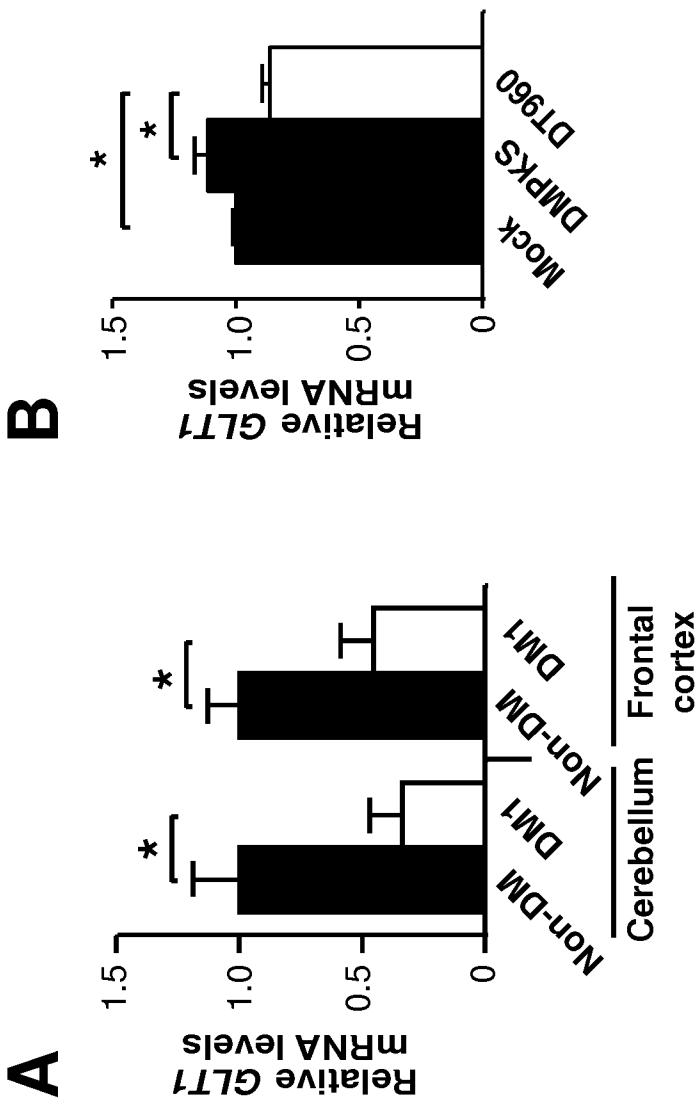


Figure 4A and 4B

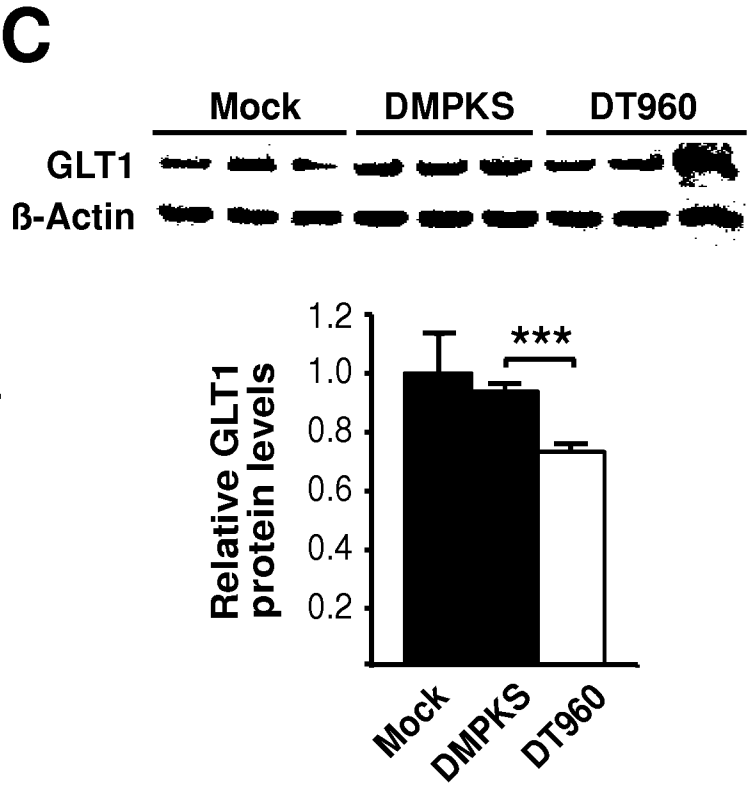


Figure 4C

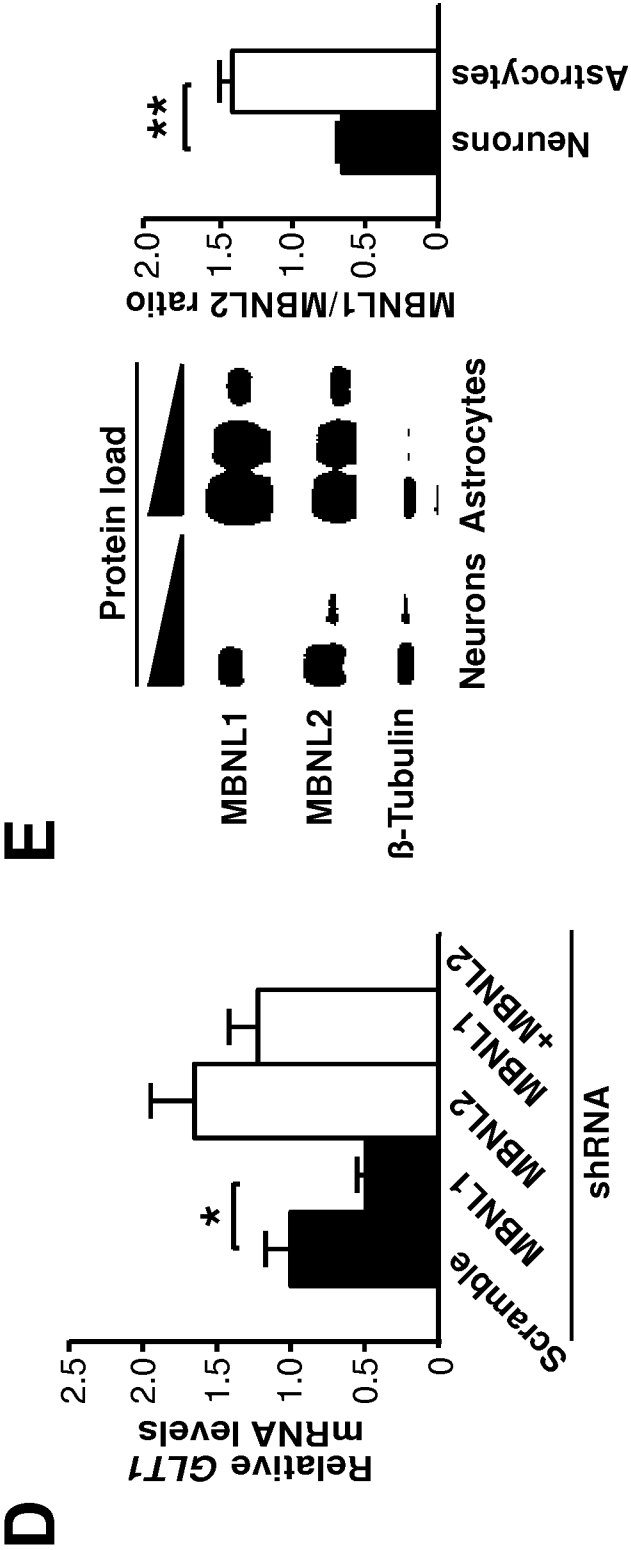


Figure 4D and 4E

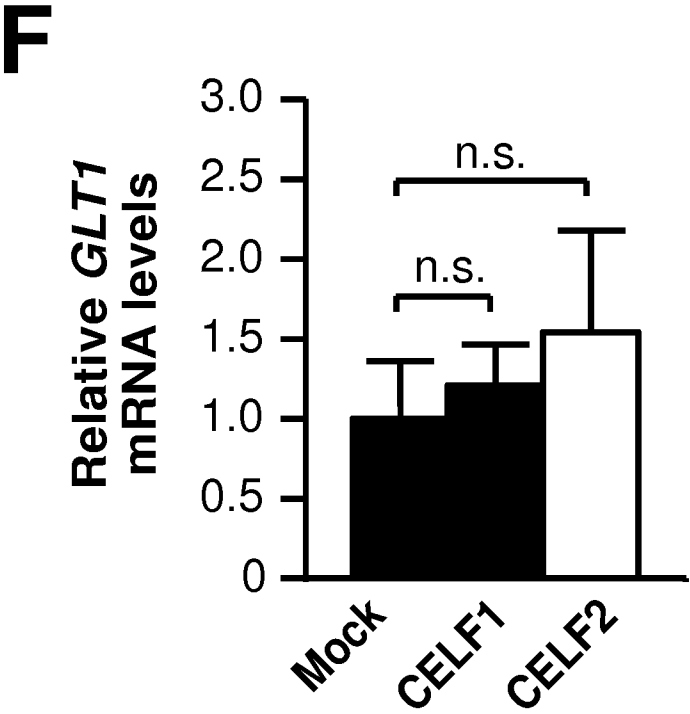


Figure 4F

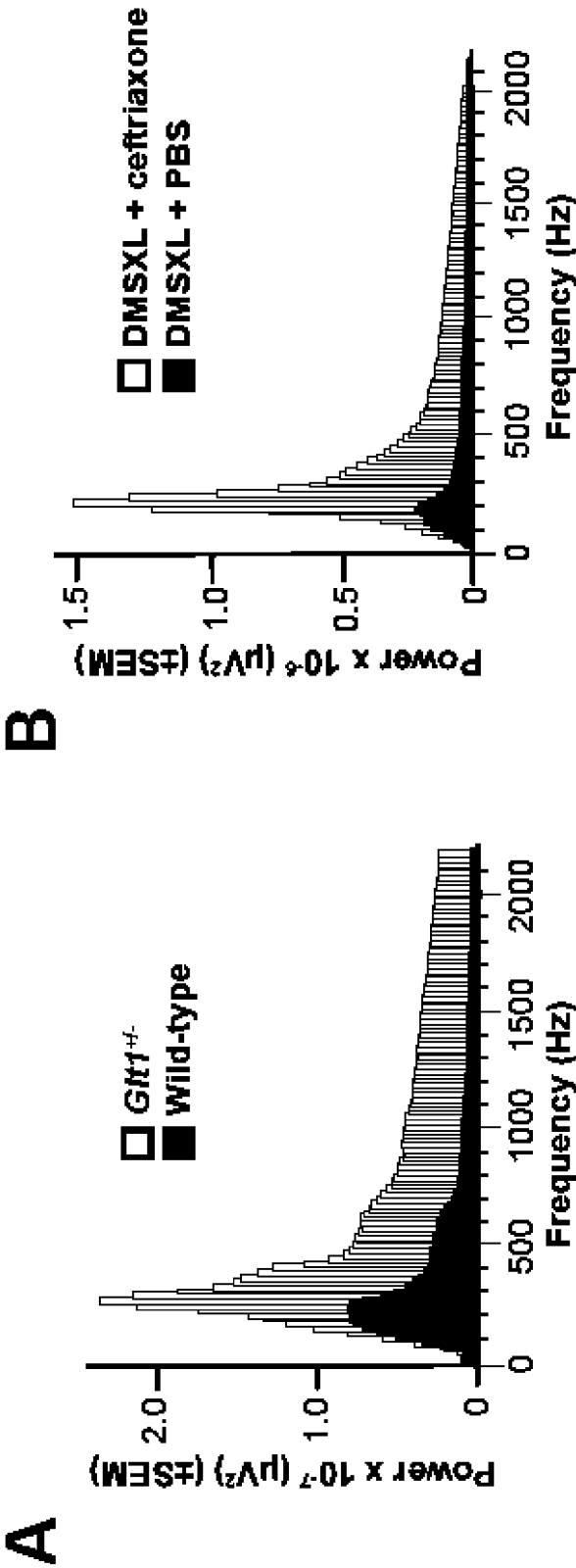


Figure 5A and 5B

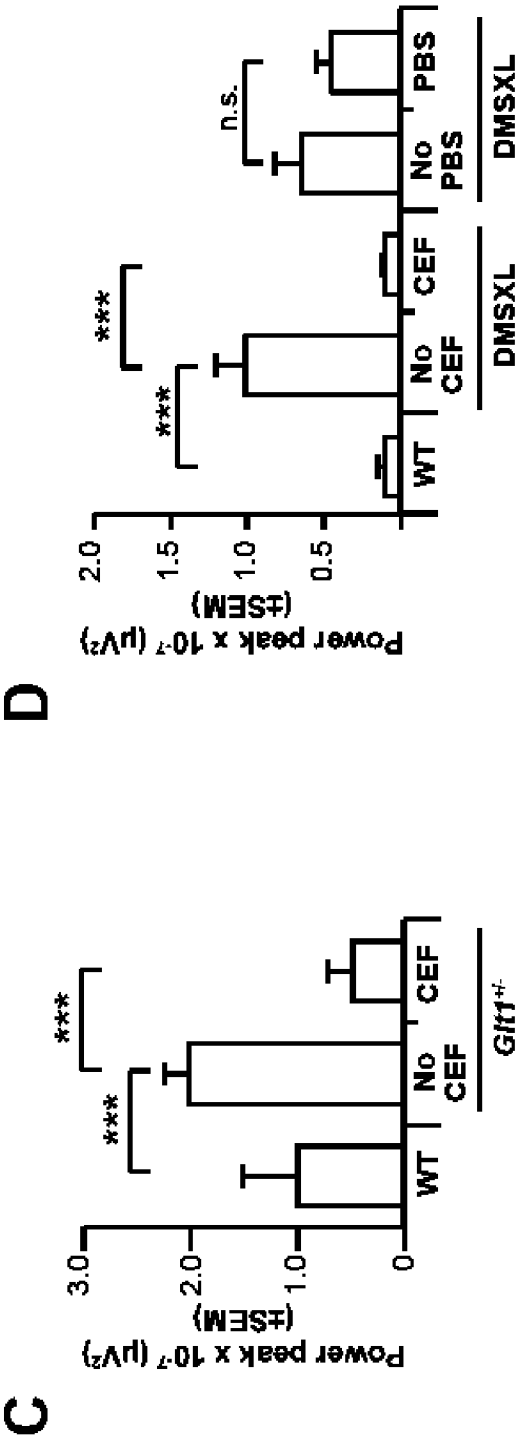


Figure 5C and 5D

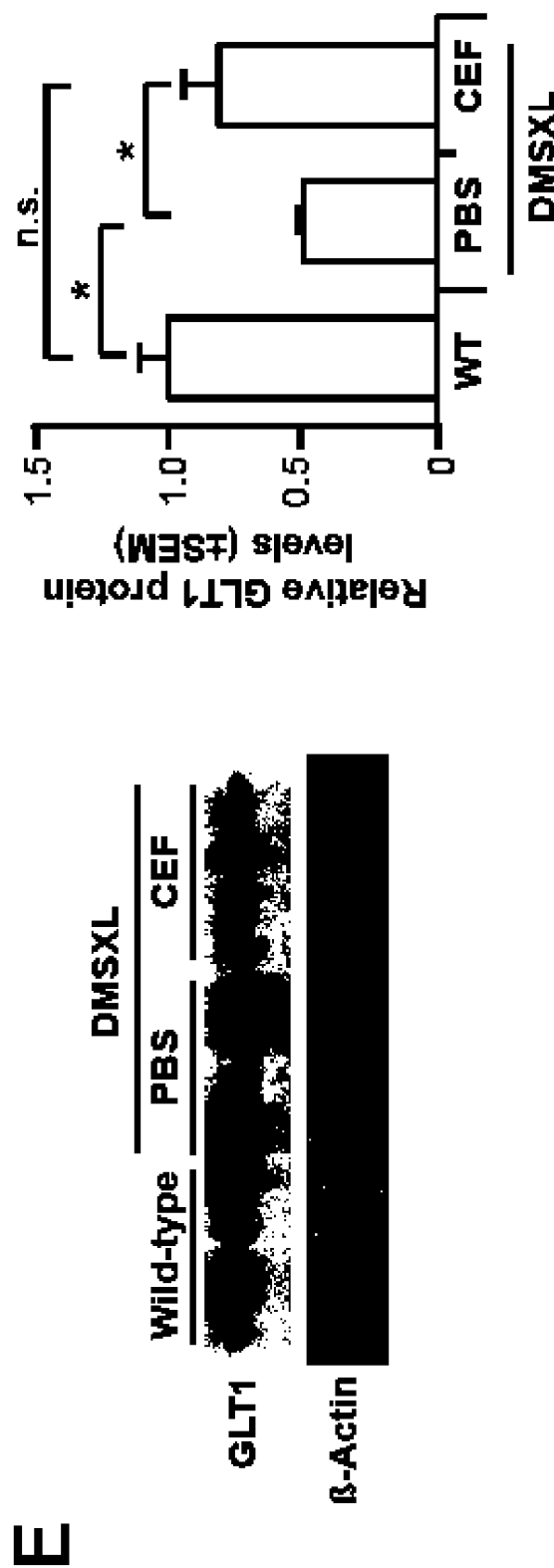


Figure 5E

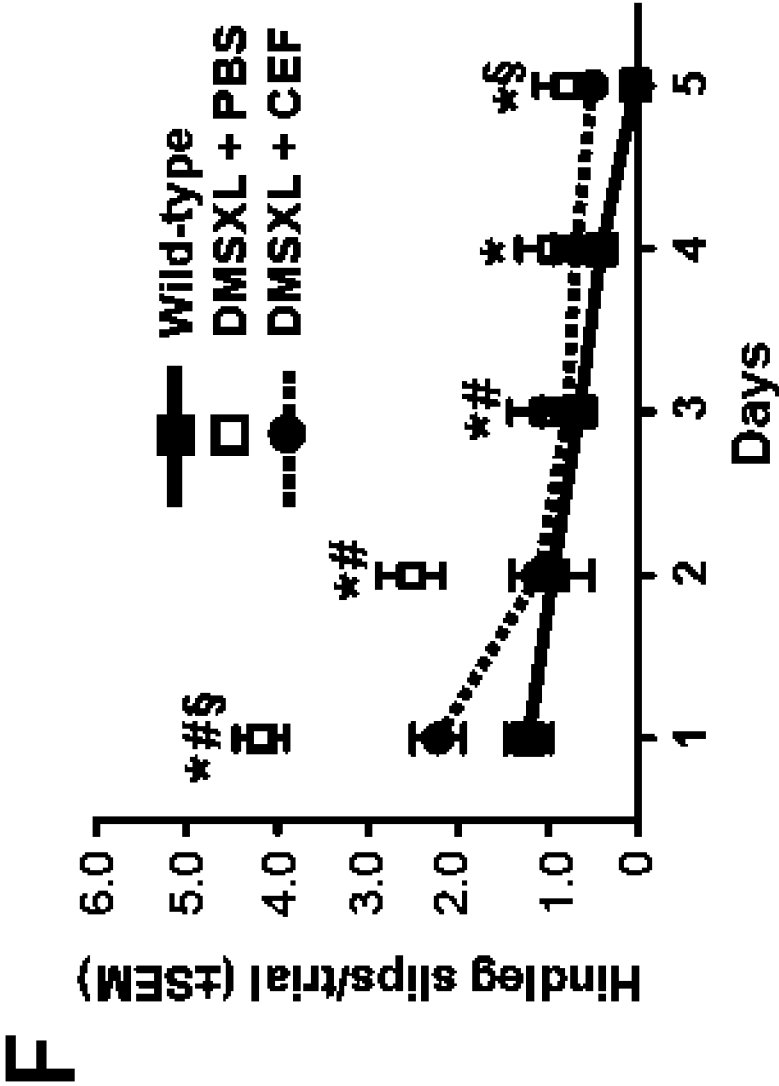


Figure 5F

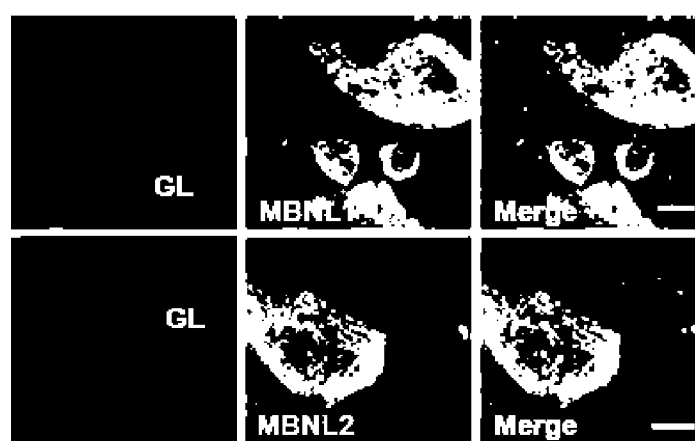


Figure 6A

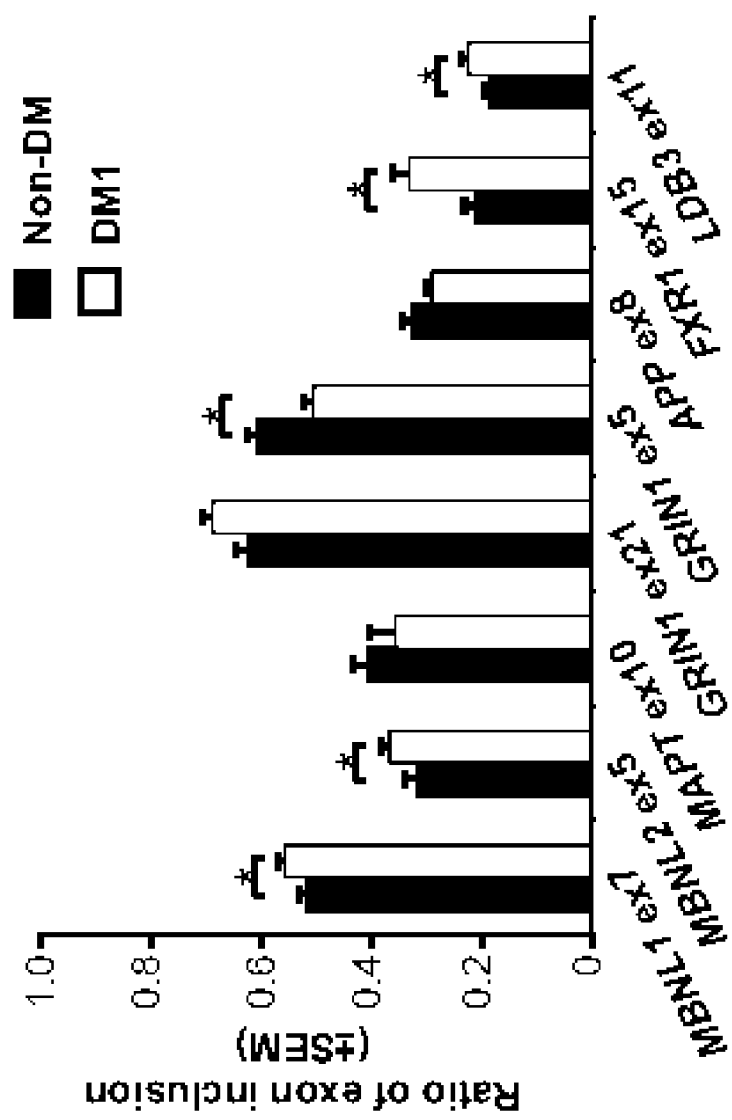


Figure 6B

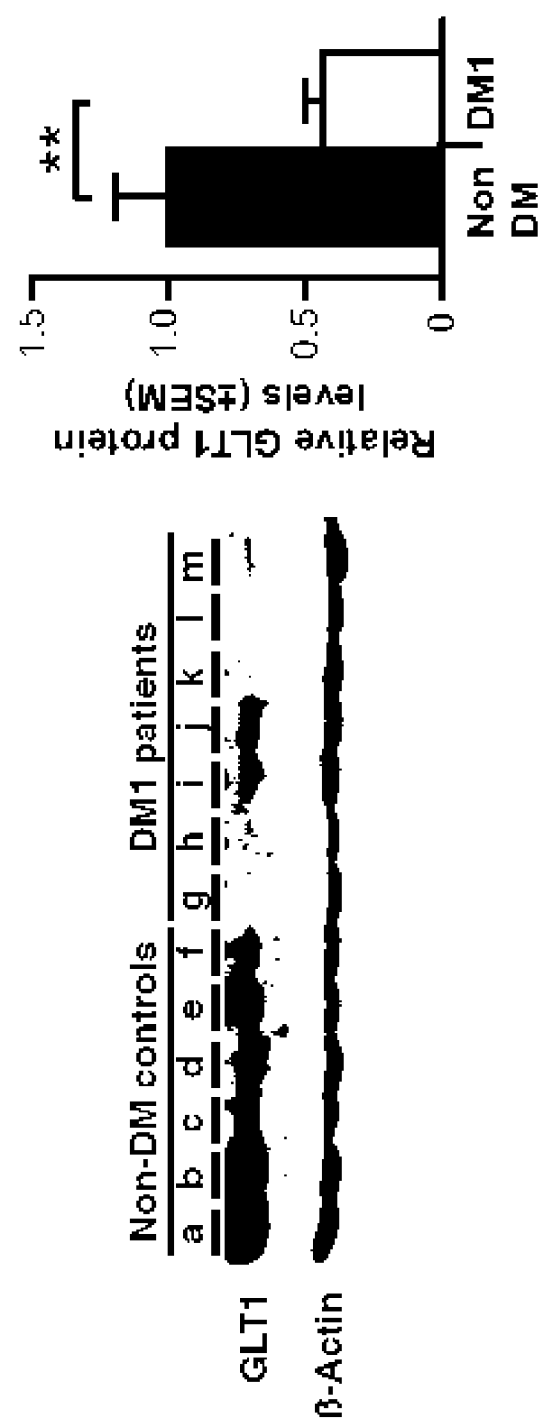


Figure 6C

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/061134

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K31/43 A61K31/496 A61K31/498 A61K31/546 A61P25/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. HOFER ET AL: "Recurrent Erysipelas in Myotonic Dystrophy Type 1 with IgG Deficiency", EUROPEAN NEUROLOGY., vol. 57, no. 4, 1 January 2007 (2007-01-01), pages 236-237, XP055223612, Switzerland ISSN: 0014-3022, DOI: 10.1159/000101288	1-3
Y	the whole document	1-4
X	WO 2010/067078 A2 (WISTA LAB LTD [SG]; CLUNAS SCOTT [GB]; STORRY JOHN MERVYN DAVID [GB];) 17 June 2010 (2010-06-17) claims 51, 60 ----- -/-	1



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

17 June 2016

Date of mailing of the international search report

27/06/2016

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Ansaldo, M

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/061134

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2014/197536 A2 (UNIV TEMPLE [US]) 11 December 2014 (2014-12-11) cited in the application the whole document	1,2,4
Y	----- WO 2006/108055 A1 (UNIV YALE [US]; CORIC VLADIMIR [US]; KRYSTAL JOHN H [US]; SANACORA GER) 12 October 2006 (2006-10-12) claims 1, 6, 10 -----	1-3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/061134

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010067078 A2	17-06-2010	AU 2009326173 A1	17-06-2010
		BR PI0922326 A2	05-01-2016
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		EP 2201963 A1	30-06-2010
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		WO 2006108055 A1	12-10-2006