Neurobiological correlates of behavioural alterations in mouse models of MBNL2 deficiency and pharmacological interventions: relevance for myotonic dystrophy type 1

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A totes les persones que m'han acompanyat al llarg d'aquest camí…

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sense ells no hauria arribat fins aquí

“Nobody said it was easy
No one ever said it would be this hard”

ColdPlay – The scientist

“Tot bé, tooot béééé”

Jeli Bossman – Mi mosquetero fiel

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Abstract

Myotonic dystrophy type 1 (DM1) is a rare disease characterized by muscular defects, as well as, cognitive and mood alterations. Although these neurophysiological disturbances are very debilitating, there is lack of data on the neurobiological mechanisms involved, and no specific treatment is available. Here, we investigated the neurobiological correlates underlying MBNL2 loss-of-function using two transgenic mouse lines: (i) constitutive *Mbnl2* knockout (KO) mice and (ii) tissue-specific KO mice, where *Mbnl2* expression is selectively deleted in the glutamatergic neurons of the forebrain. We demonstrated that both models exhibit long-term memory recognition deficits and a depressive-like state associated with increased microglia and altered neurochemical levels. Moreover, chronic treatments with the psychostimulant methylphenidate and the atypical antidepressant mirtazapine attenuated these behavioural alterations through a reduction of pro-inflammatory microglial overexpression and restoring the levels of several neurotransmitters and its receptors. Together, these data reveal new insights into the neurobiology of DM1 and provide evidence that methylphenidate and mirtazapine could be novel potential candidates to alleviate the debilitating symptoms related to the central nervous system in patients with DM1.

Resumen

La distrofia miotónica tipo 1 (DM1) es una enfermedad rara caracterizada por defectos musculares, así como por alteraciones cognitivas y afectivas. Aunque estas alteraciones neurofisiológicas son muy debilitantes, faltan datos sobre los mecanismos neurobiológicos involucrados, y no hay un tratamiento específico disponible. Aquí, investigamos los correlatos neurobiológicos subyacentes a la pérdida de función de MBNL2 utilizando dos líneas de ratones transgénicos: (i) ratones knockout (KO) constitutivos del gen *Mbnl2* y (ii) ratones KO condicionales con una deleción específia del gen *Mbnl2* en las neuronas glutamatérgicas del cerebro anterior. Los resultados demostraron que ambos modelos exhiben déficits de reconocimiento de memoria a largo plazo y un estado depresivo, asociado con un aumento de la microglia y niveles neuroquímicos alterados. Además, los tratamientos crónicos con el psicoestimulante metilfenidato y el antidepresivo atípico mirtazapina, atenuaron estas alteraciones del comportamiento mediante una reducción de la sobreexpresión microglial proinflamatoria y el restablecimiento de los niveles de varios neurotransmisores y sus receptores. Juntos, estos datos aportan nuevos conocimientos sobre la neurobiología de DM1 y proporcionan evidencia de que el metilfenidato y la mirtazapina podrían ser nuevos candidatos potenciales para aliviar los síntomas debilitantes relacionados con el sistema nervioso central en pacientes con DM1.

Abbreviations

5-HT	Serotonin
AAV	Adeno-associated virus
ADHD	Attention deficit hyperactivity disorder
ASOs	Antisense oligonucleotides
BDNF	Brain-derived neurotrophic factor
CDM1	Congenital myotonic dystrophy 1
CELF	CUG-Elav like family
Cho	Choline
CNS	Central nervous system
Cr	Creatine
CTG	Cytosine-thymine-guanine
CTG^{exp}	CTG expanded
CUGBP	CUG binding protein
CUG^{exp}	CUG expanded
DA	Dopamine
DAT	Dopamine transporter
DBH	Dopamine beta-hydroxylase
DG	Dentate gyrus
DM	Dystrophia myotonica
DM1	Myotonic dystrophy type 1
DM2	Myotonic dystrophy type 2
DMPK	Myotonic dystrophy protein kinase
EDS	Excessive daytime sleepiness
EPM	Elevated plus maze

EZM	Elevated zero maze
FDA	Food and Drug Adminsitration
fMRI	Functional magnetic resonance imaging
FST	Forced swim test
GABA	Gamma aminobutyric acid
GFAP	Glial fibrillary acidic protein
Gln	Glutamine
Glu	Glutamate
GSK3β	Glycogen synthase kinase-3 beta
HPC	Hippocampus
HSA	Human skeletal actin
Iba1	Ionized calcium binding adaptor molecule 1
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
iPS	Induced pluripotent stem cells
iRNA	RNA intereference
KO	Knockout
LTP	Long-term potentiation
MBNL	Muscleblind-like
MBNL1	Muscleblind-like protein 1
MBNL2	Muscleblind-like protein 2
MBNL3	Muscleblind-like protein 3
miRNA	micro RNA
mPFC	Medial prefrontal cortex
MRI	Magnetic resonance imaging
mRNA	Messenger RNA

MRS	Magnetic resonance spectroscopy
NA	Noradrenaline
NAA	N-acetylaspartate
NET	Noradrenaline transporter
NeuN	Neuronal nuclei
NFTs	Neurofibrillary tangles
NOR	Novel object recognition
NRF2	Nuclear factor erythroid 2-related factor 2
NSAIDs	non-steroidal anti-inflammatory drug
PKC	Protein kinase C
RBPs	RNA-binding proteins
REM	Rapid eye movement
RNAi	interference RNA
siRNA	small interference RNA
TNF-α	Tumor necrosis factor Alpha
TST	Tail suspension test
UTR	Untranslated region
WT	Wild-type
ZNF9	Zinc finger protein 9

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INTRODUCTION

1. Overview of Myotonic Dystrophy

Myotonic dystrophy (dystrophia myotonica, DM) is an autosomal dominant disorder with a progressive multisystemic phenotype. DM is primarily characterized by muscle weakness and myotonia, but also by an extra-muscular involvement including cardiac arrhythmias, insulin resistance and central nervous system (CNS) abnormalities. Based on the genetic alterations, DM is classified in two main forms: (i) myotonic dystrophy type 1 (DM1) also known as Steinert's disease, caused by increased CTG (cytosine-thymine-guanine) expanded (CTG^{exp}) repeats in the 3' untranslated region (UTR) of the *dystrophia myotonica protein kinase* (*DMPK*) gene on chromosome 19q13.3 (Brook et al., 1992) and (ii) myotonic dystrophy type 2 (DM2), a milder form, resulting from a CCTG intronic expansion in the *zinc finger protein 9* (*ZNF9*) gene, also known as *CNBP* on chromosome 3q21.3 (Figure 1) (Liquori et al., 2001). At the molecular level, when these genes are transcribed, pathogenic transcripts are formed (CUG^{exp} or $CCUG^{exp}$) which are retained in the nuclei of cells as ribonuclear aggregates, named foci. These aggregates in hairpin loop structures disrupt several RNA-binding proteins (RBPs) such as the muscleblind-like (MBNL) family, which normally regulate the alternative splicing transition from fetal to adult isoforms (Lee and Cooper, 2009).

Strong evidence supports that RNA toxicity plays a major role in disease pathology due to microsatellite expansions in noncoding regions that can be transcribed into pathogenic transcripts, modifying the bioavailability of splicing factors, altering the alternative splicing processes and enhancing fetal mRNA isoforms. Thus, dysfunctional or inappropriate protein expression patterns with deleterious effects on adult tissues contribute to the pathogenesis of DM1 (Sznajder and Swanson, 2019).

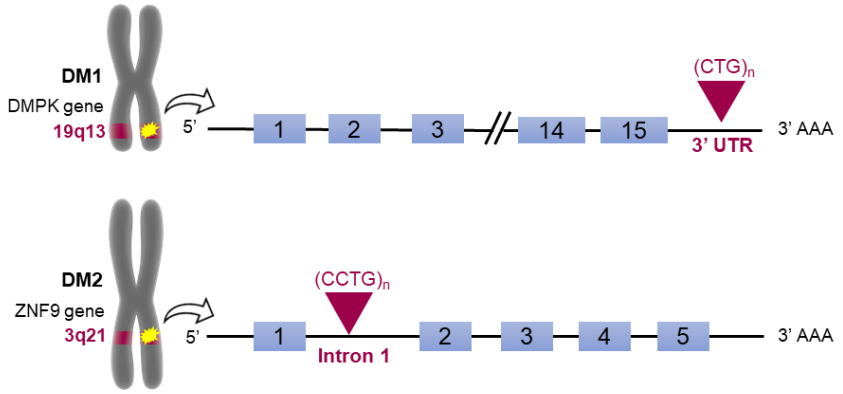

Figure 1. Genes involved in myotonic dystrophy type 1 (DM1) and type 2 (DM2). On the top, the location of CTG repeats (DM1) in the 3' untranslated region (UTR) of the *DMPK* gene. On the bottom, the location of CCTG repeats (DM2) in the intron 1 of the *ZNF9* gene. (Blue boxes represent exons).

DM1 symptoms often include myotonia, muscle weakness and wasting, along with cognitive deficits with impairments in attention, memory, visuospatial and executive functions (Rubinsztein et al., 1997; Meola and Sansone, 2007; Minnerop et al., 2011; Gallais et al., 2017). Moreover, longitudinal studies have reported many debilitating neuropsychological symptoms with excessive daytime sleepiness, cognitive decline, depression, anxiety and apathy (Thornton, 2014; Minier et al., 2018).

Although these data highlight the multitude of incapacitating symptoms in patients with DM1 (Laberge et al., 2009; Heatwole et al., 2012), to date there is no effective treatment for the muscular or the neurological alterations observed in DM1 (Ashizawa and Sarkar, 2011; Udd and Krahe, 2012; Thornton, 2014).

2. Genetics of Myotonic Dystrophy type 1 (DM1)

Despite being a rare disease, DM1 is the most common form of muscular dystrophy in adulthood with a worldwide prevalence of 1 in 8000 people (Harper et al., 2001; Lee and Cooper, 2009; Suominen et al., 2011). DM1 belongs to a group of disorders called microsatellite expansion disorders. Microsatellite repeats are short repeat sequences of 2-10 nucleotides with variable sizes among normal individuals and have important roles in genome evolution. So far, more than 40 neurological disorders are caused by microsatellite repeat expansions including Huntington disease, fragile X syndrome, DM1 and DM2, and amyotrophic lateral sclerosis (Pearson et al., 2005; Batra et al., 2010; La Spada and Ranum, 2010; Rohilla and Gagnon, 2017).

These microsatellite pathologies lead to defects in the alternative splicing of many primary transcript precursors or messenger RNA (mRNA). Alternative splicing is a regulated process by which functional mRNA can be generated through multiple isoforms of a single pre-mRNA. During this gene expression process, the introns are spliced out and exons can be merged in different mRNA variants generating many protein isoforms. During mammalian development, these splicing events are necessary for different biological processes and play a critical role in the remodelling of tissues in different developmental stages and throughout lifetime (Blencowe, 2006; Kalsotra and Cooper, 2011; Irimia and Blencowe, 2012). In DM1 tissues, the normal developmental splicing pattern is disrupted, resulting in the expression of fetal protein isoforms in adult tissues (Figure 2).

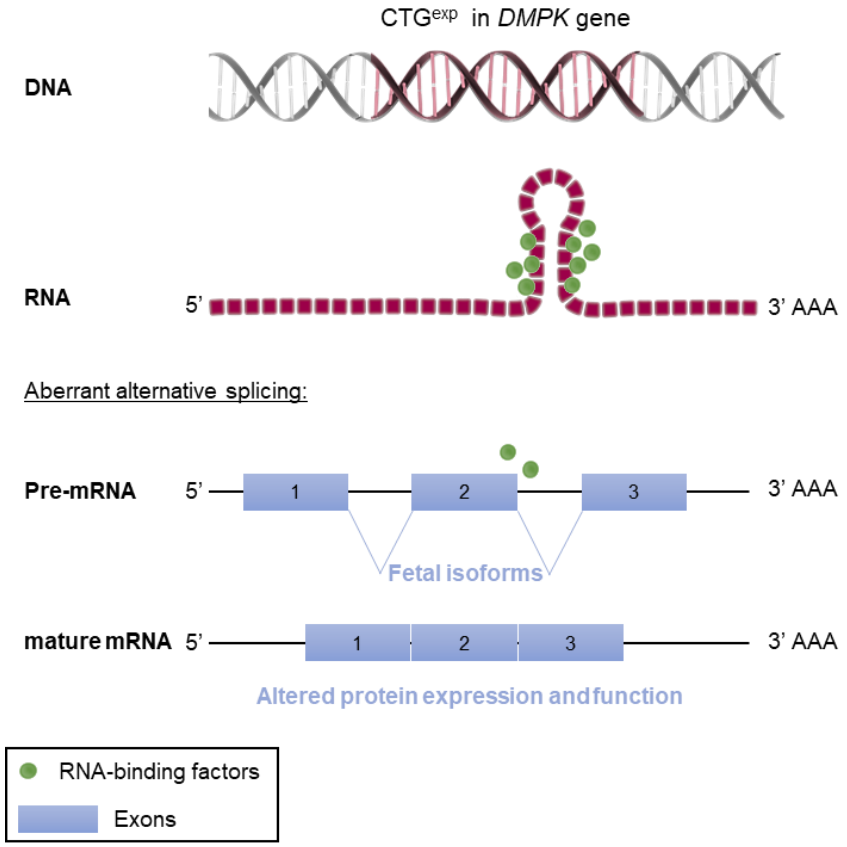

Figure 2. Schematic representation of spliceopathy in myotonic dystrophy type 1. Expanded CTG (CTG^{exp}) repeats in DNA leads to toxic RNA transcripts forming hairpin-like structures that sequester RNA-binding factors. This sequestration in nuclear foci prevents the normal processing of pre-mRNAs by the RNA-binding factors. They can not switch to an adult pattern resulting in aberrant alternative splicing inducing fetal-like splicing patterns (Adapted from Martí and Estivill, 2013).

2.1. Genotype-Phenotype correlation

In the general population, healthy people with normal DMPK alleles have 5 to 35 CTG^{exp} repeats. DM1 patients with the pre-mutation allele comprise ranges between 35 to 50 triplet repeats, which are asymptomatic but have a higher risk of having children with larger expanded repeats (Martorell et al., 2001). In DM1 patients, CTG^{exp} repeats greater than 50 have been associated with disease manifestations. Once in the range of disease, the clinical phenotype is highly variable, but in general, larger expanded repeats are mostly associated with an earlier age of onset and more severe symptoms (Harley et al., 1993; Logigian et al., 2004), while smaller pathological repeats are often correlated with a milder phenotype (Arsenault et al., 2006). Therefore, disease severity correlates with repeat size, and is inversely proportional to the age of onset of symptoms. (Figure 3). DM1 phenotypes can be classified as congenital, childhood- or juvenile-onset, adult- and late-onset (see section 3).

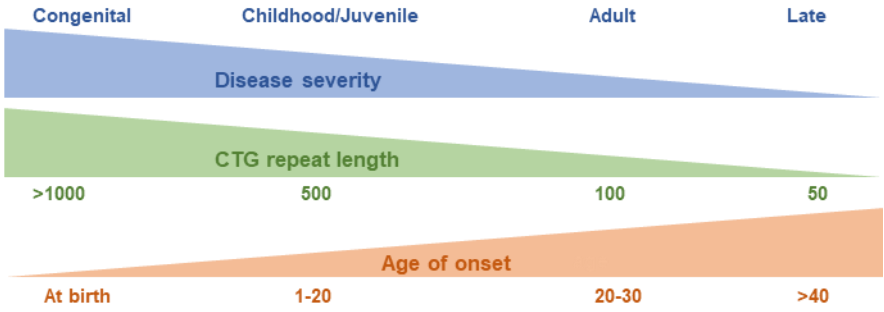

Figure 3. Genotype-phenotype correlations in patients with DM1. Larger CTG repeats (in green) are correlated with an earlier age of onset (in orange) and more severe symptoms (in blue). On the left range, CTG repeat length greater than 1000 appears at birth with the congenital form. By contrast, in the range on the right, CTG repeats around 50 are related to the late phenotype that usually appears after 40 years of age.

2.2. Somatic instability and genetic anticipation

Pathological mutations in DM1 occur at high frequency in both germline and somatic cells (Brouwer et al., 2009). This is because expanded CTG repeats are very unstable (Pearson et al., 2005; Meola and Cardani, 2015). As a result of this instability, a single individual may have different repeat sizes in cells and tissues, called somatic mosaicism. This somatic mosaicism is age-dependent and tissue-specific, and a likely contributor to the progressive nature of DM1 symptoms (Morales et al., 2012). Notably, the repeat length is always much larger in muscle DNA than that observed in blood (Thornton et al., 1994; Monckton et al., 1995). As disease symptoms are most prominent in post-mitotic tissues such as skeletal muscle, heart and brain, it is thought that an abnormal DNA repair mechanism may be responsible for repeat instability (Dion, 2014).

It is also important to note that the expanded CTG repeats in DM1 increase in size from generation to generation, leading to a dramatic intergenerational instability. This is due to expansions in germ lines giving rise to the phenomenon called “genetic anticipation”, which is the occurrence of an increase disease severity, and an earlier age of onset in successive generations due to an increase in repeat length during parent-to-child transmission (Figure 4). Thus, offspring of a parent with DM1 may inherit longer repeats than those present in the transmitting parent.

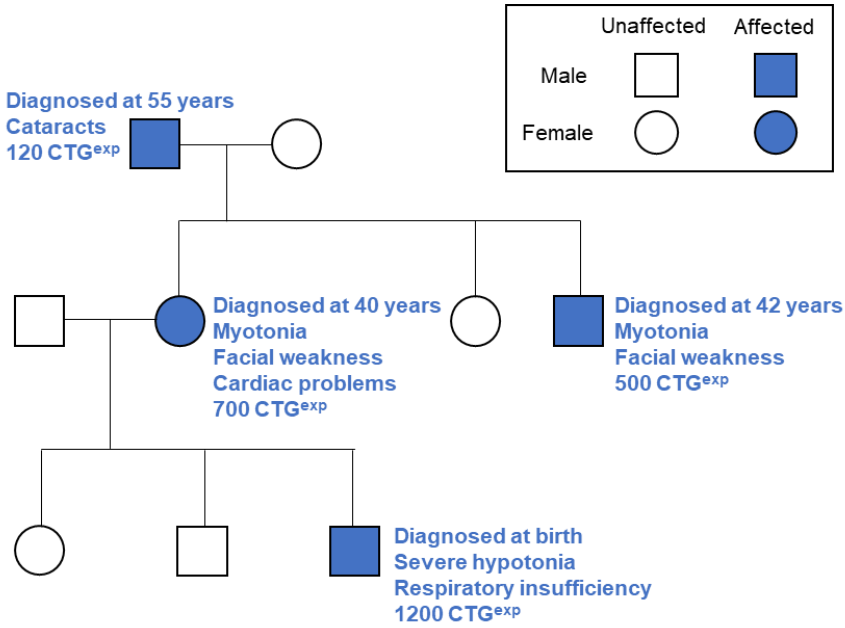

Figure 4. Family pedigree pattern of myotonic dystrophy type 1 (DM1). Example of myotonic dystrophy type 1 with an autosomal dominant inheritance with increasing severity and earlier age of onset from generation to generation due to genetic anticipation.

Because of the anticipation event, the most severe phenotype of DM1 usually appears in new-borns with the congenital form: CDM1 (De Temmerman et al., 2004; Rakocevic-Stojanovic et al., 2005; Udd and Krahe, 2012). In CDM1, 99% of infants almost always inherit the expanded DMPK allele from the mother (Martorell et al., 2007). However, paternally inherited anticipation has also been described in the congenital form of DM1, but is rare (de Die-Smulders et al., 1997; Zeesman et al., 2002; Di Costanzo et al., 2009).

Therefore, the size of CTG repeats and the parental sex of the transmitting mutation are two main factors that determine the severity of the anticipation event (Dean et al., 2006; Pratte et al., 2015).

2.3. Molecular pathomechanisms

The identification of causal mutations in individuals with DM1 has led to some hypotheses concerning the molecular pathways underlying this disease. Specifically, 3 pathogenic mechanisms have been proposed that link the expansions of CTG repeats with clinical manifestations: (i) *DMPK* haploinsufficiency; (ii) an alteration of nearby genes; and (iii) RNA-induced toxicity (Figure 5).

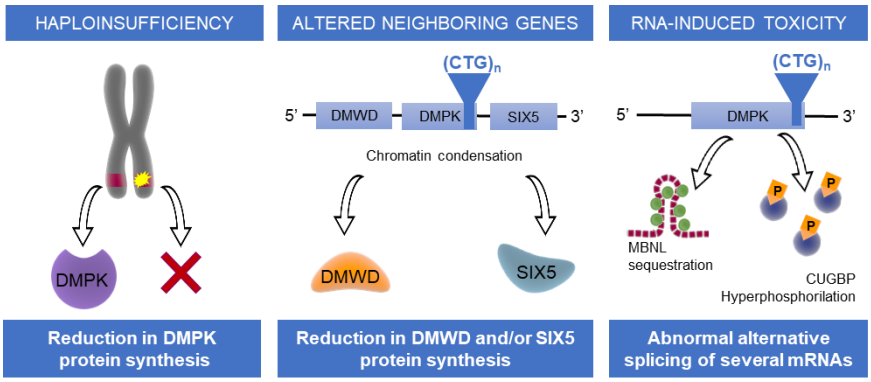

Figure 5. Representation of 3 potential pathogenic mechanisms involved in myotonic dystrophy type 1 (DM1). Mutant DMPK allele result in a reduction in DMPK protein synthesis (due to haploinsufficiency) as well as a reduction in DMWD and/or SIX5 protein expression (due to altered neighbouring genes), leading to DM1 phenotypes. The expanded CTG repeats form a stable hairpin structure sequestering MBNL proteins and stabilizing CUGBP via hyperphosphorylation (due to RNA-induced toxicity) (Modified from Mateos-Aierdi et al., 2015).

In the haploinsufficiency hypothesis, the reduction in the amount of protein synthesized (i.e. *DMPK*) is attributed to the detrimental effect of having only one wild-type copy of the gene in the organism, since the other copy contains the CTG^{exp} repeats (Fu et al., 1992). Since DM1 is an autosomal dominant disorder, nuclear retention of the mutant RNA could theoretically result in a 50% of reduction in DMPK protein. However, several studies in mouse model lines indicate that the multisystemic

phenotype of DM1 is not caused by haploinsufficiency. Thus, *Dmpk* knockout (KO) mice, in which DMPK protein is fully removed, only develop mild myopathy without myotonia and muscle degeneration (Reddy et al., 1996; Berul et al., 1999). These data indicate that the loss of DMPK function may contribute to the cardiac pathology in DM1, but that haploinsufficiency may be not the primary mechanism that explains all the DM1 alterations.

The second hypothesis proposes altered expression of genes located nearby to the genes responsible for the disease could contribute to the pathomechanism of DM1. In accordance, several studies have identified a reduced expression of both upstream (*DMWD),* and downstream (*SIX5)* genes flanking *DMPK* in DM1 patients (Klesert et al., 1997; Thornton et al., 1997). Nonetheless, *Six5* KO mice did not recapitulate the multisystem phenotype, despite exhibiting a greater susceptibility to develop cataracts and cardiac conduction abnormalities (Klesert et al., 2000; Sarkar et al., 2000; Wakimoto et al., 2002). Therefore, the loss of SIX5 function may underlie the development of the cataract phenotype in DM1 but does not reproduce major characteristic features of the disease.

During the last decade, RNA-induced toxicity has been proposed as a new pathogenic mechanism for diseases caused by expanded repeat sequences, such as Huntington disease (La Spada and Taylor, 2010), Fragile X syndrome (Swanson and Orr, 2007) and DM1 and DM2 (Mankodi et al., 2000, Osborne et al., 2009; Gomes-Pereira et al., 2011).

In DM1, expanded mutations are located within a non-coding region, thus the protein sequence of DMPK cannot be altered, but it could have detrimental effects at the RNA level. Preclinical studies revealed that the expression of ribonuclear foci in muscle fibers was the main factor for the

development of muscular features of DM1 in transgenic HSA^{LR} mice (carrying 250 CTG repeats in the 3'UTR of *human skeletal actin* (HSA) gene), and in transgenic DM300 mice (carrying 300 CTG repeats in the 3'UTR of *DMPK* gene) (Mankodi et al., 2000; Seznec et al., 2001). It has been described that mutant transcripts form hairpin-shaped secondary structures, which interfere with the MBNL family, resulting in their sequestration and subsequent loss-of-function (Miller et al., 2000; Tian et al., 2000; Mooers et al., 2005). This family of proteins has a key role in the regulation of the fetal-adult transition, and its disruption causes splicing abnormalities in patients with DM1 (Osborne et al., 2009; Yuan et al., 2007). The involvement of MBNL proteins in the pathomechanism of DM1 was supported by the generation of transgenic mouse lines with MBNL deficiencies, including myotonia in *Mbnl1* KO mice (Kanadia et al., 2003), CNS impairment in *Mbnl2* KO mice (Hao et al., 2008; Charizanis et al., 2012), and heart and muscle deficits in *Mbnl1/Mbnl2* double KO mice (Lee et al., 2013). Taken together, the toxicity of RNA seems to be crucial for promoting the multisystem phenotype seen in patients with DM1.

2.4. Ribonuclear foci

A hallmark feature in DM1 is the accumulation of mutated *DMPK* transcripts in ribonuclear inclusions called foci, which are trapped into the nucleus of cells disrupting a subset of proteins through MBNL sequestration or CELF1 upregulation (Pettersson et al., 2015). Ribonuclear foci were first detected in muscle biopsies (Taneja et al., 1995), but have also been observed in post-mortem human DM1 brains, mainly in neuronal cells of the cerebral cortex, hippocampus, dentate gyrus and thalamus (Jiang et al., 2004). These inclusions were also reported in human DM1 induced pluripotent stem cells (iPS) (Denis et al., 2013). Some evidence

suggests that nuclear foci are directly involved in disease pathogenesis through a mechanism that involves sequestration of MBNL proteins and mis-regulation of alternative splicing (Jiang et al., 2004). In fact, several studies reported co-localization of nuclear foci with MBNL proteins (Fardaei, 2001; Fardaei et al., 2002; Mankodi et al., 2001), consistent with the ability of these proteins to directly interact with expanded CUG repeats (Miller et al., 2000; Konieczny et al., 2014). Interestingly, ribonuclear foci formation has also been detectd in brains of transgenic DMSXL mice carrying more than 1000 CTG repeats (Huguet et al., 2012), and in neuronal cells derived from human embryonic stem cells (Marteyn et al., 2011). Figure 6 shows how toxic RNA foci and CUG^{exp}-associated RBPs can deregulate mRNA processing events in DM1 cells.

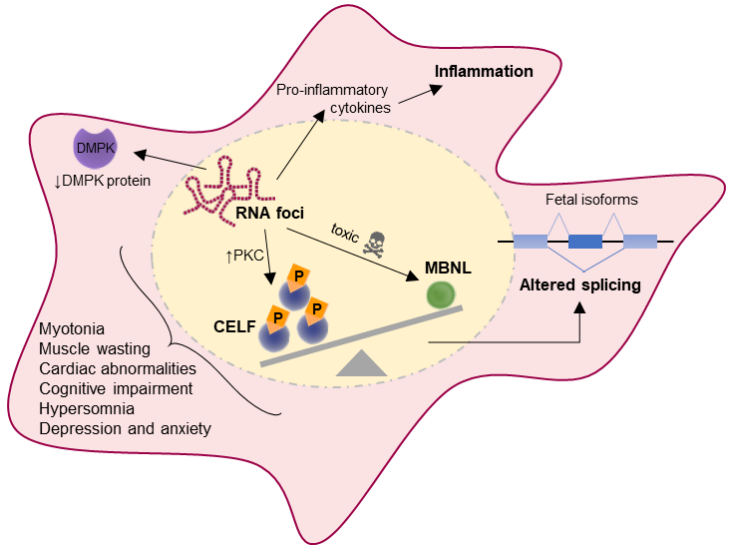

Figure 6. Cell pathways and signalling cascades deregulated by toxic RNA foci in DM1 cells. The expression of toxic RNA foci reduces DMPK protein expression, sequesters MBNL proteins and upregulates CELF by PKC activation. This results in altered splicing events with fetal isoforms. Finally, increased expression of pro-inflammatory cytokines indicates ongoing inflammation in DM1 (Modified from Braz et al., 2018).

2.5. RNA binding-proteins

RNA-binding proteins play an important role in post-transcriptional events promoting splicing, polyadenylation, mRNA stabilization, translation or degradation (Peltz et al., 1993; Ruiz-Echevarria and Peltz, 2000; Cuchalová et al., 2010). Two main families of RBPs have been recognized as key factors in the pathogenesis of DM1 consistent with their ability to interact and bind with CUG^{exp} repeats (Paul et al., 2006, 2011): (i) the CUG-binding protein (CUG-BP, also known as CELF) and (ii) the MBNL family protein. These proteins are antagonistic splicing regulators promoting fetal and adult splicing patterns, respectively, that are mis-regulated in DM1 tissues. However, the individual roles of CELF and/or MBNL remaine poorly understood (Figure 7) (Ho et al., 2004; Kuyumcu-Martinez et al., 2007; Lee and Cooper, 2009; Wang et al., 2015).

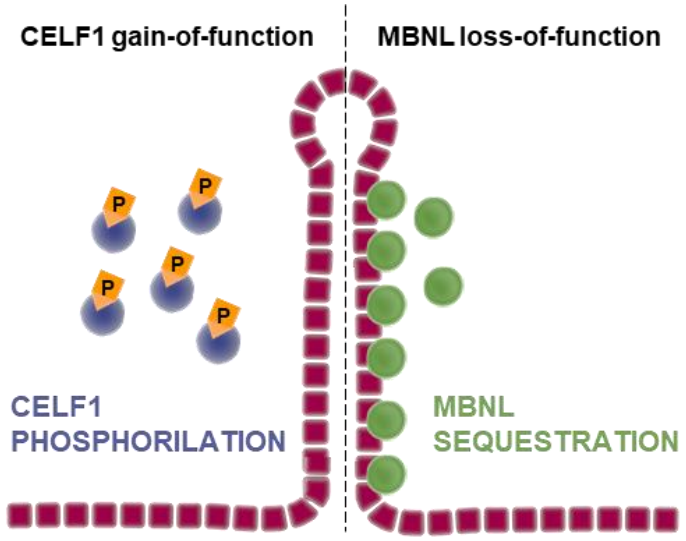

Figure 7. CELF1 gain-of-function and MBNL loss-of-function in DM1 pathogenesis. Hairpin structure formed by expanded CUG repeats sequesters MBNL proteins and upregulates and stabilizes CELF1 protein through hyperphosphorilation (Adapted from Lee and Cooper, 2009).

2.5.1. CUG-Binding Protein

CUG-BP is a member of the Elav-like family (also known as CELF) involved in the regulation of alternative splicing of several pre-mRNAs, which has been directly involved in the multisystemic phenotype of patients with DM1 (Ladd and Pliam, 2001; Osborne et al., 2009). They are proteins present in the nucleus and the cytoplasm with high affinity for UG-rich motifs rather than expanded CUG repeats, so they are not sequestered in the ribonuclear foci.

In DM1 patients, CELF1 undergo an abnormal activation via protein kinase C (PKC) which phosphorylates CELF1, increasing its levels and stability mostly in skeletal muscle and heart tissues (Tian et al., 2000; Charlet-B et al., 2002; Kuyumcu-Martinez et al., 2007). Moreover, increased levels of CELF2 have also been detected in brains of some patients with DM1 (Dhaenens et al., 2011). Currently, several lines of CELF transgenic mice have been reported (see section 5).

2.5.2. Muscleblind-like protein family

The muscleblind-like (MBNL) protein family is required for muscle development, adult photoreceptor differentiation and growth of the embryonic peripheral nervous system (Kania et al., 1995; Artero et al., 1998; Bargiela et al., 2014). In mammals, three genes with different expression patterns have been identified: *MBNL1*, *MBNL2* and *MBNL3* (Miller et al., 2000; Fardaei et al., 2002; Kanadia et al., 2003). The expression profiles of MBNL proteins are tissue specific. MBNL1 is primarily expressed in skeletal muscle and heart, while MBNL2 is predominant in the brain. In contrast, MBNL3 expression is mainly found in placenta during embryonic development, and in adult skeletal muscle

when there is muscle regeneration due to damage (Fardaei et al., 2002; Lee et al., 2007; Poulos et al., 2013; Goodwin et al., 2015). This family of proteins plays a key role in the regulation of alternative splicing of specific pre-mRNAs during developmental transition by promoting adult patterns, and their splicing mis-regulation disrupts the conversion of fetal to adult protein isoforms (Lin et al., 2006; O'Rourke and Swanson, 2009; Kalsotra and Cooper, 2011; Han et al., 2013). Thus, MBNL1 regulates splicing patterns during muscle and heart development (Lin et al., 2006; Kalsotra et al., 2008), whereas MBNL2 plays a similar role in the CNS (Figure 8) (Charizanis et al., 2012; Goodwin et al., 2015).

In DM1 patients, MBNL proteins accumulate in the ribonuclear foci due to their high binding affinity to CUG^{exp} repeats, and become sequestered (Miller et al., 2000). Because of this, MBNL function is compromised resulting in the manifestation of the typical features of DM1. Hence, MBNL1 loss-of-function has been related with myotonia, cardiac conduction defects and insulin resistance (Ho et al., 2004; Dansithong et al., 2005), while MBNL2 loss-of-function has been associated to CNS-dysfunction, including cognitive decline, hypersomnia, depression and anxiety. In fact, studies using relevant *Mbnl* KO mouse models reproducing several phenotypes consistent with DM1 have supported the contribution of MBNL loss-of-function to DM1 pathogenesis (see section 5).

In this thesis, we focused on investigating DM1 neuropathology associated with MBNL2 deficiency using a multidisciplinary approach, examinig the behavioural, molecular, neurochemical and neurophysiological correlates in two transgenic mouse lines lacking the *Mbnl2* gene.

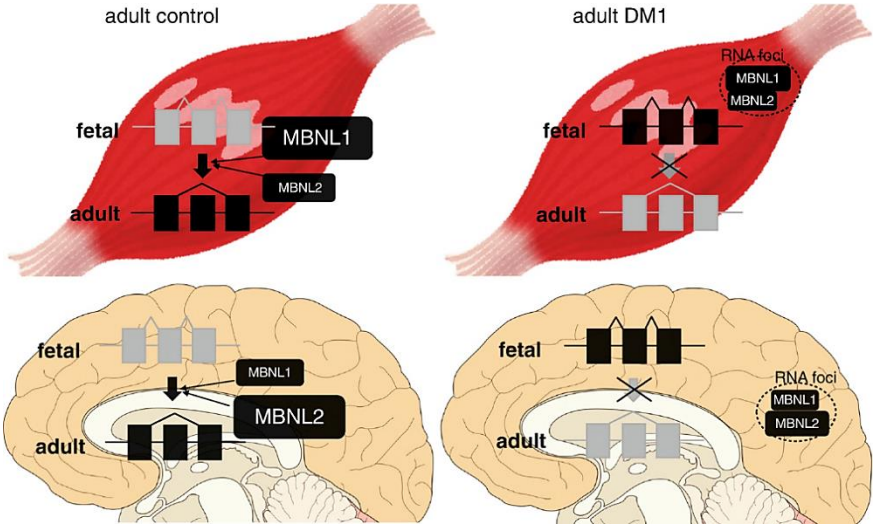

Figure 8. Contribution of MBNL1 and MBNL2 to the regulation of alternative splicing during fetal-adult transition in the skeletal muscle and brain. In a healthy adult, MBNL1 is predominantly expressed in the skeletal muscle and MBNL2 in the brain. In a DM1 patient, the RNA foci that sequesters MBNL proteins leads to several mis-splicing events with myotonia and muscle weakness in the muscle, and brain-specific splicing changes in the brain (taken from Kimura et al., 2018).

3. Pathophysiological features of DM1

DM1 is a multi-system condition that impacts nearly every organ of the body. The main feature of this disease is myotonia, a delay in relaxation after muscle contraction, along with skeletal muscle weakness and atrophy. However, patients also develop neuropsychiatric symptoms, resistance to insulin, gastrointestinal symptoms and cataracts. This progressive disorder imposes a great burden on patients and their families, and the non-muscle symptoms increase the negative impact of the disease on their quality of life (Laberge et al., 2009; Heatwole et al., 2012).

To date, DM1 is classified into 4 clinical subtypes according to age at the onset of symptoms: congenital (CDM1), childhood/juvenile, adult and late-onset forms (de Antonio et al., 2016; Ho et al., 2015) (See Table 1 for summarize).

CDM1 (>1.000 CTG) mainly consists of severe hypotonia at birth, often with a high frequency of respiratory insufficiency, mental retardation and progressive muscular dystrophy (Campbell et al., 2013; Echenne et al., 2013). Affected new-borns have an inverted V-shaped upper lip, also named “carp mouth”, a sign of severe facial weakness, which leads to difficult breast feeding. All CDM1 patients develop learning disabilities and entail special needs.

The childhood- and juvenile-onset forms (50-1.000 CTG) are often misdiagnosed in affected adolescents or children due to the absence of neurological problems. These forms are less severe than CDM1, but still display a variable degree of cognitive and learning impairment, particularly with altered psychosocial function, dysarthria (difficulty

articulating sounds and words), muscle degeneration and excessive sleepiness (Steyaert, 2000; Ho et al., 2019).

The core features of adult-onset DM1 (150-200 CTG) are associated with abnormal muscle relaxation related to myotonia, progressive muscle weakness and atrophy, accompanied by extra-muscular symptoms like cardiac arrhythmias, cataracts, CNS impairment, excessive daytime sleepiness, gastrointestinal problems and insulin resistance (Thornton, 2014; Mejersjö and Kiliaridis, 2017; Gourdon and Meola, 2017). The late-onset form (50-100 CTG) is characterized by a milder phenotype with less muscle contribution including mild myotonia and early cataracts (Arsenault et al., 2006). Moreover, an age-related cognitive involvement can be present (Modoni et al., 2004).

Table 1. Summary of myotonic dystrophy type 1 phenotypes, clinical findings and CTG repeat length.

Subytpe	Age of onset	Clinical findings	CTG length
Congenital (CDM1)	At birth	Hypotonia Respiratory failure Intellectual disability	>1.000
Childhood/Juvenile onset	1-20	Facial weakness and myotonia Cardiac conduction abnormalities Low IQ	100-1.000
Adult-onset	20-30	Weakness and myotonia Cardiac conduction defects Insulin resistance Cataracts Cognitive impairment	50-1.000
Late-onset	>40	Mild weakness and myotonia Cataracts Aging-related cognitive decline	50-100

3.1. Peripheral Phenotype

The cardinal symptoms in adult-onset DM1 are skeletal muscle weakness, atrophy and myotonia with a distal pattern, leading to difficulties in performing tasks requiring fine manual dexterity.

Myotonia is defined as a delay muscle relaxation after a voluntary muscle contraction caused by muscle hyperexcitability. However, handgrip myotonia and strength may improve with repeated contractions called the "warm-up" phenomenon (Logigian et al., 2005). From a molecular point of view, it has been indicated that myotonia is caused by an incorrect splicing of the chloride channel 1 due to mis-regulated MBNL1 and CUGBP1 (Meola et al., 2013).

Muscle wasting and weakness is the second most prominent clinical feature in DM1, affecting social activities, employment, and many aspects of their patients' daily life. Muscle wasting also affects eye movement, speech, swallowing and breathing. Moreover, DM1 patients exhibit a typical facial appearance known as "hatchet face" or "myopathic face", mainly caused by weakness and atrophy of the facial muscles and palpebra elevators. The molecular mechanisms of muscle wasting have not yet been identified. Other peripheral features present in DM1 include ocular manifestations with an early onset of cataracts, cardiovascular involvement characterized by arrhythmias and cardiac conduction defects (Groh et al., 2012) and endocrine and gastrointestinal dysfunction (Ørngreen et al., 2012; Rönnblom et al., 1996).

There are not many studies focusing on gender differences among patients with DM1, but recently it has been reported that women lose less grip strength and have greater dexterity than men in upper limb performance

(Raymond et al., 2017), and that men have higher morbidity and mortality than women with DM1 (Dogan et al., 2016).

DM1 is a chronic disease due to progressive muscle wasting and weakness, dysphagia, respiratory failure, or fatal arrhythmias. Although currently there is no cure for DM1, care therapy is focused on treatments designed to provide symptomatic relief and support. To date, only mexiline, a sodium channel blocker, can be considered relevant to treat myotonia. However, this drug has a potential pro-arrhythmic effect, and thus, its use is contraindicated in DM1 patients with a high risk of cardiac pathology (Logigian et al., 2010).

3.2. Central Nervous System Alterations

While most research has focused on the neuromuscular features of DM1, patients and families report that CNS-related sympthomatology is usually the most challenging and difficult to deal with (Antonini et al., 2006; Bugiardini et al., 2013; Dogan et al., 2016). Although CNS alterations in DM1 are well stablished, they are not well characterized. The major and most frequent symptoms include cognitive deficits, depression (Winblad et al., 2010), anxiety-linked behaviours (Meola et al., 2003; Van der Velden et al., 2019), emotional deficits (Bungener et al., 1998), excessive daytime sleepiness (Gallais et al., 2014), and fatigue (Winblad and Lindberg, 2019).

3.2.1. Cognitive function

Cognition is defined as the mental process of acquiring knowledge and understanding throught experience, thought and senses. It comprises a broad range of intellectual functions such as attention capacity, stimulus perception, memory and executive functions, all needed in everyday scenarios.

In the adult-onset form of DM1, global cognitive function is usually impaired. These cognitive alterations include deficits in executive functions, attention and visuo-spatial abilities, suggesting a frontal dysexecutive syndrome (Rubinsztein et al., 1998; Meola and Sansone, 2007; Romeo et al., 2010; Okkersen et al., 2017), and some studies have reported the progressive nature of these impairments (Gallais et al., 2017; Sansone et al., 2007; Winblad et al., 2016). Factors such as age-related decline or motor problems could affect cognitive performance in these patients and should be ruled out in clinical studies as possible confounding issues (Modoni et al., 2004; Gaul et al., 2006). Moreover, patients with DM1 also exhibit social cognition deficits. They often show reduced ability to recognize facial expressions or infer mental states from other people (Kobayakawa et al., 2010, 2012; Serra et al., 2016a; Winblad et al., 2016).

3.2.2. Affective alterations

Affective alterations are common neuropsychiatric features in DM1. Depression is one of the main health problems in DM1 due to its high prevalence and incapacitating effects (Antonini et al., 2006; Minier et al., 2018; Van der Velden et al., 2019). Winblad et al. (2010) identified clinical depression in 32% of DM1 patients. This depressive condition ranged from mild to moderate and was mainly associated with early stages of the disease, and a higher educational level (Koenigs et al., 2008; Winblad et al., 2010). Morever, higher levels of anxiety, apathy, reduced initiative and fatigue are also present in DM1 patients, and may be linked to depression (Antonini et al., 2006; Van der Velden et al., 2019).

3.2.3. Excessive daytime sleepiness and fatigue

Excessive daytime sleepiness (EDS) and fatigue are prominent complaints among DM1 patients with a significant impact on the patient's quality of life (Heatwole et al., 2012). EDS is a common and incapacitating feature of DM1 with a prevalence in some populations that reaches 88% (Ciafaloni et al., 2008; Heatwole et al., 2012). EDS is characterized by hypersomnolence and usually occurs in situations of monotony or when no attention is being paid, not during activity (Laberge et al., 2013). Furthermore, rapid eye movement (REM) sleep deregulation and increased nighttime sleep propensity has been described (Laberge et al., 2009; Heatwole et al., 2012). Fatigue is a major complaint reported by DM1 patients and may be unrelated to sleep quality (Angelini and Tasca, 2012; Heatwole et al., 2012). It involves central and peripheral components and may be due to CNS dysfunction, muscle weakness, depression or thyroid dysfunction (Baldanzi et al., 2017).

There are very few studies investigating potential therapeutic interventions for CNS-related disturbances in DM1. Up to now, only modafinil, a compound normally used for excessive somnolence, has shown some efficacy for treating EDS and fatigue (MacDonald et al., 2002). As cognitive impairment and depression have been associtated with worse quality of life in DM1 patients, there is an impending need to improve these problems (Antonini et al., 2006; Laberge et al., 2013; Rakocevic-Stojanovic et al., 2014). Some non-pharmacological interventions have been effective for improving cognitive functions in DM1 patients, such as cognitive behavioural therapy (Okkersen et al., 2018; Winblad and Lindberg, 2019). However, there are currently no medications available to treat both the cognitive alterations and depressive state associated with DM1.

4. Neuropathological correlates of DM1

4.1. Histopathological evidence

A variety of histopathological features have been observed in post-mortem brain samples of DM1 patients. These neuropathological studies documented neuronal loss, neurofibrillary degeneration and abnormal TAU protein expression in brain tissues.

4.1.1. Neuronal loss

Reduced neuronal densities have been reported in specific areas of the brainstem in DM1 patients. In particular, primary degeneration of cathecolaminergic and serotonin neurons in medullary and rahpe nuclei, repectively, has been considered as a possible neuropathological correlate of hypersomnolence and hypoventilation (Ono et al., 1998a, 1998b). Moreover, neuronal loss in the superficial layer of the frontal and parietal cortices and in deep layers of the occipital cortex has also been reported (Mizukami et al., 1999), which may contribute to several behavioral and cognitive problems observed in patients with DM1.

4.1.2. Neurofibrillary degeneration

Neurofibrillary degeneration is an age-related process that occurs during normal aging and is abnormally increased in neurodegenerative diseases. It consists of intracellular aggregates of insoluble fibers and hyperphosphorylated microtubule-associated tau proteins, which form neurofibrillary tangles (NFTs) (Sergeant et al., 2001; Buée et al., 2010). Some studies indicate that the accumulation of NFTs may be key for the development and progression of brain dysfunction (Maurage et al., 2005;

Winblad et al., 2008). Post-mortem studies have described significant changes in specific tau isoforms in the hippocampus, entorhinal and temporal areas of DM1 brain tissue (Vermersch et al., 1996; Sergeant et al., 2001; Maurage et al., 2005). Moreover, tauopathy in DM1 brain tissue was associated with larger CTG^{exp} repeats (Sergeant et al., 2001).

4.2. Structural and functional alterations in DM1 brains

In the last decade, a significant number of imaging studies have confirmed DM1 as a brain disease. Magnetic resonance imaging (MRI) studies in DM1 patients showed white matter hyperintense lesions mainly located in frontal and temporal lobes (Di Costanzo et al., 2001; Romeo et al., 2010; Minnerop et al., 2011, 2018; Wozniak et al., 2014). In addition, callosal body atrophy has been described in adult-onset form of DM1 along with degradation of major pathways in the limbic system (Minnerop et al., 2011). Paradoxically, significant positive correlations have been found between white matter integrity and motor function, depression and hypersomnia/fatigue (Minnerop et al., 2011). In accordance, DM1 patients with longer disease duration, and presence of white matter lesions, exhibited fewer depressive symptoms (Winblad et al., 2010).

Gray matter alterations in DM1 have been observed in the cortex and in subcortical structures, such as the thalamus and the basal ganglia (Antonini et al., 2004; Weber et al., 2010; Minnerop et al., 2011; Baldanzi et al., 2016). Other abnormalities like dilated ventricles, could also be attributed to reduced grey matter (Schenider-Gold et al., 2015; Zanigni et al., 2016). On a structural level, global brain atrophy is linked with impaired motor functions such as planning and execution actions (Mitsuoka et al., 2003),

whereas white matter disruption has been associated with cognitive deficits including working memory, executive function, visuo-spatial impairment and attention deficits (Weber et al., 2010; Wozniak et al., 2013, 2014; Caso et al., 2014; Baldanzi et al., 2016).

Functional MRI (fMRI) studies have revealed changes in resting state connectivity in DM1 (Serra et al., 2014, 2016) with altered cerebral cortical circuits, mainly in white matter regions (Minnerop et al., 2011). Increased activity is found in motor regions during the performance of a motor task, while in DM1 patients with myotonia, there was no activation of these motor areas, indicating that there is no involvement of brain function in the development of myotonia (Caramia et al., 2010; Toth et al., 2015). In resting-state MRI studies, abnormal connectivity networks were described in regions related to personality profiles and social cognition (Serra et al., 2016a, b).

Altogether, correlation analysis between brain pathological changes and cognitive function indicate that complex neural networks, involving numerous CNS structures and mechanisms related to plasticity, neurodevelopment and neurodegeneration are involved in DM1. Therefore, it is important to carry out long-term studies to determine if the symptoms vary (neurodegeneration) or remain stable (neurodevelopmental) over time. In this sense, Winblad et al. (2016) reported that cognitive decline in adult-onset DM1 correlated with earlier onset and longer disease duration, indicating a neurodegenerative process.

Complementing MRI studies, *in vivo* proton magnetic resonance spectroscopy (^{1}H-MRS) allows analysing alterations in metabolite concentrations in different brain regions. The most commonly measured metabolites are N-acetylaspartate (NAA), choline (Cho), and creatine (Cr).

NAA is a marker of neuronal function since it is expressed in the cytosol, axons and synaptosomes of neurons, and a decrease in this metabolite correlates with a neuronal dysfunction or neuronal loss. Cho is often used as a glial marker, which predicts a cell proliferation or gliosis process, and Cr is the compound peak used as control housekeeping expressed everywhere in the brain. Since estimating absolute levels of metabolites *in vivo* is difficult, the content is measured in relation to other molecules.

Previous studies reported altered NAA/Cho and NAA/Cr ratios in adult-onset DM1 patients, showing a decrease in NAA production in temporoparietal grey matter, and a more pronounced attenuation in frontal white matter (Hashimoto et al., 1995; Akiguchi et al., 1999; Vielhaber et al., 2006; Takado et al., 2015). Accordingly, post-mortem DM1 brains exhibited cerebral neuronal loss along with myelin abnormalities (Ogata et al., 1998; Mizukami et al., 1999). Moreover, a significant increase in glutamine, and reduced glutamate in the frontal cortex has been also observed (Takado et al., 2015) These abnormalities in the glutamatergic system of DM1 patients, could be related to a synaptic dysfunction, as previously demonstrated in post-mortem brains (Charizanis et al., 2012; Hernandez-Hernandez et al., 2013).

5. Preclinical models of DM1

The generation of animal models that faithfully reproduce a particular disease presents a unique opportunity not only to understand the mechanisms involved, but also to screen, identify and evaluate new pharmacological therapies. Several animal models of DM1 have been developed and have provided information to associate disease mutations with the resulting phenotypes.

To model the disease, invertebrate and vertebrate animals have been used. The fruit fly *Drosophila melanogaster* and the zebrafish model *Danio rerio* have been widely utilized as model organisms to study RNA toxicity of expanded CUG repeats (de Haro et al., 2006; Garcia-Lopez et al., 2008; Plantié et al., 2015). However, the mouse has proven to be more useful for gaining deep insight into DM1 pathomechanism (Mankodi et al. 2000, Seznec et al., 2000, Gomes-Pereira et al., 2007; Timchenko et al., 2004; Ward et al., 2010). Here, we will summarize the data regarding two different categories: (i) models of toxic RNA expression and (ii) models of altered RBPs such as MBNL sequestration or CELF1 overexpression (see Table 2 for summary).

To assess the contribution and toxicity of foci formation to the multisystem pathology observed in patients with DM1, several animal models have been generated. *Drosophila* models have managed to reproduce genetic, molecular and histological aspects of the disease and have been used as a tool to discover new components in the pathogenesis pathway of the disease, as well as potential drugs for the treatment of the disease (Garcia-Lopez et al., 2008; Garcia-Lopez et al., 2011; Fernandez-Costa et al., 2013; Garcia-Alcover et al., 2014).

Many mouse models have been developed to investigate the underlying mechanisms of DM1, but only a few transgenic lines showed CNS-related phenotypes. These include DM300 and DMSXL mice, and the inducible mouse model EpA960. These CTG-expressing lines in the CNS recapitulated molecular, behavioral and neurochemical characteristics, thus becoming robust models of the pathology of the CNS of DM1.

On the other hand, the use of the HSA^{LR} mouse is restricted for the investigation of skeletal muscle pathology in adults, despite not recapitulating muscle wasting and weakness (Mankodi et al., 2000). However, this mouse model corroborates that promoting a specific muscle expression of CUGexp repetitions is enough to trigger a DM1-like muscle degeneration phenotype and provides crucial evidence of the toxicity of RNA protein aggregates in DM1 disorder.Moreover, the characterization of molecular and physiological phenotypes resulting from CELF1 upregulation and MBNL sequestration in mice, regardless of expanded CTG repeats, has illustrated the central role of these two protein families of splicing regulators in the pathogenesis of DM1. These mouse lines recapitulated most of the phenotypic features of DM1, indicating that foci itself are not required for DM1 pathogenesis.

Mouse models of CELF1 upregulation have shown that the toxicity of CELF1 is sufficient to reproduce phenotypes such as those observed in congenital DM1, but due to their limitations of high mortality and difficult reproduction, inducible lines of CELF1 overexpression in skeletal muscle were developed (Ward et al., 2010) and the heart (Koshelev et al., 2010) reproducing functional and molecular abnormalities observed in patients with DM1. Therefore, CELF proteins can also regulate the alternative splicing of transcripts involved in neuronal function and may contribute to

the etiology of neurological disorders already described in patients with DM1 (Ladd, 2013).

On the other hand, the expression of increased CTG^{exp} repeats also induces the sequestration of the three MBNL proteins in the RNA foci (Miller et al., 2000; Mankodi et al., 2001; Fardaei et al., 2002). The involvement of these proteins in DM1 pathology was confirmed by the generation of KO mouse lines through the depletion of *Mbnl* genes alone, or by inactivating multiple *Mbnl* genes at once. These mouse models allowed to establish the functional role of the MBNL family and its contribution to DM1 molecular pathogenesis.

Regarding MBNL1, the generation of the constitutive *Mbnl1* KO mice revealed a crucial role of MBNL1 in the specific control of the fetal-adult transition in the muscle (Suenaga et al., 2012). However, did not develop muscle weakness. Therefore, the recruitment of other MBNL proteins may be necessary to fully replicate the multisystemic DM1 phenotype. In line with the hypothesis that MBNL1 downregulation causes the major muscular-phentoypes of DM1 and aberrant splicing events, Kanadia et al. (2006) used adeno-associated virus (AAV) to overexpress MBNL1 in transgenic HSA^{LR} mice, and rescued myotonia and adult splicing events (Kanadia et al., 2006). MBNL2 contribution was also tested in the *Mbnl2* KO mouse model which showed marked CNS phenotypes, along with brain-specific splicing changes (Charizanis et al., 2012). These results suggest that the main pathological changes in DM1 brains are attributable to the expression of toxic RNA, with the subsequent MBNL2 sequestration, and deregulation of specific alternative splicing events essential for normal CNS function in adults.

Moreover, MBNL3 loss of function was also examined using the *Mbnl3* KO mice which showed pathologies associated with age, such as insulin resistance, heart defects and cataracts, suggesting that loss of MBNL3 function may contribute to premature aging (Choi et al., 2016). Despite finding significant phenotypes in the depletion lines of a single *Mbnl* gene, they do not faithfully recapitulate all the features of DM1. It could be probably due to the compensatory mechanisms of the other *Mbnl* isoforms (Lee et al., 2013). For example, in *Mbnl1* KO mouse, the expression of *Mbnl2* is upregulated and MBNL2 protein binds to target transcripts that are normally regulated by MBNL1 (Lee et al., 2013). Therefore, MBNL-like compound KO mice have been generated to recreate a most human-like DM1. The double KO of *Mbnl1* and *Mbnl2* mouse was lethal in embryonic stages, suggesting a crucial role of these proteins during development (Lee et al., 2013). These findings revealed that the simultaneous deletion of various MBNL proteins is a critical event in the pathogenesis of DM1.

Altogether, by generating and characterizing individual and compound *Mbnl* KO mouse models, it has been shown that simultaneous sequestration of MBNL proteins are crucial for the development of clinical features of DM1. Due to limited availability and technical difficulties of working with human DM1 tissue, constitutive and conditional *Mbnl* KO mouse models allow for the investigation of altered RNA processing in multiple tissues, cell types and developmental phases.

In this thesis, we used *Mbnl2* KO mice to examine more in depth the behavioural, neurochemical and neurophysiological correlates of a constitutive loss of MBNL2 in the brain. In addition, we investigated the progression of these alterations, and studied whether gender differences existed.

Table 2. Summary of the most relevant transgenic animal models of myotonic dystrophy type 1.

MODELS OF RNA TOXICITY	
Drosophila$_{480}$	Expressing 480 CTG repeats in mushroom bodies leading to muscle wasting and degeneration (de Haro et al., 2006; García-López et al., 2008)
Drosophila$_{960}$	Expressing 960 CTG repeats in the larval muscles leading to myoblast fusion defects, muscle hypercontraction and reduced fiber size (Picchio et al., 2013)
HSA_{LR}	Expressing 250 CTG repeats in the *Human Skeletal Actin* gene promoter. Skeletal muscle: Myotonia and muscle degeneration (Mankodi et al., 2000)
DM300	Expressing 300 CTG repeats with a high frequency of intergenerational instability. Skeletal muscle and CNS: Myotonia and altered tau protein isoforms in brain (Seznec et al., 2001; Guiraud-Dogan et al., 2007).
DMSXL	Expressing >1.000 CTG repeats in the *DMPK* locus. Muscle: Muscle deficits with disrupted motor performance (Gomes-Pereira et al., 2007; Huguet et al., 2012) Brain: Anxiety, spatial and working memory impairment, anhedonia and deficits in short-term plasticity (Hernández-Hernández et al., 2013)
EpA960	Inducible and tissue-specific mouse line expressing 960 CTG repeats in human *DMPK* gene. Heart: Cardiac pathology with arrhythmias (Wang et al., 2007) Skeletal muscle: Progressive muscle impairment (Orengo et al., 2008) Brain: Learning deficits, impaired hippocampal LTP and reduced synaptic transmission (Wang et al., 2017)
MODELS OF ALTERED RNA-BINDING PROTEINS	
CELF1 O.E	CELF1 overexpression in heart tissue and skeletal muscle. Severe growth retardation, delayed myogenesis and elevated neonatal mortality similar to congenital DM1 phenotype (Timchenko et al., 2004; Ho et al., 2005)
MBNL1 O.E	MBNL1 overexpression in skeletal muscle. Phenotype: Improved skeletal muscle (Chamberlain and Ranum, 2012)
Mbnl1 KO	Inactivation of MBNL1 impacted mainly in skeletal muscle and eye. Phenotype: Myotonia, cataracts and cardiac defects (Kanadia et al., 2003)
Mbnl2 KO	Inactivation of MBNL2 impacted mainly in CNS, with no muscle affection. Phenotype: hypersomnia, learning and spatial memory impairment, decreased synaptic plasticity (Charizanis et al., 2012)
Mbnl3 KO	Inactivation of MBNL3 impacted mainly in skeletal muscle. Phenotype: impaired muscle regeneration and progressive wasting and weakness (Polulos et al., 2013)

O.E: overexpression

6. Therapeutic strategies in animal models of DM1

The investigations in animal models have improved our understanding of the molecular pathogenesis of DM1 and have opened new avenues in the search for therapeutic targets. Based on the accumulated evidence, two major therapeutic approaches, mostly for mytonia, have been conducted in animal models: (i) silencing strategies to reduce RNA toxicity, and (ii) inhibiting interactions between MBNL and toxic RNA. These strategies include the use of antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), microRNAs (miRNAs) and pharmacological approaches with small molecules (see Figure 9).

6.1. Degradation of toxic RNA

In recent years, numerous works have focused their efforts on reducing the expression of mutant transcripts through their degradation (Francois et al., 2011; Gonzalez-Barriga et al., 2013). ASOs are of interest for their capacity to discriminate between mutant and normal transcripts and they are designed to disperse intranuclear foci. Preclinical studies showed that intramuscular injections of ASOs reduced foci number, and improved myotonia in HSA^{LR} (Wheeler et al., 2009) and in DMSXL mice (Mulders et al., 2009). Given that DM1 is a multisystemic disorder that affects multiple tissues and organs, many studies have been conducted to investigate the systemic effects of a new type of ASOs called “gapmer”, with a that degrades mRNA (Chan et al., 2006). These gapmer-ASOs have reached the clinical stage after showing high efficacy in HSA^{LR} (Wheeler et al., 2012), and DMSXL mice (Jauvin et al., 2017), and in monkeys (Pandey et al., 2015). Taken together, antisense technology has significant potential to target specific genes in diseases that involve increased protein or RNA function (Bennett and Swayze, 2010).

On the other hand, siRNAs exert their gene-silencing effect within the RNA interference (iRNA) pathway. Once inside a cell, siRNAs are recognized by proteins in the RNA-induced silencing complex (RISC), activating and cleaving the mRNA (Garber, 2017). Both intramuscular and systemic delivery of siRNA molecules, reduced toxic transcripts and improved muscular phenotypes in HSA^{LR} mice (Sobczak et al., 2013; Bisset et al., 2015). More recently, *in vivo* administration of miRNAs has demonstrated beneficial effects in *Drosophila* DM1 model (Cerro-Herreros et al., 2016) and its administration in quadriceps muscles in HSA^{LR} mice, upregulated MBNL protein, and rescued the molecular, cellular and functional defects of DM1-muscle pathology (Cerro-Herreros et al., 2018).

6.2. Inhibition of CUG^{exp}-MBNL interaction

Several research groups have developed therapeutic approaches based on small molecules to treat disease-related phenotypes by the inhibition of CUG^{exp}-MBNL interaction or by the upregulation of MBNL1 (Kumar, 2012; Parkesh et al., 2012; Chen et al., 2012; Ketley et al., 2014). Thus, hundred of molecules have emerged in screening assays with cell and fly DM1 models (Garcia-Alcover et al., 2014; Yildirim et al., 2015; Luu et al., 2016; Konieczny et al., 2017). Recently, the peptide ABP1 was discovered using the *Drosophila* model. This peptide suppressed the RNA toxicity by preventing hairpin formation, and reduced muscle histopathology in HSA^{LR} mice (Garcia-Lopez et al., 2011).

However, among the drugs that are already approved by the FDA (also known as drug repositioning), the most relevant are: pentamidine, actinomycin D, erythromycin and phenylbutazone. Pentamidine, was the

first small molecule identified that disrupted toxic RNA foci, released MBNL1 protein and reversed splicing defects. The beneficial effects of this antibiotic drug have been reported in HSA^{LR} mice (Warf et al., 2009) and *Drosophila* DM1 model (Chakraborty et al., 2015). Actinomycin D and erythromycin demonstrated an improvement of myotonia and splicing defects in HSA^{LR} mice (Siboni et al., 2015; Nakamori et al., 2016). Phenylbutazone, a non-steroidal anti-inflammatory drug (NSAIDs), upregulated the expression of MBNL1 protein and ameliorated splicing defects improving wheel-running activity in HSA^{LR} mice (Chen et al., 2016).

In addition, CELF proteins are also targets to consider. CELF activity is controlled by PKC and glycogen synthase kinase-3 beta (GSK3β), which are increased in skeletal muscle of DM1 patients. Thus, inhibitors of those proteins have been shown to reverse muscle and cardiac phenotypes in DM mouse models (Wang et al., 2009; Jones et al., 2012). Tideglusib, a GSK3β inhibitor, reduced CUG^{exp} levels and enhanced muscle pathology in HSA^{LR} mice and increased postnatal survival, and improved growth and neuromotor activity in DMSXL mice (Wang et al., 2016). These findings indicate that GSK3β inhibitors can correct the pathology of DM1 through several pathways.

Although all these studies provide promising results regarding potential treatment options for the muscular deficits observed in DM1, there is a lack of research focusing on possible therapeutic alternatives for the CNS deficits associated to this pathology. In this sense, the *Drosophila* fly model of DM1 expressing 480 CTG repeats in mushroom bodies, which are brain centres for associative learning, and affects only females has provided a relevant tool to screen chemical suppressors of neuronal toxicity related to CUG^{exp} RNA (Garcia-Lopez et al., 2008). This fly model

triggers a semi lethal phenotype in pupal female flies. Therefore, compounds that increase the viability of female flies, would be compounds with anti-DM1 properties. After testing 400 candidate drugs from the Prestwick Chemical Library, 10 chemical compounds with diverse mechanisms of action were shown to significantly improve the viability of female flies (Garcia-Lopez et al., 2008). These included non-steroidal anti-inflammatory agents, muscarinic, cholinergic, histamine receptor inhibitors, and monoamine uptake inhibitors.

Taking advantage of these data, in this thesis we selected the psychostimulant methylphenidate, which increases dopamine by blocking dopamine transporters (Garcia-Lopez et al., 2008), as a potential therapeutic strategy for CNS alterations in DM1 using constitutive *Mbnl2* KO mice (Article 1).

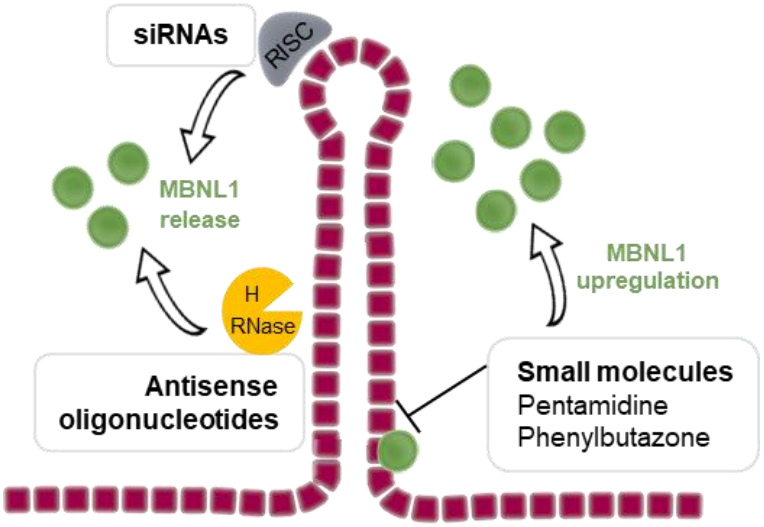

Figure 9. Therapeutic strategies for DM1. Antisense oligonucleotides and siRNAs restore MBNL function by degrading the toxic RNA of hairpin structures. Small molecules such as pentamidine and phenylbutazone increase the expression of MBNL1 by inhibiting the CUG^{exp}-MBNL interaction.

OBJECTIVES

General objective

Although extensive data support the involvement of the *Mbnl2* gene in CNS pathology in DM1, there is a lack of studies investigating the neurobiological correlates of CNS alterations related to MBNL2 deficiency. Thus, the main goal of this thesis was to investigate novel mechanisms relevant to DM1 using (i) constitutive *Mbnl2* KO mice and (ii) tissue-specific KO mice, where *Mbnl2* expression is selectively deleted in the glutamatergic neurons of the forebrain ($Mbnl2^{NEX\text{-}Cre}$). The second aim of this thesis was to find novel therapeutic avenues to alleviate the neuropsychological disturbances associated to loss of MBNL2 function.

Specific objectives

1) To study the progression of behavioural alterations in male and female mice associated to a constitutive loss of MBNL2 protein, and to determine the cellular, neurobiochemical, molecular and eletrophysiological correlates of these deficits (Article 1).

2) To evaluate the potential therapeutic use of the psychostimulant methylphenidate and elucidate its mechanisms of action in constitutive *Mbnl2* KO mice (Article 1).

3) To assess the role of the *Mbnl2* gene in forebrain glutamatergic neurons in the behavioural and brain disturbances related to DM1 and investigate the associated neurobiological correlates (Article 2).

4) To examine the potential therapeutic use of the atypical antidepressant mirtazapine and explore its mechanisms of action in tissue-specific $Mbnl2^{NEX\text{-}Cre}$ mice (Article 2).

RESULTS

ARTICLE 1

Methylphenidate Attenuates the Cognitive and Mood Alterations Observed in Mbnl2 Knockout Mice and Reduces Microglia Overexpression

Ramon-Duaso C, Gener T, Consegal M, Fernández-Avilés C, Gallego JJ, Castarlenas L, Swanson MS, de la Torre R, Maldonado R, Puig MV, Robledo P.

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ARTICLE 2

Behavioral and Neurochemical Alterations Associated with Loss of MBNL2 in the Dentate Gyrus are Rescued by Mirtazapine Treatment

Ramon-Duaso C, Rodríguez-Morató J, Selma-Soriano E, Fernández-Avilés C, Artero R, de la Torre R, Pozo O and Robledo P.

Journal of Neuroscience (under review)

Abstract

Myotonic dystrophy type 1 (DM1) is a multisystemic disorder characterized by muscle weakness and wasting and also by important central nervous system-related symptoms including impairments in executive functions, spatial abilities and increased anxiety and depression. The *Mbnl2* gene has been implicated in several phenotypes consistent with DM1 neuropathology. In this study, we developed a tissue-specific knockout mouse model lacking the *Mbnl2* gene in forebrain glutamatergic neurons to examine its specific contribution to the neurobiological perturbations related to DM1. We found that these mice exhibit long-term cognitive deficits and a depressive-like state associated with neuronal loss, increased microglia and decreased neurogenesis, specifically in the dentate gyrus (DG). Chronic treatment with the atypical antidepressant mirtazapine (3 and 10 mg/kg) for 21 days rescued these behavioral alterations, reduced inflammatory microglial overexpression, and reversed neuronal loss in this brain structure. We also show that mirtazapine re-established 5-HT_{1A} and histaminergic H_1 receptor gene expression in the hippocampus. Finally, metabolomics studies indicated that mirtazapine increased serotonin (5-HT), noradrenaline (NA), gamma-aminobutyric acid (GABA) and adenosine. These data suggest that loss of *Mbnl2* gene in the glutamatergic neurons of the DG may underlie the most relevant DM1 neurobiological and behavioral features and provide evidence that mirtazapine could be a novel potential candidate to alleviate these debilitating symptoms in DM1 patients.

Key-words: DM1; tissue-specific Mbnl2 knockout mice; glutamate; depression; cognitive deficits; neuronal loss; inflammation; dentate gyrus; adenosine; GABA; mirtazapine.

1. INTRODUCTION

Myotonic dystrophy type 1 (DM1) is a multisystemic disorder caused by an RNA gain-of-function in which pathological CTG repeats generate toxic RNAs that sequester, most prominently, the muscleblind-like (MBNL) proteins (Fardaei et al., 2002). While MBNL1 is required for skeletal and cardiac muscle development, MBNL2 is highly expressed in the central nervous system (CNS) and MBNL3 is more related to placenta and fetal stage (Miller et al., 2000; Goodwin et al., 2015). Some of the features in adult-onset DM1 include muscular symptoms, cardiac arrhythmias, insulin resistance and cerebral atrophy (Gourdon and Meola, 2017). Also, cognitive impairments, increased anxiety and depression have been reported, indicating a dysfunction of the frontal and temporo-parietal regions (Sansone et al., 2007; Modoni et al., 2008; Winblad et al., 2016; Gallais et al., 2017). Accordingly, imaging studies have confirmed the widespread alteration of cortical and subcortical regions among patients with DM1 (Cabada et al., 2017; Minnerop et al., 2018). Moreover, loss of dopamine (DA) and serotonin (5-HT) neurons, reduced N-acetylaspartate and reduced glutamate (Glu) were found in brains of DM1 patients (Ono et al., 1998a, 1998b; Vielhaber et al., 2006; Takado et al., 2015). Although these data point to deregulation of some neurotransmitter systems in the brains of DM1 patients, there is lack of evidence regarding the neurobiological mechanisms involved, and no specific treatments exist for the neuropathology associated with this disabling disease (Heatwole et al., 2012).

Only a few animal models of brain-related alterations in DM1 have been developed to investigate the neurobiological substrates of this disease (Hernández-Hernández et al., 2013; Wang et al., 2017; Charizanis et al., 2012). Moreover, studies in *Drosophila* flies expressing CUG repeats that

reproduce key features of DM1 disease, such as mis-regulated alternative splicing of muscle genes, Mbnl sequestration in the CNS, and CUG-dependent CNS alterations (de Haro et al., 2006), have been useful for screening new treatment strategies. In this sense, suppression of brain DM1-like phenotypes in this fly model has been reported using several compounds with diverse pharmacological activity (García-Lopez et al., 2008). One class of compounds that suppressed the DM1-related neurotoxicity in this fly model specifically acted on dopaminergic neurons. In accordance, we have previously reported that constitutive *Mbnl2* KO mice exhibiting cognitive impairment and affective deficits associated to DM1 neuropathology were reversed by chronic treatment with the psychostimulant methylphenidate (Ramon-Duaso et al., 2019).

In searching to identify novel candidates with therapeutic potential for DM1 brain phenotypes, in this study we employed the same model already used for previous drug screening; the transgenic *Drosophila* model to assess up to 1200 candidate drugs in functional studies. Among the chemical suppressors of DM1 toxicity found, the atypical antidepressant mirtazapine (MRZ) was selected because of its promising pharmacological profile to further investigate its potential beneficial effects in a mouse model of DM1 Since our previous data showed that constitutive *Mbnl2* KO display behavioral alterations associated with major neurobiological changes in the mPFC and HPC (Ramon-Duaso et al., 2019), here we developed a tissue-specific mouse model lacking the *Mbnl2* gene in glutamatergic neurons of the neocortex and HPC using the Cre-Lox system, and assessed the effects of MRZ on brain and behavioral alterations. MRZ is a noradrenergic and specific serotonergic antidepressant (Berton and Nestler, 2006), characterized by antagonistic activities at presynaptic α_2-adrenergic, 5-HT_2 and 5-HT_3 receptors and by a 5-HT_{1A} agonistic activity (De Boer, 1996).

Interestingly, MRZ can enhance cognitive performance in depressed and schizophrenic patients (Borkowska et al., 2007; Stenberg et al., 2011).

Hence, in this study, we first tested whether loss of MBNL2 specifically in glutamatergic neurons of the forebrain would be sufficient to provoke behavioral and neurochemical alterations associated with DM1, and whether MRZ could be a new promising treatment option for suppression of brain DM1-like phenotypes. Moreover, we investigated the mechanisms of action of MRZ by studying changes in relevant receptors and neurotransmitter systems in the mPFC and HPC applying gene expression and targeted metabolomics techniques.

2. RESULTS

2.1 MRZ Rescues Pupal Viability in the *Drosophila* CNS-DM1 model

In this study, we screened 1200 FDA-approved drugs from the Prestwick Chemical Library at the concentrations of 1 and 10 µM. Out of 1200 drugs tested, we identified 36 compounds that rescued the female *Drosophila* viability, indicating an anti-DM1 effect (Fig. 1d). These anti-DM1 compounds belonged to different therapeutic categories such as antibacterial (8%), anti-inflammatory (11 %), and others (81 %), which included antipsychotics, anticonvulsants and antidepressants. Among the antidepressants, MRZ showed potent anti-toxic effects in the 28 ºC assay (Fig. 1e), significantly rescuing pupal viability at the doses of 1 µM ($p<0.01$) and 10 µM ($p<0.05$), with respect to vehicle administration. Similarly, in the 29 ºC test (Fig. 1f), MRZ showed significant anti-DM1 effect at the doses of 0.1 and 1 µM. Due to the multifarious mechanism of action of MRZ, different from other antidepressants, its beneficial effects could have involved various neurotransmitter receptor targets. Therefore, we assessed the anti-DM1 activity of several compounds with different pharmacological profiles. Our results show that neither the 5-HT reuptake inhibitor fluoxetine, the 5-HT_{2A} receptor antagonists (ritanserin and MDL-11939), nor the 5-HT_3 receptor antagonist, granisetron showed any rescue of pupal viability (Fig. 1g).

2.2 Generation of a Tissue-Specific Mouse Model Lacking the *Mbnl2* gene in Glutamatergic Neurons

Mbnl2 constitutive knockout (KO) mice display a wide range of behavioral alterations, including impaired long-term memory, emotional working memory and increased depressive-like behavior related to DM1-CNS phenotype (Ramon-Duaso et al., 2019). MBNL2 protein is prominently expressed in the HPC (Charizanis et al., 2012), where glutamatergic

neurons are predominant. In this study, we generated a tissue-specific mouse model selectively lacking the *Mbnl2* gene in the forebrain glutamatergic neurons (*Mbnl2*$^{NEX\text{-}cKO}$) (Fig. 2a). We confirmed the cell-specific loss of MBNL2 in excitatory neurons of the forebrain using Western blot analysis of brain tissues. One way ANOVA revealed that, in brain lysates of *Mbnl2*$^{NEX\text{-}cKO}$ mice, MBNL2 protein levels were reduced by 90% in the mPFC (WT: 100±5.88, *Mbnl2*$^{NEX\text{-}cKO}$: 10.39±2.37; $F_{(1,11)}$= 885.2, $p<0.001$) and by 80% in the HPC (WT: 100±2.26, *Mbnl2*$^{NEX\text{-}cKO}$: 19.76±1.48; $F_{(1,11)}$=199.9, $p<0.001$) in comparison with WT mice (Fig. 2b). In the striatum and cerebellum, MBNL2 levels were not altered due to the low expression of NEX in these brain areas. Moreover, we quantified the immunoreactivity of MBNL2 protein by immunofluorescence staining in the mPFC and HPC of WT and *Mbnl2*$^{NEX\text{-}cKO}$. Accordingly, the analysis of MBNL2 expression in *Mbnl2*$^{NEX\text{-}cKO}$ mice showed a significant decrease of immunoreactivity in the mPFC [$F_{(1,11)}$=99.0, $p<0.001$] and HPC [$F_{(1,11)}$=17.5, $p<0.001$] with respect to WT group (Fig. 2c).

2.3 *Mbnl2*$^{NEX\text{-}cKO}$ Mice Exhibit Impaired Long-term Recognition Memory and Depressive-Like Behavior

We first performed a battery of behavioral tests in WT (n=10) and *Mbnl2*$^{NEX\text{-}cKO}$ (n=10) male mice to assess motor, cognitive and affective processes at two time points: 3 and 8 months of age. Total horizontal locomotion and number of rearings in the locomotor cages were not significantly different between groups (Fig. 3a, b). For long-term recognition memory, two-way ANOVA revealed a significant effect of genotype [$F_{(1,39)}$=84.5, $p<0.001$] and interaction [$F_{(2,39)}$=4.8, $p<0.05$], but with no significant effect of time. Subsequent post-hoc analysis revealed that *Mbnl2*$^{NEX\text{-}cKO}$ mice exhibited a greater cognitive impairment than WT mice ($p<0.001$) at both 3 and 8 months of age (Fig. 3c). For depressive-like behavior, two-way ANOVA only revealed a significant main effect of

genotype [$F_{(1,39)}$=102.8, p<0.001], indicating that *Mbnl2*$^{NEX\text{-}cKO}$ exhibited higher immobility time compared to WT at both 3 and 8 months (Fig. 3d). These data indicate that the behavioral alterations observed due to loss of MBNL2 in glutamatergic neurons of the neocortex and HPC persist in time, lasting at least up to 8 months of age.

2.4 Chronic Treatment with MRZ Reduces the Cognitive Deficits and Depressive-Like Phenotype in *Mbnl2*$^{NEX\text{-}cKO}$ Mice

The effects of chronic treatment with MRZ were tested in WT (n=10 per group) and *Mbnl2*$^{NEX\text{-}cKO}$ (n=10 per group) male mice at 4 months of age. In locomotor activity, no statistical differences were observed in total horizontal locomotion or number of rearings (Fig. 4a, b). In the NOR test, two-way ANOVA revealed significant main effects of genotype [$F_{(1,59)}$=33.4, p<0.001], treatment [$F_{(2,59)}$=21.1, p<0.001] and interaction between factors were observed [$F_{(2,59)}$=18.1, p<0.001]. The post-hoc test revealed that *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with vehicle displayed cognitive deficits in comparison with WT mice (p<0.001), and chronic treatment with MRZ significantly reversed this impairment at both doses of MRZ tested (p<0.001) (Fig. 4c). In the FST, two-way ANOVA revealed significant main effects of genotype [$F_{(1,59)}$=9.5, p<0.01], treatment [$F_{(2,59)}$=8.8, p<0.001] and interaction between factors were found [$F_{(2,59)}$=10.6, p<0.001]. Subsequent analysis revealed a significant increase in immobility time in *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with vehicle with respect to WT controls (p<0.001), indicating a depressive-like state, that was attenuated at both doses of MRZ (p<0.001) (Fig. 4d).

2.5 *Mbnl2*$^{NEX\text{-}cKO}$ Mice Show Increased Neuroinflammation in the DG of the HPC and Chronic Treatment with MRZ reverses this Effect

Microglial expression was evaluated by quantifying the Iba1 marker in the mPFC and HPC of WT (n=6 per group) and *Mbnl2*$^{NEX\text{-}cKO}$ (n=6 per group) male mice. In the mPFC and CA1 and CA3 fields of the HPC (Fig. 5a-c) the number of Iba1 positive cells was not significantly different between groups. In contrast, in the DG, two-way ANOVA showed a significant main effect of genotype [$F_{(1,35)}$=18.6, $p<0.001$], treatment [$F_{(2,35)}$=18.7, $p<0.001$] and interaction between factors [$F_{(2,35)}$=5.7, $p<0.01$] were observed. Further analysis revealed a significant increase in microglial expression in *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with vehicle compared to WT controls ($p<0.001$), and both doses of MRZ (3 and 10 mg/kg) attenuated this overexpression ($p<0.001$) (Fig. 5d). Moreover, to classify the state of the exacerbated microglia in the HPC, we performed a gene expression analysis in whole hippocampal lysates, with the pro-inflammatory marker interleukin 1 beta (IL-1β) and the anti-inflammatory marker transforming growth factor (TGF-β). For expression of IL-1β , two-way ANOVA showed a significant main effect of treatment [$F_{(2,35)}$=7.1, $p<0.01$] and interaction between factors [$F_{(2,35)}$=7.5, $p<0.01$]. Post-hoc analysis revealed an increase of transcriptional levels of IL-1β in *Mbnl2*$^{NEX\text{-}cKO}$ treated with vehicle with respect to WT mice ($p<0.01$), and both doses of MRZ reversed this inflammatory process ($p<0.01$) (Fig. 5e). However, no significant effects of treatment were observed in the gene expression of the anti-inflammatory marker TGF- β between groups (Fig. 5f).

2.6 *Mbnl2*$^{NEX\text{-}cKO}$ Mice Exhibit Neuronal Loss in the DG of the HPC and MRZ reduces this Effect

Neuronal expression was evaluated by quantifying the NeuN marker in the mPFC and HPC of WT (n=6 per group) and *Mbnl2*$^{NEX\text{-}cKO}$ (n=6 per group) male mice. No statistical differences were observed between groups in the

mPFC, CA1 and CA3 brain areas (Fig. 6a-c). In contrast, in the DG, two-way ANOVA revealed a significant interaction between factors [$F_{(2,35)}$=5.6, p<0.01], but no significant main effect of genotype or treatment. Moreover, post-hoc test revealed that vehicle-treated *Mbnl2*$^{NEX\text{-}cKO}$ mice showed a significant decrease in the number of NeuN positive cells with respect to WT mice (p<0.01) and this effect was reversed with the dose of 10 mg/kg of MRZ (p<0.05) (Fig. 6d). Given that exacerbated microglia and neuronal loss were mainly observed in the DG, we performed studies of neurogenesis and apoptosis, two processes that could be dysregulated in *Mbnl2*$^{NEX\text{-}cKO}$ mice. In BrdU immunostaining, although a tendency for less cell proliferation in vehicle-treated *Mbnl2*$^{NEX\text{-}cKO}$ mice was observed in comparison with WT mice, Two-way ANOVA only revealed a significant main effect of treatment [$F_{(2,35)}$=76.1, p<0.01], but with no main effect of genotype or interaction between factors (Fig. 6e) indicating that MRZ increased cell proliferation in both genotypes equally. In DCX expression, two-way ANOVA showed a significant main effect of treatment [$F_{(2,35)}$=40.6, p<0.001], and interaction between factors [$F_{(2,35)}$=4.6, p<0.05], with no main effect of genotype. Further analysis revealed a significant decrease of DCX integrated density in *Mbnl2*$^{NEX\text{-}cKO}$ treated with vehicle compared to WT group (p<0.001), indicating a reduction of immature cells in *Mbnl2*$^{NEX\text{-}cKO}$ mice. MRZ, however, significantly increased DCX in both genotypes to a similar extent (p<0.01) (Fig. 6f). No significant differences between groups treated with vehicle were observed in caspase-3 protein (data now shown), indicating that there is no deregulation in the cell death cycle.

2.7 MRZ Modulates the Expression of 5-HT$_{1A}$ and H$_1$ Receptors in the HPC of *Mbnl2*$^{NEX\text{-}cKO}$ mice

To gain deeper insight into the neurobiological substrates that underlie the behavioral alterations observed in *Mbnl2*$^{NEX\text{-}cKO}$ mice and the mechanisms

of action of MRZ, we evaluated the transcriptional activity of 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, 5-$HT_{3,}$ adrenergic α_{2A} and histamine H_1 receptors in the mPFC and HPC of WT (n=6 per group) and *Mbnl2*$^{NEX\text{-}cKO}$ (n=6 per group) male mice treated chronically with VEH or MRZ (3 and 10 mg/kg). In the mPFC, no significant differences were found between treatments in the expression levels of serotonin receptors (Fig. 7a-c). However, a main effect of genotype was revealed for 5-HT_{1A}R mRNA levels [$F_{(2,35)}$=24.8, p<0.001], indicating a lower expression in *Mbnl2*$^{NEX\text{-}cKO}$ mice (Fig. 7d). For alpha$_{2A}$ receptor, a significant interaction was observed between factors [$F_{(2,35)}$=3.0, p<0.05]. Post-hoc analysis revealed decreased levels of alpha$_{2A}$ receptor in *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with vehicle as compared with WT mice (p<0.05) and no significant differences between genotypes were found following MRZ treatment (Fig. 7e). Two-way ANOVA did not reveal any significant differences in the histamine H_1 receptor (Fig. 7f). In the HPC, statistical analysis revealed a significant main effect of genotype for 5-HT_{2A} [$F_{(2,35)}$=5.9, p<0.05] and for 5-HT_{2C} [$F_{(2,35)}$=8.3, p<0.01] gene expression (Fig. 7g, h). The transcriptional levels of 5-HT_3 and alpha$_{2A}$ receptors were not significantly different between groups (Fig. 7i, k). However, for 5-HT_{1A}R gene expression, a significant interaction between genotype and treatment was observed [$F_{(2,35)}$=5.8, p<0.01]. Post-hoc comparisons showed that 5-HT_{1A}R mRNA levels were significantly decreased in vehicle-treated *Mbnl2*$^{NEX\text{-}cKO}$ mice as compared to WT mice (p<0.05), and MRZ reverse this effect at the dose of 10 mg/kg (p<0.001) (Fig. 7j). For the transcriptional levels of histaminergic H_1 receptor, a significant main effect of genotype [$F_{(2,35)}$=5.1, p<0.05] and a significant interaction between genotype and treatment [$F_{(2,35)}$=12.2, p<0.001] were found. Subsequent analysis showed a significant increase of H_1 levels in *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with vehicle as compared to WT group (p<0.001), and MRZ reverse this effect at the dose of 3 mg/kg (p<0.01) and 10 mg/kg (p<0.001) (Fig. 7l).

2.8 MRZ Modulates Monoamine, GABA and Adenosine production in the HPC of *Mbnl2*$^{NEX\text{-}cKO}$ mice

To further examine possible alterations in neurotransmitter systems, we performed targeted metabolomics studies after chronic treatment (21 days) with vehicle or MRZ (3 and 10 mg/kg) in brain homogenates of mPFC and HPC of WT (n=6 per group) and *Mbnl2*$^{NEX\text{-}cKO}$ (n=6 per group) male mice. We determined the tryptophan (Trp) and tyrosine (Tyr) metabolism in order to quantify the generation of 5-HT and NA neurotransmitters, as well as, the generation of GABA, Glu, and adenosine neurotransmitters.

We quantified the ratio of 5-HT/Trp as a measure of 5-HT production, and in the mPFC, statistical analysis revealed a significant interaction between factors [$F_{(2,30)}$=4.2, $p<0.05$], but no significant main effects of genotype or treatment. Post-hoc analysis showed that chronic treatment with 10 mg/kg of MRZ significantly reduced the generation of 5-HT in WT mice compared to those treated with vehicle ($p<0.001$) (Fig. 8a). In contrast, in the HPC, two-way ANOVA showed a significant interaction in the ratio 5-HT/Trp [$F_{(2,30)}$=6.4, $p<0.01$]. Subsequent analysis exhibited a significant decrease in the production of 5-HT from its precursor Trp in *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with vehicle compared to WT mice ($p<0.05$). Moreover, MRZ at 10 mg/kg significantly reduced the generation of 5-HT in WT mice in comparison with WT group treated with vehicle ($p<0.05$) (Fig. 8f). No significant differences were observed when considering 5-HT degradation (5-HIAA/5-HT) (Data not shown).

We quantified the ratio of NA/Tyr as a measure of NA production, and the statistical analysis in the mPFC showed a significant interaction between factors [$F_{(2,30)}$=5.4, $p<0.05$] with a significant decrease of NA production in *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with vehicle compared to WT mice ($p<0.05$) while MRZ at 10 mg/kg significantly reduced the generation of NA in WT mice compared to WT group ($p<0.05$) (Fig. 8b). In the HPC, two-way

ANOVA revealed a significant main effect of genotype [$F_{(1,30)}$=17.9, p<0.001] and interaction between factors [$F_{(2,30)}$=5.9, p<0.01]. Post-hoc analysis showed an increase in NA production in *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with MRZ at 10 mg/kg in comparison with WT group (p<0.001), while this dose of MRZ reduced the production of NA in the WT group in comparison with WT mice treated with vehicle (p<0.05) (Fig. 8g).). No significant differences were observed when considering NA degradation (NE/NA) (Data not shown).

We quantified the ratio of GABA/Glu as a measure of GABA production, and the statistical analysis in the mPFC did not expose significant differences between groups (Fig. 8c). In contrast, in the HPC, a significant interaction between factors was found [$F_{(2,30)}$=4.8, p<0.05]. Subsequent analysis revealed a significant increase of GABA production in *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with MRZ 10 mg/kg in comparison with *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with vehicle (p<0.05) (Fig. 8h).

We also determined the ratio of Glu/Gln as a measure of Glu production and statistical analysis only showed a significant main effect of genotype in mPFC [$F_{(1,30)}$=5.0, p<0.05] and HPC [$F_{(1,30)}$=28.3, p<0.001], indicating that *Mbnl2*$^{NEX\text{-}cKO}$ mice had lower generation of Glu compared to WT group.(Fig 8d, i). The statistical analysis of adenosine concentrations showed a main effect of genotype [$F_{(1,30)}$=4.4, p<0.05] in the mPFC due to an increase in WT mice treated with 10 mg/kg of MRZ, with no main effect of treatment or interaction between factors (Fig. 8e). In the HPC, a significant interaction was found [$F_{(2,30)}$=4.4, p<0.05], with no main effects of genotype or treatment. Post-hoc analysis revealed a significant reduction of adenosine levels in *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with vehicle in comparison with WT group (p<0.05) and the dose of 10 mg/kg of MRZ reversed this effect (p<0.05) (Fig. 8j).

3. DISCUSSION

Our previous work demonstrated that constitutive deletion of *Mbnl2* in mice resulted in cognitive impairments and depressive-like behavior, that persisted up to 5 months of age, and recapitulated the neuropsychological disturbances observed in DM1 (Ramon-Duaso et al., 2019). The present findings reveal that conditional loss of the *Mbnl2* gene specifically in forebrain glutamatergic neurons of the PFC and HPC provoked: (i) long-lasting DM1-related behavioral disturbances, and (ii) neuronal and glial alterations in the DG and receptor/neurotransmitter changes in the HPC. Accordingly, deregulation of neural networks between mPFC and HPC has been shown to occur in DM1 patients (Romeo et al., 2010), and is associated to alterations in cognition and affective functions (Sampath et al., 2017; Richardson and Adams, 2018). Several clinical studies have shown that most patients with depression, including DM1 patients (Nätterlund and Ahlström, 2001), report significant deficits in executive function, memory and attention (Russo et al., 2015; Kaser et al., 2017). Although both cognitive dysfunction and depression represent a major burden for patients and family, there are no medications currently approved for treating the complex CNS symptomatology seen in DM1 patients.

Our *in vivo* screening results in a transgenic *Drosophila* showed that the atypical antidepressant, MRZ was able to rescue female pupal viability, suggesting anti-CNS-related toxicity in this DM1 fly model. To explore the mechanisms of action of MRZ, we treated flies with compounds known to be targets of MRZ. The results revealed that neither the SSRI, fluoxetine nor the 5-HT_{2A}R antagonists (ritanserin and MDL-11939), or the 5-HT_3R antagonist, granisetron induced anti-toxicity effects when tested individually. These findings suggested that the effects of MRZ could be due to a combinatory action on all of these targets, or on other neurotransmitter receptors that were not tested in the assay. Therefore, we examined the

effects of MRZ on the behavioral and neurochemical alterations observed in the *Mbnl2*$^{NEX\text{-}cKO}$ mouse model, selecting two low doses (3 and 10 mg/kg) from our pilot study that did not reduce locomotor activity in WT mice, to ensure the lack of confounding sedative effects in our behavioral studies. Indeed, some clinical trials have shown that small doses of MRZ may induce excessive sedation, while other studies have not reported this effect (Anttila and Leinonen, 2001). Our data in *Mbnl2*$^{NEX\text{-}cKO}$ mice showed that MRZ reversed both cognitive impairment and depressive-like behavior without affecting locomotor activity.

The behavioral changes in *Mbnl2*$^{NEX\text{-}cKO}$ mice were associated with exacerbated microglia expression specifically in the DG of the HPC in comparison with WT mice. Moreover, the transcriptional levels of the pro-inflammatory cytokine, IL-1β, were enhanced in the HPC of these mice, while no alterations were found in the gene expression of TGF-β, an anti-inflammatory cytokine. Interestingly, previous studies have reported a microglia enhancement in DM1 brains (Satoh et al., 2016), attributable to a high prevalence of white matter damage in this disease (Itoh et al., 2010). In addition to increased microgliosis, *Mbnl2*$^{NEX\text{-}cKO}$ mice exhibited significant neuronal loss, decreased neurogenesis, and significantly fewer proliferating and immature neurons in the DG of the HPC. These findings are consistent with the diffuse brain damage observed in DM1 patients (Mizukami et al., 1999; Itoh et al., 2010; Takado et al., 2015), and with the data showing that both depression and cognitive deficits are related to altered neurogenesis in the HPC (Cao et al., 2004; Papakostas and Culpepper, 2015). While the behavioral alterations observed in the tissue-specific *Mbnl2*$^{NEX\text{-}cKO}$ model are similar to those observed in constitutive *Mbnl2* mice (Ramon-Duaso et al., 2019), the associated changes in neuronal and glial cells show several differences in both models. Hence, in the constitutive KO we observed more extended microgliosis, including the

mPFC and the entire HPC, but no neuronal loss. In the conditional KO however, microgliosis was restricted to the DG of the HPC, where neuronal loss was also present. These findings imply that loss of MBNL2 constitutively induces more wide-spread brain inflammation than a tissue-specific deletion in glutamatergic neurons. Moreover, the specific effects observed in the DG indicate that this structure is particularly sensitive to the lack of MBNL2 protein, leading to the observed behavioral deficits. In accordance, studies in rats have demonstrated that the DG is required to mediate pattern separation (Kesner et al., 2004), a central mechanism for novelty detection within an environment and for learning and memory (Bakker et al., 2008). In fact, it has been described that DG-lesioned rats are not able to discriminate between the familiar and novel object (Hunsaker et al., 2008). Also, impaired adult neurogenesis in the DG has been related to depressive states (Jacobs et al., 2000). Overall, our data reveal that lack of MBNL2 protein in the DG (inducing neuronal loss, decreased neurogenesis, and inflammatory microglia activation) is sufficient to cause cognitive deficits and depression-like behavior.

Notably, chronic treatment with MRZ attenuated microglial and pro-inflammatory cytokine overexpression, and neuronal loss in *Mbnl2*$^{\text{NEX-cKO}}$ mice, and increased neurogenesis in the DG of WT and *Mbnl2*$^{\text{NEX-cKO}}$ mice. Thus, by restoring neuronal and glial function in the DG of the HPC, MRZ may reverse the depressive-like behavior and cognitive impairment in *Mbnl2*$^{\text{NEX-cKO}}$ mice. In accordance, cellular adaptations in the HPC after repeated antidepressant administration have been shown to play an important role in their behavioral effects (Malberg et al., 2000; Santarelli et al., 2003). Moreover, chronic treatment with MRZ decreases plasma levels of pro-inflammatory markers in depressed patients (Tulner et al., 2011), and reestablishes the equilibrium between physiological and pathological levels of cytokines in the brain (Müller and Schwarz, 2007; Sutcigil et al., 2007).

Since loss of MBNL2 protein in forebrain glutamatergic neurons lead to neuronal loss and microglia enhancement in *Mbnl2*$^{NEX\text{-}cKO}$ mice, we next investigated more specifically which types of neuronal receptors and neurotransmitters were altered, and whether MRZ administration could reverse these changes. In the first place, we examined the expression changes of serotonergic, noradrenergic, and histaminergic receptors, which are known targets of MRZ. Our data showed a significant reduction in 5-HT_{1A}R gene expression, and an increase in H_1R transcriptional levels specifically in the HPC of *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with vehicle. Decreased 5-HT_{1A}R gene transcription in these mice could be related to neuronal loss found in the HPC, where they are highly expressed. Moreover, these receptors are crucially involved in depression, anxiety and memory processes (Le François et al., 2008). On the other hand, an increase in H_1R may be associated to inflammatory processes, since these receptors contribute to the effects of histamine on microglial activation following neural death (Rocha et al., 2016). Interestingly, MRZ increased 5-HT_{1A}R and reduced H_1R gene expression in the HPC of *Mbnl2*$^{NEX\text{-}cKO}$ mice. Thus, the neuroprotective effects of MRZ in the HPC of *Mbnl2*$^{NEX\text{-}cKO}$ mice may be due to a reduction in microglia activation through its action on H_1R.

Secondly, we determined changes in the production of brain neurotransmitters and their metabolites in HPC and mPFC homogenates using targeted metabolomics. We found that *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with vehicle presented a significantly lower production of 5-HT and adenosine in the HPC. Accordingly, loss of 5-HT neurons has been reported in DM1 patients (Ono et al., 1998b). As expected by its mechanisms of action, chronic treatment with MRZ at 10 mg/kg increased 5-HT and NA production (Anttila and Leinonen, 2001). Interestingly, we also found that this dose of MRZ increased GABA and adenosine production in the HPC of *Mbnl2*$^{NEX\text{-}cKO}$ mice. This result is in line with previous studies showing

that MRZ increases GABA and reduces Glu concentrations in nucleus accumbens homogenates in a rat model of chronic mild stress (Kamal et al., 2013). While no evidence is currently available as to the effects of MRZ on adenosine neurotransmission, our data suggests that this compound may be increasing adenosine in the HPC to modulate neuronal and glial stability. Indeed, adenosine acting on A_1 receptors reduces excitatory transmission, and is neuroprotective in brain injuries (Ribeiro et al., 2003).

Together, these data reveal that the beneficial effects of MRZ may be associated with re-establishing receptor function in known targets, such as $5HT_{1A}R$ and H_1R, and also by alternative mechanisms involving GABA and adenosine neurotransmission in the HPC

In conclusion, using the *Mbnl2*$^{NEX\text{-}cKO}$ mouse model we revealed that granule cells of the DG and surrounding glial cells are markedly sensitive to loss of MBNL2 protein leading to brain-related DM1 impairments. These results provide new insights into the neurobiological mechanisms related to brain phenotypes in DM1. In addition, we provide evidence that MRZ could be a potential new treatment to counteract the neurodegenerative process, and the cognitive and affective disorders observed in DM1, through a mechanism of action involving known targets, such as 5-HT and histaminergic receptors, but also through a novel mechanism of action involving GABA and adenosine neurotransmission.

4. MATERIALS AND METHODS

4.1 *Drosophila* Transgenes

The *Drosophila mushroom body-Gal4 (MB-Gal4)* line (Tettamanti et al., 1997) was obtained from Dr. J.D. Armstrong *(*Institute of Biomedical and Life Sciences, University of Glasgow, United Kingdom) and *the yw* line was obtained from Bloomington *Drosophila* Stock Center (Indiana University). The recombinant lines *yw; +; UAS-CTG480* and *yw; +; UAS-CTG480 UAS-GFP* are described in Garcia-Lopez et al. 2008. Flies were maintained at 25 ºC on standard nutritive medium unless stated otherwise. X chromosome-linked targeted expression of 480 CTG repeats to the mushroom bodies (MB) originates a temperature-sensitive viability phenotype that affects only females while males serve as an internal control (Fig. 1a-c). Thus, at 29 ºC no adult females hatch from the pupa whereas at 28 ºC a small percentage of females are able to emerge (Garcia-Lopez 2008). We used this DM1 fly model to identify drugs that significantly modify CUG repeat-induced toxicity as candidate therapies against DM1.

4.2 Generation of Conditional *Mbnl2* Knock-out Mice (*Mbnl2*$^{NEX\text{-}cKO}$)

To induce a tissue-specific deletion of *Mbnl2* gene in forebrain glutamatergic neurons, *Mbnl2*$^{fl/fl}$ conditional mice (kindly provided by Dr. M. Swanson, University of Florida) were crossed with mice expressing a *Cre* recombinase under the neuronal differentiation 6 (NeuroD6) gene promoter (also known as NEX: kindly provided by Dr. K. Nave, Max Planck Institute) (see Suppl. Fig.S1). NEX-Cre transgene mice (Goebbels et al., 2006) were kindly shared by Klaus-Armin Nave (Max Planck Institute of Experimental Medicine, Göttingen, Germany). Each breeding cage consisted of two homozygous female mice (genotype: *Mbnl2*$^{fl/fl}$) with one hemizygous male mouse (genotype: Cre$^{+/-}$). Mice were genotyped using a PCR-based strategy to detect the presence of the LoxP flanked allele

and the integration of the *Cre* transgene. The genotyping primers are shown in Supplementary Table S1. Mice were bred in the animal facilities of the PRBB to generate WT and *Mbnl2*$^{NEX\text{-}cKO}$ littermates. All mice were grouped-housed and maintained in a temperature (21 ± 1°C) and humidity (50 ± 10%) controlled environment in a room with a 12 h light/dark cycle (lights on at 8 am and off at 8 pm) with food and water available *ad libitum.* All the behavioral experiments were performed during the light phase of the light/dark cycle by a trained observer that was blind to experimental conditions. Animal procedures were adhered to the standard guidelines of the National Institutes of Health 1995; European Communities Directive (86/609 EEC) and approved by the local ethical committee (CEEA-PRBB). After behavioral assessment, mice were euthanized by cervical dislocation to obtain brain areas for western blot, quantitative PCR and targeted metabolomics or transcardial perfused for immunohistochemical analysis. For the timeline of experimental procedures see Supplementary Figure S2.

4.3 Drugs and treatments

Drosophila i*n vivo* drug screening was performed as follows: two *yw;+ ; UAS-CTG480* females were crossed with one *MB-Gal4* male and placed in vials containing nutritive media supplemented with the compounds from the Prestwick Chemical Library (PCL; 1200 compounds) (http://www.prestwickchemical.com/libraries-screening-lib-pcl.html) at 1 µM final concentration. The crosses were cultured for two days at 25ºC in 96-well trays, where the first column contained vials of the control crosses (two *yw* females and one *MB-Gal4* male) and the twelfth column was for crossings of two *yw; +; UAS-CTG480* females and one *MB-Gal4* male treated with vehicle (DMSO). Offspring were shifted to 28 or 29 ºC till hatching and the number and sex of F1 adult progeny was scored. Compounds were tested per triplicate and those showing the capacity to

rescue any female viability at 29 ºC were considered primary hits. Males were used as internal controls to discard toxic drugs.

MRZ (Alomone Labs) of a purity of 99.5% was dissolved in a vehicle solution (5% DMSO, 5% Tween80 and 90% saline) and administered intraperitoneally (i.p.) in a volume of 0.1 ml/10 g of body weight. To determine the optimal doses of MRZ to be used for chronic administration, we first carried out a dose-response curve in locomotor studies. Male WT and *Mbnl2*$^{NEX\text{-}cKO}$ littermates were treated once daily and during 5 consecutive days with vehicle (VEH) or 1, 3, 10 and 30 mg/kg of MRZ. Thus, we evaluated the effects of acute (after a single administration) and subchronic (after following 5 days) treatment by placing the mice in the locomotor cages during 1 hour. At both time points, only the highest dose of MRZ, 30 mg/kg, significantly reduced the locomotor activity in comparison with VEH administration (See Supplementary figure S2). Thus, to avoid the sedative effects of the drug, WT and *Mbnl2*$^{NEX\text{-}cKO}$ were treated with VEH or MRZ (3 and 10 mg/kg) once daily for 21 days. See Supplementary Figure S2 for a timeline of experimental procedures.

4.4 Behavioral assessment

4.4.1 Locomotor activity assessment

To evaluate locomotor responses in WT and *Mbnl2*$^{NEX\text{-}cKO}$ male mice, mice were tested in individual locomotor boxes (9 x 20 x 11 cm; Imetronic, Bordeaux, France) equipped with two lines of infrared beams in low-lighting conditions (20 lux). Total horizontal activity and the number of rearings (standing on rear limbs) were analyzed during a 30 min-period.

4.4.2 **Novel Object Recognition (NOR)**

Long-term recognition memory was assessed using the novel object recognition (NOR) test with the V-maze at 15 lux as previously described

(Busquets-Garcia et al., 2013). Briefly, this test consists of 3 phases of 9 min each (habituation, familiarization and test). The first day, mice were habituated to the empty maze. The second day, mice were placed again in the maze and 2 identical objects were presented. Twenty-four hours later, one of the familiar objects was replaced with a novel object and the time exploring both objects (novel and familiar) was measured. Object exploration was defined as the orientation of the nose towards the object and touching it with the nose. A discrimination index (DI) was used as outcome of cognitive behavior. A higher DI score represents a greater recognition memory.

4.4.3 Forced Swim Test (FST)

Depressive-like behavior was evaluated using the forced swim test (FST) (Porsolt et al., 1977). Briefly, mice were placed for 6 min into a transparent Plexiglas cylinder filled with water (24 ± 1°C), and the total immobility time was measured for the last 4 min of the test. Immobility was considered when the animal made no movements to escape. An increase in immobility is associated with a higher depressive-like state.

4.5 Western blotting

To validate the strategy of the tissue-specific *Mbnl2*$^{NEX\text{-}cKO}$ mouse line, we performed western blots in cortical, hippocampal, striatal and cerebellar brain areas to quantify the MBNL2 protein. A general immunoblot analysis was followed as previously described (Abad et al., 2019). Briefly, to extract total protein, tissues were dounce-homogenized at 4ºC in 30 volumes of RIPA buffer (Roche Diagnostics, Barcelona, Spain). Equal amounts of protein (40 μg) were loaded onto a 10% acrylamide gel and transferred to Immobilion-P PVDF sheets (Millipore, Darmstadt, Germany). Membranes were blocked for 1h at room temperature with 5% skimmed milk and incubated 2 h at room temperature with mouse anti-Mbnl2 (1:500, Sta. Cruz

Technologies; sc-136167). Then, membranes were incubated with the corresponding secondary antibody mouse anti-GAPDH (1:5.000, Sta. Cruz Technologies; sc-32233). The detection was visualized by chemiluminescence ChemiDoc XRS methodology and protein expression levels were quantified by QuantityOne software (Bio-Rad, Madrid, Spain). The optical density of the bands was normalized to the amount of housekeeping control GAPDH and expressed as a percentage of control. The whole list of antibodies used in these experiments is shown in Supplementary Table S2.

4.6 Immunofluorescence studies

Forty-eight hours after the behavioral assessment, mice were anesthetized by an i.p. injection of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) and transcardially perfused with 4% paraformaldehyde. Coronal sections of 30 µm containing the cingulate, prelimbic and infralimbic areas of the mPFC (from bregma +2.71 to +1.70 mm) and the CA1, CA3 and dentate gyrus (DG) of the HPC (from bregma -1.22 to -2.46 mm) were obtained using a cryostat (Leica CM3050 S). The expression of ionized calcium binding adapter molecule 1 (Iba1) and neuron specific nuclear protein (NeuN) was determined by immunofluorescence studies as described previously (Ramon-Duaso et al., 2019). The primary and secondary antibodies used are list in Supplementary Table S2. Moreover, to analyze proliferation-related markers, we used 5-bromo-2'-deoxyuridine (BrdU) to label the proliferating population that incorporated BrdU during the S phase of the cell cycle and the microtubule-associated protein doublecortin (DCX) to label immature neurons (up to four weeks), which may have started to develop a differentiated neuronal morphology (Luján et al., 2018). For BrdU labeling, mice were treated with a single *i.p.* injection of BrdU at the dose of 100 mg/kg (Sigma, St.Louis, MO) after chronic treatment with MRZ and behavioral assessment. For experimental

procedures see Supplementary Figure S2. A DNA denaturation step in 2 M HCl for 15 minutes at 37ºC was required only for BrdU staining (Wojtowicz and Kee, 2006). Images were obtained using a Nikon Eclipse Ni-E microscope. Six to eight representative images from each mouse were analyzed defining a region of interest (ROI) that remained constant in all experimental groups. All images were processed using the ImageJ software (NIH). The quantification of BrdU and DCX positive cells from the subgranular zone (SGZ) of the HPC was performed as previously described (Saravia et al., 2019).

4.7 Quantitative Real-Time PCR analysis

We determined the transcriptional levels of several neurotransmitter receptors, including 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, 5-$HT_{3,}$ adrenergic α_{2A} and histamine H_1. This procedure was performed as previously described (Duart-Castells et al., 2018) with minor modifications. Total RNA isolation was carried out by RNeasy Mini Kit (Tissue; QIAGEN) and 300 ng of each sample was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was performed using the PowerUp SYBR Green Master Mix and analyzed by QuantStudio™ 12K Sequence Detection System (Applied Biosystems). Primers used are listed in Supplemental Table S3. mRNA expression was normalized to the relative expression value of β-actin, as endogenous housekeeping gene. Gene expression was determined using the comparative cycle threshold (Ct) method and the results are reported as fold change compared with the control.

4.8 Metabolomics by LC-MS/MS

For the targeted metabolomics study, we used brain homogenates from the mPFC and HPC of WT and *Mbnl2*$^{NEX\text{-}cKO}$ male mice that had been treated chronically with vehicle, MRZ 3 and 10 mg/kg during 21 days. We applied

two previously reported methods focused on the quantification of neurotransmitters and related compounds in mice brain homogenates. Forty-eight hours after the behavioral assessment, a group of mice were sacrificed, and polar neurotransmitters (GABA, glutamate, and adenosine) were determined as previously described (Olesti et al., 2019a). Given the short half-life of monoamines (Cransac et al., 1996), another group of mice was sacrificed 1 hour after the last vehicle or MRZ (3 mg/kg and 10 mg/kg during 21 days) administration, and the production of NA and 5-HT was assessed (Olesti et al., 2019b). For analysis of polar neurotransmitters, an aliquot of 10 µL of brain homogenate was transferred into a glass tube and was spiked with 50 µL of the internal standard mixture. Then, 130 µL of acetonitrile were added to precipitate the proteins. After centrifugation (5 min; 4000 rpm; 2650 g) the supernatant was transferred to a microvial and directly injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system. A 1:100 dilution was required for the analysis of GABA, glutamine (Gln), glutamate (Glu) and adenosine. For the analysis of monoamines, an aliquot of 200 µL of HPC or mPFC was transferred into a glass tube, was spiked with 100 µL of internal standard mixture, and was dried under nitrogen gas. Then, it was reconstituted with 50 µL of $NaHCO_3$ 100 mM buffer (pH = 10.5) and was derivatized using 100 µL of dansyl chloride solution (1 mg/mL in acetone; 60ºC; 20 min). The solvent was then transferred to a microvial and was directly injected into the LC-MS/MS system. In both methods, the LC-MS/MS system consisted on an Acquity UPLC system (Waters Associates) for the chromatographic separation coupled to a triple quadrupole (Xevo TQ-S micro) mass spectrometer provided with an orthogonal Z-spray-electrospray interface (ESI) (Waters Associates, Milford, MA, USA). Analytes were determined by a Selected Reaction Monitoring (SRM) method by acquiring two transitions for each compound. Besides the response of the analytes, ratios between selected metabolites were also considered in order to evaluate not only the levels of

neurotransmitters, but also their formation and degradation. As an example, the ratio 5-HT/Trp was selected as indicative of 5-HT biosynthesis whereas the ratio 5-HIAA/5-HT was considered for the evaluation of 5-HT degradation. MassLynx software V4.1 and TargetLynx XS were used for data management.

4.9 Statistical Analysis

In *Drosophila* studies, *in vivo* drug screening at 28 ºC was analyzed using Fisher's exact test, in which it was assumed that the number of males is equal to the expected number of females in case the rescue was complete. One-way ANOVA was used to confirm the specific depletion of the *Mbnl2* gene in the generation of the new tissue-specific mouse line. Two-way ANOVA was used to analyze the behavioral data with genotype (WT and *Mbnl2*$^{NEX\text{-}cKO}$) as a between-subjects factor, and age (3 and 8 months of age) as a within-subjects factor. The effects of chronic treatment with MRZ were analyzed using 2-way ANOVAs with genotype and dose as between-subject factors. Subsequent Bonferroni's Post-hoc test was used when required. All data were analyzed using SPSS and GraphPAD Prism 8.0 software. Comparisons were statistically significant when the level of significance was $p<0.05$.

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FIGURE LEGENDS

Figure 1. *In vivo* drug screening using the *MB-Gal4 Drosophila* line. (a) Representative images of control males, expressing no repeats in the mushroom bodies (MB) and emerging from pupae (normal fly). DM1 females are unable to completely emerge from the pupal stage (minus compound). DM1 females rescued by the oral administration of defined drugs (plus compound). (b) Confocal image of the MB structure marked by GFP reporter under the control of MB-Gal4 driver (green) and a graphical representation of the left lobe of the MBs. (c) 96-well trays were used for the screening of 1200 compounds. (d) Compounds showing anti-DM1 effects in the *Drosophila* model: the table indicates the number of hits from each therapeutic group. Pharmacological studies with mirtazapine (MRZ) at different concentrations: the number of adults which emerged from pupae with standard nutritive media supplemented with DMSO (control) or with the indicated concentrations of MRZ. (e) At 28 ºC in the control DMSO treatment, a small percentage of females were able to emerge (n=15), whereas following MRZ (1 and 10 µM) an increase in female offspring was detected (n=20 and n=14, respectively). (f) At 29 ºC, no female pupae hatched in the non-treated group, while MRZ (0.1 and 1 µM) rescued at least 1 adult female. (g) The atypical antidepressant MRZ, with a multimodal activity, had anti-DM1 activity, while closely related compounds with different pharmacological profiles did not show a significant rescue. Hit compounds were required to score at least 5 males in each of the 3 replicates to discard toxicity issues. * $p<0.05$, ** $p<0.01$ (Fisher's Exact test).

Figure 2. Generation and validation of conditional knock-out strategy of *Mbnl2*$^{NEX\text{-}cKO}$ mice. (a) Generation of conditional Mbnl2$^{NEX\text{-}cKO}$ mouse line. On the right, expression pattern of NEX-Cre promoter. (b) Western blot analysis of MBNL2 protein levels in the mPFC, HPC, striatum and cerebellum in WT (n=6) and Mbnl2$^{NEX\text{-}cKO}$ (n=6) mice. The panels on the right represent the bands of relative intensity of MBNL2 (upper) and GAPDH (lower) used as a loading control. (c) Quantification of MBNL2 immunoreactivity in the PFC and HPC of WT (n=6) and Mbnl2$^{NEX\text{-}cKO}$ (n=6) mice. The panels on the right show representative images of MBNL2 intensity (green). Scale bar=500 µm. Images were taken at x4 magnification. Values are the mean + SEM. *** $p<0.001$ vs. WT group (one-way ANOVA).

Figure 3. Behavioral assessment of motor, cognitive and depression-like state in male WT and *Mbnl2*$^{NEX\text{-}cKO}$ mice at 3 and 8 months of age. No significant alterations were observed between groups in locomotor activity by determining (a) horizontal locomotion and (b) number of rearings. (c) Long-term recognition memory was impaired in the *Mbnl2*$^{NEX\text{-}cKO}$ mice, showing lower rates of discrimination index at 3 and 8 months of age compared to WT mice. (d) Depressive-like state was increased in *Mbnl2*$^{NEX\text{-}cKO}$ mice, presenting greater immobility times at 3 and 8 months of age than WT mice. Values are the mean + SEM. *** $p<0.001$ vs. WT group (2-way ANOVA followed by Bonferroni's post-hoc analysis). $$$ $p<0.001$ (main effect of genotype).

Figure 4. Behavioral assessment of motor, cognitive and depression-like state at 5 months of age following the chronic treatment with mirtazapine (MRZ). WT (n=10 per group) and *Mbnl2*$^{NEX\text{-}cKO}$ (n=10 per group) mice were treated during 21 days with vehicle, 3 and 10 mg/kg of MRZ, and behavioral testing was performed one week after the last

administration. No statistical differences were observed in locomotor activity by determining (a) horizontal locomotion and (b) number of rearings. (c) The discrimination index was decreased in *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with vehicle and both doses of MRZ reversed this cognitive impairment. (d) Immobility time was increased in *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with vehicle and both doses of MRZ attenuated this depressive-like state. Values are the mean + SEM. *** $p<0.001$ vs. WT group (genotype effect). ### $p<0.001$ vs. VEH of same genotype (treatment effect) (2-way ANOVA followed by Bonferroni's post-hoc analysis).

Figure 5. Chronic treatment with mirtazapine (MRZ) attenuates the enhanced microglial phenotype in the hippocampus (HPC) of *Mbnl2*$^{NEX\text{-}cKO}$ mice. Quantification of Iba1 immunofluorescence in WT (n=6 per group) and *Mbnl2*$^{NEX\text{-}cKO}$ (n=6 per group) mice. No statistical differences were observed in microglial expression in the (a) mPFC, (b) CA1 and (c) CA3 regions of the HPC. (d) In the dentate gyrus (DG), an enhanced microglial expression was found in *Mbnl2*$^{NEX\text{-}cKO}$ treated with vehicle and both doses of MRZ attenuated this overexpression. The transcriptional levels of (e) interleukin 1 beta (IL-1β), a pro-inflammatory cytokine, were exacerbated in *Mbnl2*$^{NEX\text{-}cKO}$ treated with vehicle and both doses of MRZ reversed this cytokine production, whereas no differences were observed between groups in the gene expression of (f) transforming growth factor (TGF- β), an anti-inflammatory cytokine. The Y-axis shows the mRNA expression as a fold change compared with the control group (WT VEH). On the bottom, representative images of Iba1 positive cells (red) and DAPI counterstaining (blue) of the DG region. Scale bar = 200 µm. Values are the mean + SEM. * $p<0.05$, *** $p<0.001$ vs. WT group (genotype effect). ## $p<0.01$, ### $p<0.001$ vs. VEH of same genotype (treatment effect) (2-way ANOVA followed by Bonferroni's post-hoc analysis).

Figure 6. Chronic treatment with mirtazapine (MRZ) reverses the neuronal loss in the hippocampus (HPC) of *Mbnl2*$^{NEX\text{-}cKO}$ mice. Quantification of NeuN immunostaining in WT (n=6 per group) and *Mbnl2*$^{NEX\text{-}cKO}$ (n=6 per group) mice. No statistical differences were observed in neuronal expression in the (a) mPFC, (b) CA1 and (c) CA3 regions of the HPC. (d) In the dentate gyrus (DG), a significant decrease in NeuN positive cells was found in *Mbnl2*$^{NEX\text{-}cKO}$ treated with vehicle, and the dose of 10 mg/kg of MRZ reversed this neuronal loss. On the right, representative images of NeuN positive cells (green). Scale bar = 200 µm. Adult neurogenesis was assessed in the subgranular zone (SGZ) of the HPC by (e) cell proliferation where MRZ increased the number of BrdU positive cells in WT and *Mbnl2*$^{NEX\text{-}cKO}$ mice and by (f) doublecortin (DCX) expression where *Mbnl2*$^{NEX\text{-}cKO}$ treated with vehicle exhibited a decrease in DCX integrated density, and MRZ increased this immature neuronal expression in WT and *Mbnl2*$^{NEX\text{-}cKO}$ mice. On the bottom, representative images of BrdU positive cells (green dots) and DCX positive cells (red marker). Values are the mean + SEM. **p<0.01, ***p<0.001 vs. WT group (genotype effect). #p<0.05, ##p<0.01, ###p<0.001 vs. VEH of same genotype (treatment effect). +++ (main effect of treatment). (2-way ANOVA followed by Bonferroni's post-hoc analysis).

Figure 7. Chronic treatment with mirtazapine (MRZ) modulates the expression of 5-HT$_{1A}$ and H$_1$ receptors in the hippocampus (HPC) of *Mbnl2*$^{NEX\text{-}cKO}$ mice. Transcriptional levels of MRZ receptor targets were evaluated in WT (n=6 per group) and *Mbnl2*$^{NEX\text{-}cKO}$ (n=6 per group) mice. In mPFC, no significant differences were observed in the gene expression of (a) 5-HT$_{2A}$, (b) 5-HT$_{2C}$ and (c) 5-HT$_3$ serotonin receptors. The transcriptional levels of (d) 5-HT$_{1A}$ receptor were reduced in all treatment groups of *Mbnl2*$^{NEX\text{-}cKO}$ compared to WT groups. Decreased levels of (e) alpha$_{2A}$ receptor were revealed in *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with vehicle

as compared with WT mice. (f) Histaminergic H_1 receptor was not altered in any experimental condition. In the HPC, a main effect of genotype between WT and *Mbnl2*$^{NEX\text{-}cKO}$ mice was found in the gene expression of (g) 5-HT_{2A} and (h) 5-HT_{2C} serotonin receptors. No significant differences between groups were observed in the transcriptional levels of (i) 5-HT_3 and (k) alpha$_{2A}$ receptors. Importantly, (j) 5-HT_{1A} receptor was significantly reduced in the *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with vehicle, and the dose of 10 mg/kg of MRZ increased its expression. Moreover, the (l) histaminergic H_1 receptor was exacerbated in the *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with vehicle, and both doses of MRZ attenuated this expression. The Y-axis shows the mRNA expression as a fold change compared with the control group (WT VEH). Values are the mean + SEM. *p<0.05, ***p<0.001 vs. WT group (genotype effect). #p<0.05, ##p<0.01 vs. VEH of same genotype (treatment effect). $ (main effect of genotype). (2-way ANOVA followed by Bonferroni's post-hoc analysis).

Figure 8. Chronic treatment with mirtazapine (MRZ) increases monoamine, GABA and adenosine production in the hippocampus (HPC) of *Mbnl2*$^{NEX\text{-}cKO}$ mice. Brain metabolites were quantified in WT (n=6 per group) and *Mbnl2*$^{NEX\text{-}cKO}$ (n=6 per group) mice. In the mPFC and HPC, 10 mg/kg of MRZ significantly reduced the generation of (a,f) 5-HT/Trp and (b,g) NA/Tyr in WT mice compared to WT group treated with vehicle. Moreover, NA and 5-HT generation were reduced in *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with vehicle, in mPFC and HPC respectively. In (c) GABA/Glu no significant alterations were found in mPFC, while in hippocampal homogenates (h) GABA production was increased in *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with MRZ 10 mg/kg. In the ratio (d, i) Glu/Gln a significant main effect of genotype was found in both areas, showing that *Mbnl2*$^{NEX\text{-}cKO}$ mice had lower production of Glu compared to WT group. A main effect of genotype was also found in the levels of (e) adenosine within

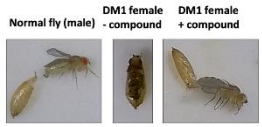
the mPFC, whereas in the HPC (j) adenosine levels were reduced in *Mbnl2*$^{NEX\text{-}cKO}$ mice treated with vehicle and the dose of 10 mg/kg of MRZ reversed this effect. Data is expressed as metabolic ratios (area of the analyte/area of internal standard). Values are the mean + SEM. *p<0.05, ***p<0.001 vs. WT group (genotype effect). #p<0.05, ##p<0.01 vs. VEH of same genotype (treatment effect). $ (main effect of genotype). (2-way ANOVA followed by Bonferroni's post-hoc analysis).

Suppl. Fig. S1. Generation of Tissue-Specific *Mbnl2*$^{NEX\text{-}cKO}$ Mouse Line. Two sequential crosses were made: First we outcrossed (shaded in blue) a brain-specific *Cre* transgene (NEX-Cre$^{+/-}$) mouse strain with homozygous *Mbnl2* loxP-flanked mice (*Mbnl2*$^{fl/fl}$). In the F1 generation, around the 50% of the offspring was hemizygous for the *Cre* transgene and for the loxP-flanked allele (Cre$^{+/-}$; Mbnl2$^{fl/+}$) and we backcrossed them (shaded in green) with the homozygous *Mbnl2* loxP-flanked mice (*Mbnl2*$^{fl/fl}$). In the N1 generation, approximately 25% of the progeny was hemizygous for the C*re* transgene and homozygous for the loxP-flanked allele (Cre$^{+/-}$; Mbnl2$^{fl/fl}$), resulting in our conditional knockout mice (*Mbnl2*$^{NEX\text{-}cKO}$) and around 25% was also homozygous for the loxP-flanked allele but was a non-carrier for the *Cre* transgene (Cre$^{-/-}$; Mbnl2$^{fl/fl}$), resulting in our control group (wild-type mice). To maintain the colony of our experimental groups (shaded in orange), we crossed the *Mbnl2*$^{NEX\text{-}cKO}$ mice with the wild-type mice.

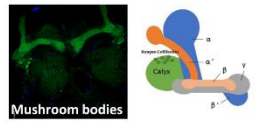
Suppl. Fig. S2. Timeline of experimental procedures following chronic treatment with mirtazapine. (a-b) Schematic representation of the experimental design for behavioral procedures. (c) Horizontal locomotion during 1 hour of male WT mice treated daily during 5 consecutive days with vehicle (VEH) or 1, 3, 10 and 30 mg/kg of mirtazapine. ***p<0.001 vs vehicle (Bonferroni following one-way ANOVA).

FIGURE 1.

a



b



c

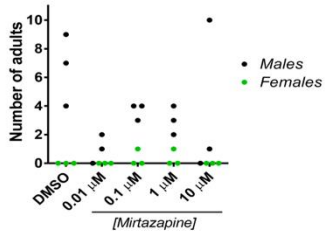
d

Compounds showing anti-DM1 effect	
Therapeutic Group	**Number of hits (%)**
Antibacterials	3 (8 %)
Anti-inflammatories	4 (11 %)
Unknown	2 (6 %)
Others	27 (75 %)

e

Mirtazapine treatment	Number of adult males	Number of adult females
0 µM (DMSO)	82	15
0.01 µM	32	13
0.1 µM	49	6
1 µM	34	20 **
10 µM	32	14 *

f



g

Drug	Rescue	Target
Mirtazapine	+	5-HT_2, 5-HT_3, α_2 adrenergic and histamine H_1 receptor antagonists 5-HT_{1A} receptor agonist
Fluoxetine	-	Blocks serotonin reuptake
Ritanserin	-	5-HT_{2A} receptor antagonist
Granisetron	-	5-HT_3 receptor antagonist
MDL-11939	-	5-HT_{2A} receptor antagonist

FIGURE 2.

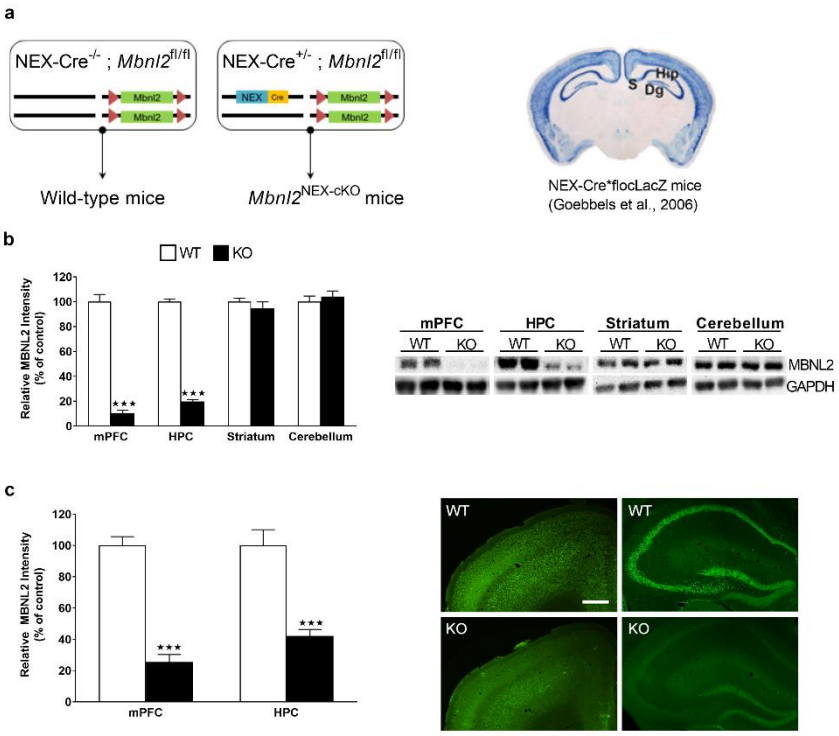

a
NEX-Cre-/- ; Mbnl2fl/fl
NEX-Cre+/- ; Mbnl2fl/fl
Mbnl2
NEX
Cre
Wild-type mice
Mbnl2NEX-cKO mice
Hip
S
Dg
NEX-Cre*flocLacZ mice
(Goebbels et al., 2006)
b
WT
KO
Relative MBNL2 Intensity (% of control)
mPFC
HPC
Striatum
Cerebellum
MBNL2
GAPDH
c
Relative MBNL2 Intensity (% of control)
WT
KO

FIGURE 3.

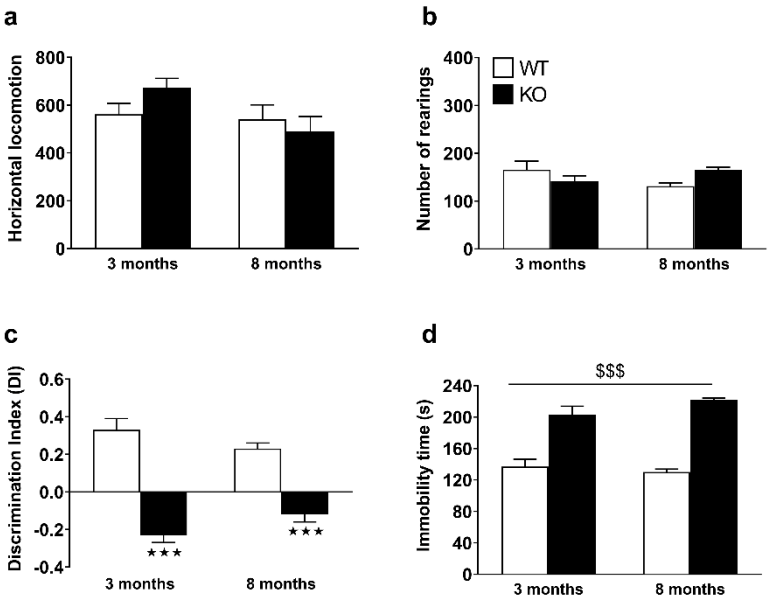

a
Horizontal locomotion
800
600
400
200
0
3 months
8 months
b
Number of rearings
400
300
200
100
0
WT
KO
3 months
8 months
c
Discrimination Index (DI)
0.6
0.4
0.2
0.0
-0.2
-0.4
★★★
★★★
3 months
8 months
d
$$$
Immobility time (s)
240
200
160
120
80
40
0
3 months
8 months

FIGURE 4.

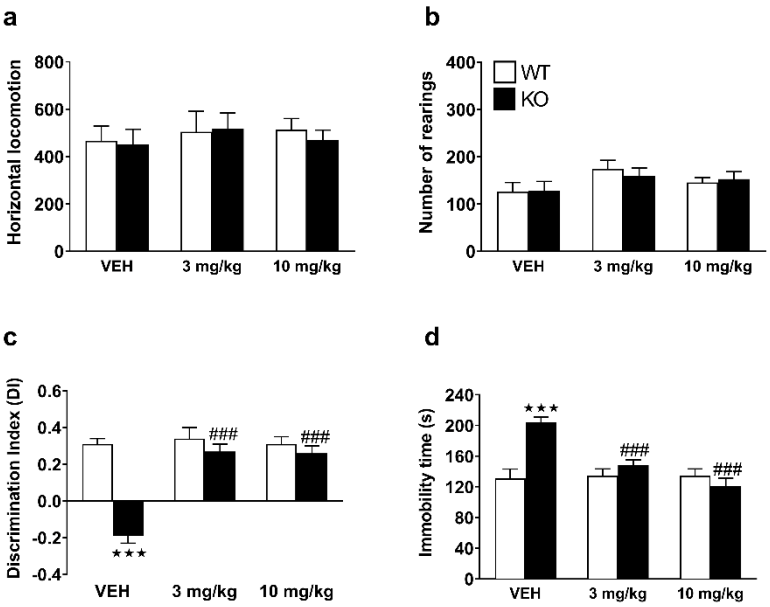

a
Horizontal locomotion
800
600
400
200
0
VEH
3 mg/kg
10 mg/kg
b
Number of rearings
400
300
200
100
0
WT
KO
VEH
3 mg/kg
10 mg/kg
c
Discrimination Index (DI)
0.6
0.4
0.2
0.0
-0.2
-0.4
###
###
★★★
VEH
3 mg/kg
10 mg/kg
d
Immobility time (s)
240
200
160
120
80
40
0
★★★
###
###
VEH
3 mg/kg
10 mg/kg

FIGURE 5.

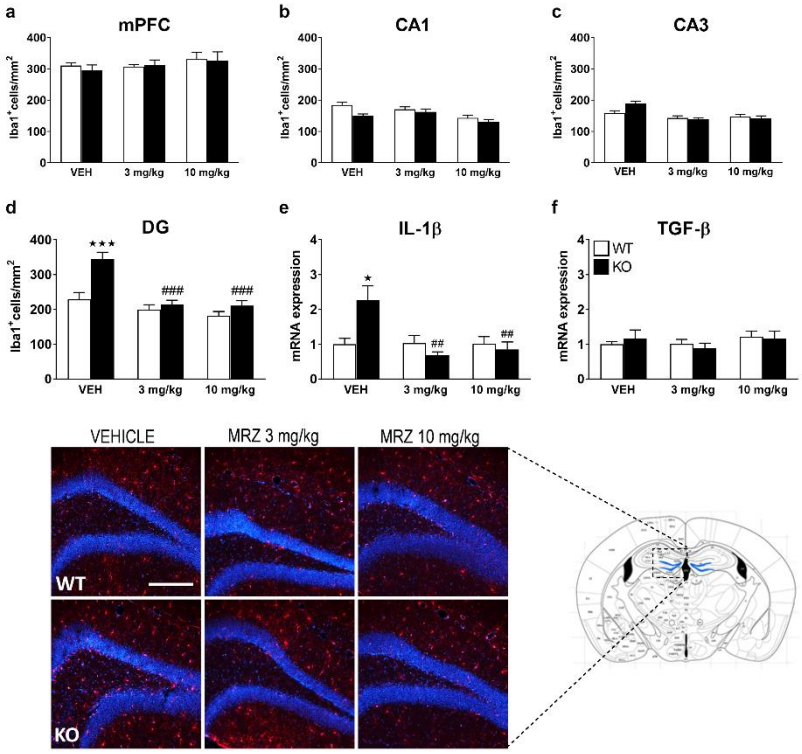

a
mPFC
b
CA1
c
CA3
Iba1+cells/mm2
VEH
3 mg/kg
10 mg/kg
d
DG
e
IL-1β
f
TGF-β
mRNA expression
WT
KO
VEHICLE
MRZ 3 mg/kg
MRZ 10 mg/kg

FIGURE 6.

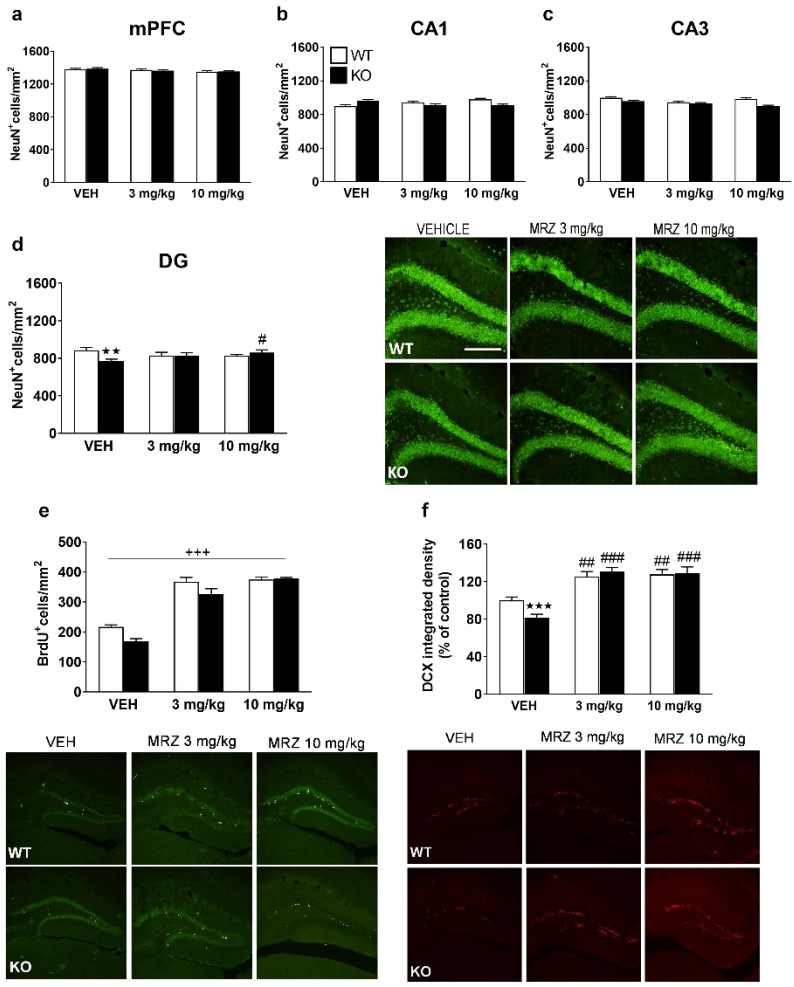

a
mPFC
b
CA1
c
CA3
WT
KO
NeuN+ cells/mm2
VEH
3 mg/kg
10 mg/kg
d
DG
VEHICLE
MRZ 3 mg/kg
MRZ 10 mg/kg
WT
KO
e
BrdU+ cells/mm2
f
DCX integrated density (% of control)

FIGURE 7.

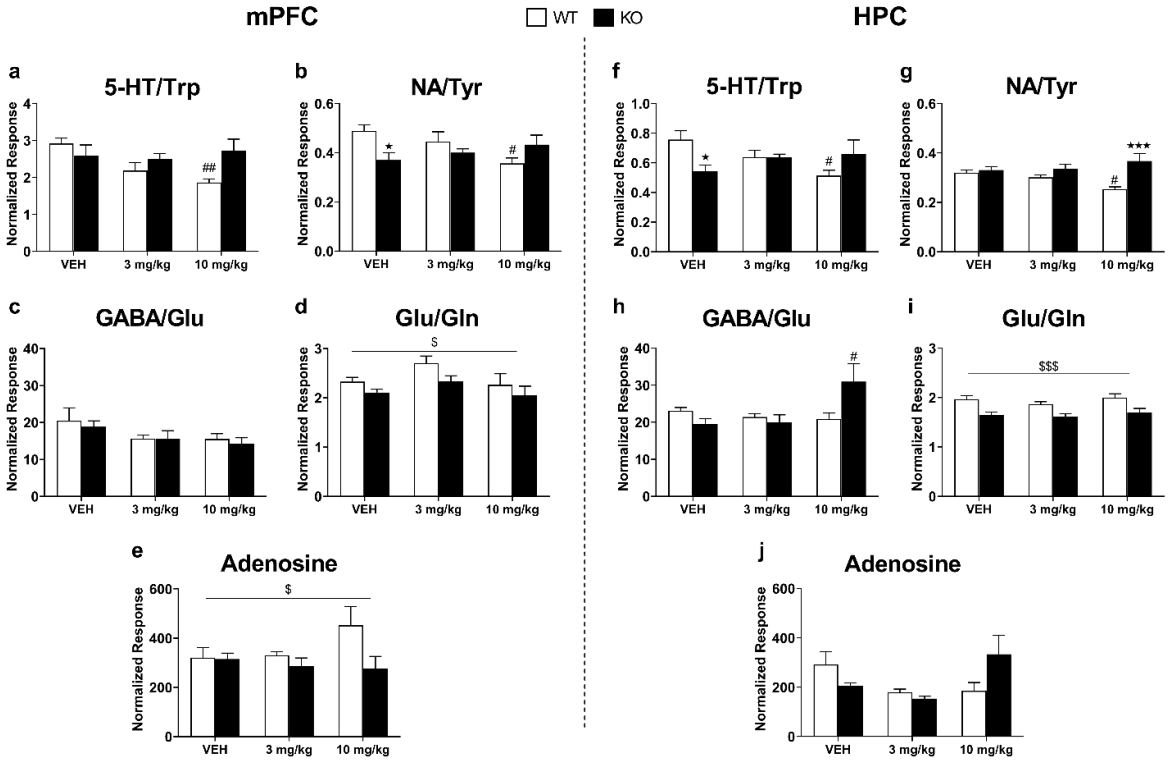
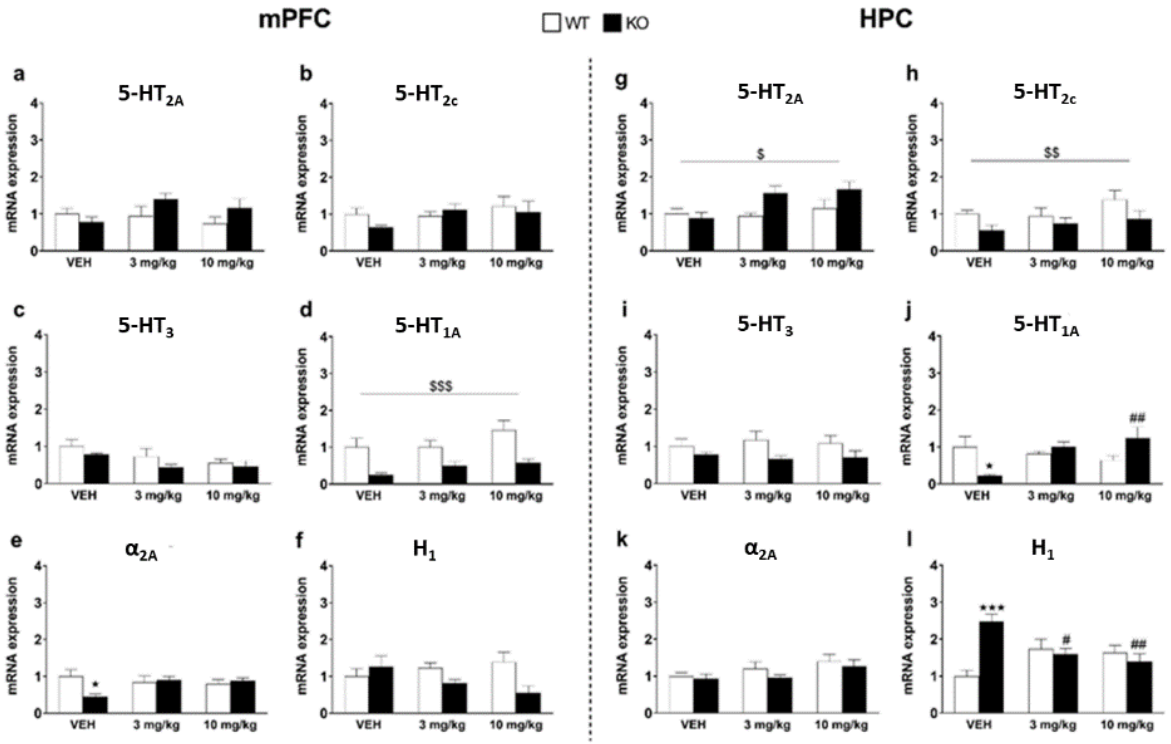

mPFC
WT
KO
HPC
a
5-HT/Trp
b
NA/Tyr
f
5-HT/Trp
g
NA/Tyr
c
GABA/Glu
d
Glu/Gln
h
GABA/Glu
i
Glu/Gln
e
Adenosine
j
Adenosine
Normalized Response
VEH
3 mg/kg
10 mg/kg

FIGURE 8.


mPFC
WT
KO
HPC
a
5-HT2A
b
5-HT2c
g
5-HT2A
h
5-HT2c
c
5-HT3
d
5-HT1A
i
5-HT3
j
5-HT1A
e
α2A
f
H1
k
α2A
l
H1
mRNA expression
VEH
3 mg/kg
10 mg/kg
$
$$
$$$
*
***
#
##

SUPPLEMENTARY MATERIAL

Behavioral and Neurochemical Alterations Associated with Loss of MBNL2 in the Dentate Gyrus are Rescued by Mirtazapine Treatment

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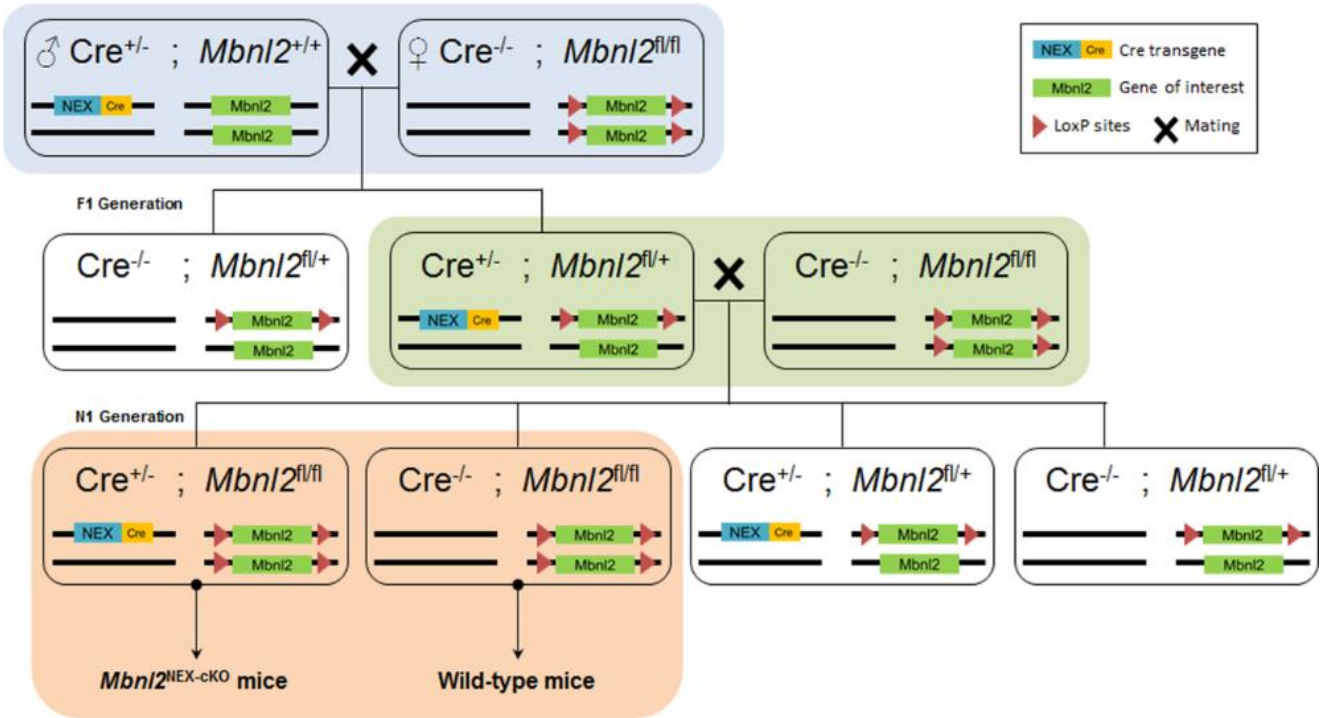
[6]CIPF-INCLIVA joint unit.

Content:

SUPPLEMENTARY FIGURE S1



SUPPLEMENTARY FIGURE S2.

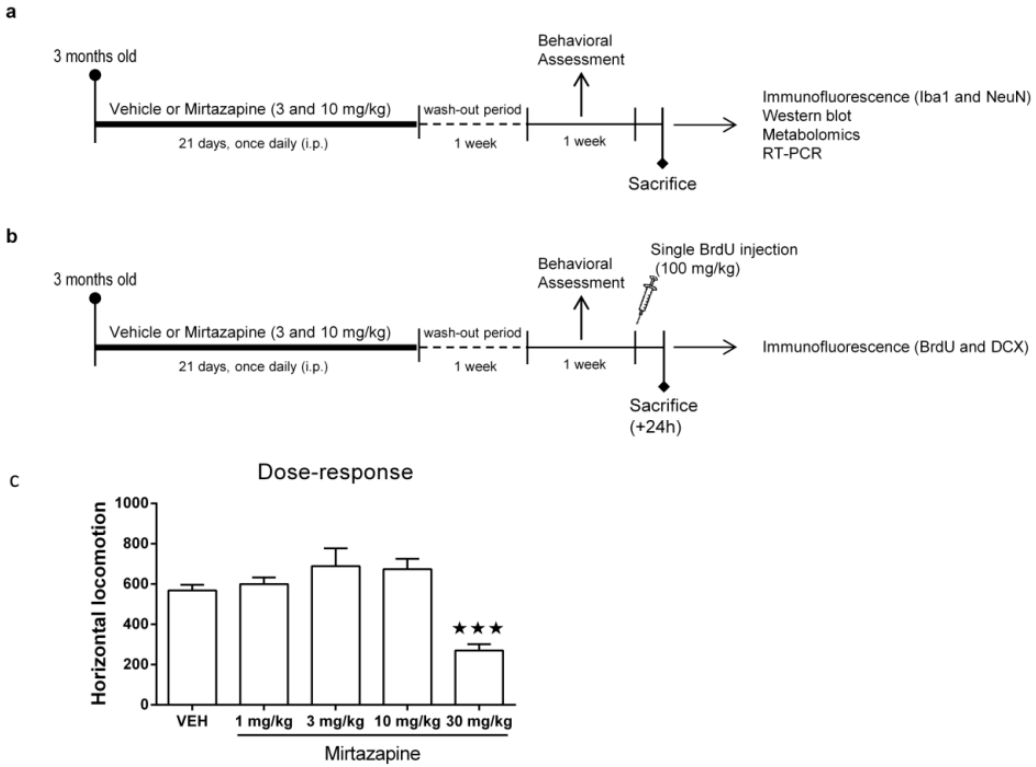


TABLE S1. PCR Primers for *Mbnl2*$^{NEX\text{-}Cre}$ Mouse Conditional Knockout Generation

Genotyping conditional *Mbnl2* allele		
Forward primer (5'-3')	**Reverse primer (5'-3')**	**Product size (bp)**
GTAGGGCTCTCAAGGAGA GCACTGCATTGAGC	AATGTCAAACCAGACCAG AAATACACCACCATG	216
CGCCTTCTATCGCCTTCTT GACGAGTTCTTCTGAG		525
Genotyping NEX-Cre allele		
Forward primer (5'-3')	**Reverse primer (5'-3')**	**Product size (bp)**
GAGTCCTGGAATCAGTCTT TTTC	AGAATGTGGAGTAGGGT GAC	770
	CCGCATAACCAGTGAAAC AG	525

TABLE S2. List of Antibodies Used in Immunofluorescence Procedures.

Primary Antibodies	Company	Reference	Dilution
Iba1	Wako	019-19741	1:500
NeuN	Millipore	MAB377	1:200
BrdU	Abcam	ab6326	1:200
DCX	Santa Cruz Biotechnology	sc-8066	1:200
Secondary Antibodies	**Company**	**Reference**	**Dilution**
Anti-mouse IgG, HRP-linked	Cell Signaling	7076	1:10.000
Alexa Fluor 555 Anti-Rabbit IgG	Thermo Scientific	A-32794	1:500
Alexa Fluor 488 Anti-Mouse IgG	JacksonImmuno Research	715-545-150	1:500
Alexa Fluor 488 Anti-Ratt IgG	Abcam	ab150157	1:500
Alexa Fluor 555 Anti-Goat IgG	Thermo Scientific	A-32816	1:500

TABLE S3. List of Mouse RT-PCR Primers Used for *Mbnl2*$^{NEX\text{-}Cre}$ Gene Expression Analysis.

GenBank Accession Number	Name	Forward primer (5'-3')	Reverse primer (5'-3')
NM_007393.5	Beta-actin	TCCATCATGA AGTGTGACGT	GAGCAATGAT CTTGATCTTCA T
NM_008308.4	5-HT_{1A} receptor	GATCTCGCTC ACTTGGCTCA	ACTCGATGCA CCTCGATCAC
NM_172812.3	5-HT_{2A} receptor	ATGCAGTCCA TCAGCAACGA	AATGTACCGTG AGAAGGCGG
NM_008312.4	5-HT_{2C} receptor	GCTGGACCGG TATGTAGCAA	GCTTTCGTCCC TCAGTCCAA
NM_001099644.1	5-HT_3 receptor	GACTGCTCAG CCATGGGAAA	CAGGGTGATG CTGTAAGCGA
NM_001317126.1	Histamine H_1 receptor	CTGTGGCTGC TATGCCCGTA	AGAAATCGGA GGCGGACTCT
NM_007417.4	alpha $_{2A}$ adrenergic receptor	GCACAACTTT GGAAGTCCCG	TTTCCAACTCC GTCGCTACC
NM_008361.4	interleukin 1 beta	GAAGAGCCCA TCCTCTGTGA CT	GTTGTTCATCT CGGAGCCTGT AG
NM_011577.2	transforming growth factor beta 1	CCCGAAGCGG ACTACTATGC	ATAGATGGCG TTGTTGCGGT

DISCUSSION

1. *Mbnl2* KO mice as models of CNS-related alterations in DM1

DM1 is classified as a muscular dystrophy, but the development and maintenance of brain function are also deeply affected in this disorder. RNA-induced toxicity has been proposed as the main pathological mechanism involved in DM1. RNAs containing CUG^{exp} repeats are retained and accumulated forming ribonuclear foci in the nucleus of cells disrupting a subset of binding proteins required for normal adult CNS function; the MBNL protein family. Based on previous studies, MBNL2 was proposed as the main splicing regulator disrupted in the CNS responsible for DM-associated abnormalities in the brain (Charizanis et al., 2012). In that study, it was reported that *Mbnl2* KO mice did not exhibit muscle pathology, but recapitulated several phenotypes consistent with DM1 neuropathophysiology, such as learning and spatial memory deficits, decreased synaptic plasticity and impaired hippocampal LTP. More recently, these findings were supported by histopathological studies, where it was shown that MBNL2 was sequestered in the HPC and the frontal cortex in post-mortem DM1 brains (Goodwin et al., 2015). However, there is still a lack of evidence regarding the cellular and molecular mechanisms that underlie the neuropsychological deficits related to MBNL2 loss of function. Moreover, there is no data as to whether theses alterations are progressive or sex-dependent.

Therefore, during this thesis we used two transgenic mouse lines of MBNL2 deficiency to investigate the neurobiological substrates involved in the behavioural alterations related to DM1 phenotypes, and to find novel pharmacological interventions to reverse them. In the first part, we used male and female constitutive *Mbnl2* KO mice (Article 1).

The advantages of this mouse model were two-fold: First, loss of MBNL2 protein in the entire brain permitted the comprehensive examination of the neurobiological and behavioural underpinnings. Second, the lack of muscular deficits in these mice allowed testing them in complex behavioural paradigms without counfounding motor effects. One of the limitations we encountered when working with constitutive *Mbnl2* KO mice was maintaining high levels of breeding. Indeed, KO mice offspring showed significantly lower weights than their WT littermates, and quite a few of them died before weaning. This could be because mothers reject smaller offspring focusing on those that are more likely to survive or it could be due to an extreme hyperactivity followed by enhanced seizure susceptibility (<10%), as already described in *Mbnl2* KO and DMSXL mice (Charizanis et al., 2012). To curtail these difficulties, for the second part of this thesis, we developed a new conditional tissue-specific KO mouse model, where the *Mbnl2* gene was deleted selectively in the glutamatergic neurons of the forebrain (Article 2). The reasoning behind the development of this model streamed from the fact that most of the neurochemical changes found in constitutive *Mbnl2* KO mice were observed in the mPFC and the HPC, where MBNL2 protein is highly expressed (Charizanis et al., 2012; Goodwin et al., 2015). In addition, alterations in the glutamatergic system had been previously reported in patients with DM1 (Takado et al., 2015), and in DMSXL mice (Sicot et al., 2017). To generate this mouse line, we used the Cre-lox system based on the recombination of a Cre recombinase enzyme, in this case the NEX-Cre, with a pair of short target sequences called LoxP sequences that floxed the gene of interest, in our case the *Mbnl2* gene. Therefore, by crossing a mouse that expresses the NEX-Cre promoter with a mouse that has floxed the *Mbnl2* gene, in a three-breeding scheme, we obtained our conditional tissue-specific mouse model of *Mbnl2* KO.

2. Relevant phenotypes in *Mbnl2* KO mice

In this thesis we focused mainly on assessing cognitive and affective functions. For this purpose, we used different behavioural tests to examine long-term memory, emotional working memory and depressive- and anxiety- like states in both *Mbnl2* KO mouse models. A detailed summary of the behavioral tests carried out during this thesis is shown in Table 3.

To verify that *Mbnl2* KO mice did not present any motor deficits in our hands, we first assessed locomotor activity using both the open field test and locomotor activity cages. In the first study, *Mbnl2* KO male mice displayed higher horizontal locomotion in the open-field tes than their WT littermates at 3 months of age, but this effect was attenuated at 5 months of age (Article 1). Moreover, KO female mice showed a greater horizontal activity than male KO mice, suggesting that female mice may have a greater sensitivity to new environments. In the second study, tissue-specific *Mbnl2* KO mice displayed similar levels of horizontal and exploratory activity in locomotor cages at 3 and 8 months compared to WT mice (Article 2). Taken together, the locomotor activity in both mouse models, excludes the presence of a muscular pathology or muscle deficits. Importantly, since our studies were conducted in *Mbnl2* KO mice with a maximum of 8 months of age, we cannot exclude the possibility that loss of MBNL2 expression may contribute to skeletal muscle pathology beyond this age.

Cognitive performance was evaluated using the novel object recognition test in both KO mouse models. Constitutive KO mice showed progressive impairments in long-term memory from 3 to 5 months of age that were more profound in male than female mice. Additionally, male constitutive KO mice, also exhibited working memory deficits in the active-avoidance task, indicating alterations in emotional working memory processing (Article 1).

In line with our results, previous studies have described cognitive impairment in patients with DM1 (Caso et al., 2014; Fujino et al., 2018; Labayru et al., 2019), and also a mild impairment of working memory in DMSXL mice was also described in the Morris water maze (Hernandez-Hernandez et al., 2013). In female KO mice, long-term recognition memory was not so prominent as in male KO mice. Interestingly, in the conditional KO mice memory deficits were more intense and persisted over time up to 8 months of age (Article 2). These findings indicate that the tissue-specific deletion of *Mbnl2* gene in neocortex and HPC is more deleterious in terms of memory recognition than the elimination of this gene in the entire brain, suggesting differential compensatory mechanisms in both mice models.

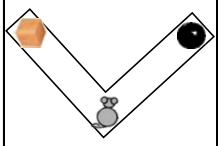
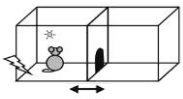
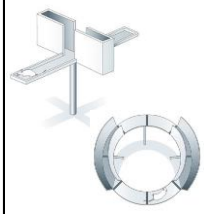
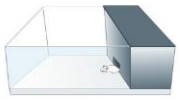
Affective alterations, including depression-like behaviour, anxiety-like states, and sleep disturbances were evaluated using several different paradigms (see Table 3). Both mouse models of MBNL2 deficiency, showed a depressive-like phenotype in the forced swim (FST) and tail suspension tests (TST) that persisted over time (Article 1 and 2), but female KO mice did not show a depression-like state. Previous studies have shown depressive-like symptoms in patients with DM1 (Winblad et al., 2010; Van der Velden et al., 2019) and signs of anhedonia in DMSXL mice (Hernandez-hernandez 2013).

Moreover, since sleep disturbances may contribute to depressive states in DM1 patients (Phillips et al., 1998), we evaluated the circadian activity patterns in constitutive male *Mbnl2* KO mice (Article 1). In agreement with the human data, we found that the pattern of circadian activity was interrupted in KO mice. They presented less horizontal activity during the dark cycle (activity period) with greater exploratory rearings during the light cycle (resting period). These results are in line with previous data showing an increase in REM sleep propensity in the same the constitutive

Mbnl2 KO model (Charizanis et al., 2012) and depressive-like behaviours in mouse models with disrupted circadian rhythms (Landgraf et al., 2016). Athough increased anxiety is a well-stablish feature in patients with DM1 (Van del Velden et al., 2019), our models of MBNL2 deficiency failed to reproduce this affective disorder, despite using three different tests to evaluate anxiety-like state in constitutive KO mice. These results indicate that lack of MBNL2 alone is not crucially implicated in anxiety processes. In fact, few studies have reproduced anxiety-like behaviour or motivational deficits in mouse models of DM1 including DMSXL and *Mbnl1* KO mice (Hernandez-Hernandez et al., 2013; Matynia et al., 2010).

Taken together, these data revealed that (i) the cognitive and mood disturbances associated with the loss of *Mbnl2* gene are progressive and more prominent in male than female constitutive *Mbnl2* KO mice (Article 1), and that (ii) loss of *Mbnl2* gene specificically in the glutamatergic neurons of the forebrain is sufficient to induce DM1-related behavioural disturbances (Article 2).

Table 3. Behavioural tests used during this thesis:

Behavioural Tests	Assessment	Description	Brain regions involved	Representation
Novel object recognition (NOR)	Recognition learning and memory	Based on the innate preference of mice for novelty. Consists of 3 phases: 1st) Habituation to the empty maze, 2nd) familiarization to 2 identical objects placed in each arm of the maze and 3rd) one of the objects is replaced by a new one, and the exploration time with each of the objects is measured and represented as a Discrimination Index (DI), where the highest scores reflect a great memory of recognition.	Perirhinal cortex Hippocampus	
Active-avoidance task	Emotional working memory	Mice are placed in a two-way shuttle box and learn to avoid an aversive foot shock (unconditioned stimulus) with the presentation of a light as a conditioned stimulus.	Prefrontal cortex Amygdala Dorsal striatum	
Forced swim test (FST)	Depressive-like behaviour	Mice are placed individually in a cylinder filled with water during 6 minutes. The total immobility time is measured during the last 4 minutes. Enchanced immobility represent a state of behavioural despair characteristic of depression.	Prefrontal cortex Hippocampus Amygdala	
Tail suspension test (TST)	Depressive-like behaviour	Mice are individually suspended by the tail with an adhesive tape during 6 minutes. The total time spent immobile is measured during the last 4 minutes. Enchanced immobility represent a state of behavioural despair characteristic of depression.	Prefrontal cortex Hippocampus Amygdala	
Phecomp cages	Circadian rhythm	Mice are individually placed in experimental chambers. Each box registers horizontal locomotion, rearing activity and resting time. After an habituation phase of several days, the parameters of circadian activity are measured.	-	
Elevated pluz maze (EPM) and elevated zero maze (EZM)	Anxiety-like behaviour	EMP consists of a maze with 4 arms set in a cross, whereas EZM is comprised of a ring-shaped runway. Both are elevated 30 cm above the fround. It has 2 opposite closed arms (with vertical walls) with 2 open arms (unprotected and highly illuminated). The number of entries and time spent in each arm are measured. A more anxious mouse will spend less time in open arms.	Hippocampus Amygdala Limbic regions	
Light-dark box	Anxiety-like behaviour	One compartment is small and dark, while the otheris larger and illuminated. The total number of squares crossed and number of entries in each compartments was measured. A more anxious mouse will spend less time in thr brighly zone.	Hippocampus Amygdala Limbic regions	
Open field test	Locomotor response Anxiety	Mice are placed in a squared brighlty arena with surrounding walls that prevent escape. The exploratory behaviour and general activity is measured by counting the number of rearings and number of squares crossed during a 5-min period.	-	
Locomotor activity	Locomotor response	Mice are tested in individual locomotor boxes equipped with two lines of infrared beams in low-lighting conditions (20 lux). Total horizontal activity and the number of rearings were analyzed during a 30 min-period.	-	

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3. Neuronal and glial changes associated to MBNL2 loss of function

Previous studies have reported neuropathological changes with neuronal loss (Ono et al., 1998a, b; Mizukami et al., 1999), and diffuse brain damage (Itoh et al., 2010) in brains of patients with DM1. In addition, cognitive deficits and depression have been related to an altered adult hippocampal neurogenesis (Cao et al., 2004) and several neuropsychiatric disorders, such as Alzheimer's disease, have been associated with a chronic exacerbation of microglia (Donzis and Tronson, 2014; Charlton et al., 2018). Thus, we evaluated glial alterations and neuronal loss by quantifying brain biomarkers of astrocytes (GFAP), microglia activation (Iba1) and neuronal specific marker (NeuN) by immunofluorescence techniques in different brain structures of *Mbnl2* KO mice models.

Microglia cells are the major resident immune cells in the CNS that play dynamic roles in the healthy brain. Microglia can rapidly respond to patgological insults like brain trauma or infection by changing their morphology from a ramified, inactive state to an amoeboid reactive state. Through the secretion of pro- or anti-inflammatory cytokines they induce a range of effects that may contribute either to detrimental effects on surrounding neurons, or to neuronal protection (Wake et al., 2011; Nayak et al., 2014; Stephenson et al., 2018; Subhramanyam et al., 2019) (Figure 10). Through the release of cytokines and growth factors, microglia play a crucial role in the crosstalk among brain cells such as astrocytes with neurons, as well as with regulatory immune cells (von Bernhardi et al., 2016).

Moreover, during development and neurogenesis, interactions between microglia and neurons are fundamental to shape the patterns of neural activity circuits (Wake et al., 2011). Hence, neuroinflammation has been

described as a complex immune response designed to protect the body from pathogens, toxins or injuries, and it has been linked to depression (Jeon and Kim, 2016) and cognitive dysfunction (Heneka et al., 2015; Hein and O'Banion, 2012).

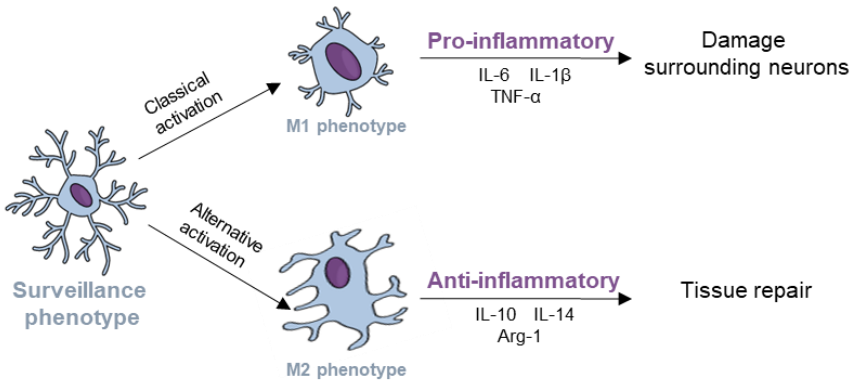

Figure 10. Schematic representation of the different morphology of microglial cells. Upon a pathological damage, microglial cells shift from the surveillance phenotype to a reactive state. Following a classical activation, M1 phenotype, microglial cells expressed pro-inflammatory cytokines, chemokines and neurotoxic factor, while in the M2 phenotype, microglia generally produce anti-inflammatory neuroprotective factors (Adapted from Subhramanyam et al., 2019).

Although the behavioral alterations observed were similar in the two models of MBNL2 deficieny, the associated changes in glial and neuronal cells differed between them. Firstly, in the constitutive KO mice, we observed a greater widespread microgliosis in areas such as the mPFC, HPC and striatum at 6 months of age, but no neuronal loss was detected in any brain area. On the other hand, tissue-specific KO mice exhibited a restricted microgliosis in the DG of HPC, accompanied by neuronal loss and reduced neurogenesis. In both mouse models, we found an increased expression of IL-1β, indicating that the overexpression of microglia in these brain areas was mainly pro-inflammatory. These findings are consistent with abnormal

upregulation of pro-inflammatory cytokines reported in patients with DM1 with increased plasma levels of IL-6 and TNF-α (Johansson et al., 2000; Mammarella et al., 2002). Interestingly, it was described that hippocampal IL-1β overexpression leads to memory impairment in mice (Hein et al., 2010). Notably, no astrogliosis was detected in any of the two mouse models studied, maybe because astrocytes are less sensitive and respond later than microglia to moderate pathological changes in the CNS (Liddelow et al., 2017).

The changes in glial and neurons observed in the mPFC and HPC in *Mbnl2* KO mice were associated with cognitive impairments and a depressive phenotype. The cortex and the HPC are two key structures in the regulation of learning, memory, attention and emotion (Meola and Sandone, 2007; Winblad et al., 2010; Cabada et al., 2017). In fact, both HPC and perirhinal cortex are required in object recognition memory (Cohen et al., 2013), and studies in rats have shown that the integrity of the DG is crucial for discriminating between familiar and novel objects (Hunsaker et al., 2008). Recently, an 11-year longitudinal follow-up study revealed a specific cognitive decline in the domains of visuospatial and visual memory in patients with DM1 (Labayru et al., 2019). Accordingly, neuroimaging studies showed the involvement of frontotemporal cortices and HPC in patients with DM1 (Romeo et al., 2010; Minnerop et al., 2011; Caso et al., 2014), where several genes were found to be mis-regulated in post-mortem brains (Sergeant et al., 2001; Jiang et al., 2004; Furuta et al., 2018).

Overall, the loss of MBNL2 in the constitutive mouse model suggests the presence of an initial pathological state consisting in a more generalized brain inflammation, but no neuronal loss. On the other hand, the inflammation together with neuronal loss and reduced neurogenesis observed in the conditional KO mice, specifically in the DG of the HPC,

indicate that this structure is particularly sensitive to MBNL2 downregulation. In addition, these data reveal that the MBNL2 loss of function in the DG is sufficient to induce cognitive and depressive-like symptoms associated with DM1.

4. Neurochemical alterations related to MBNL2 loss of function

Few studies have examined neurochemical signalling in DM1 brains. Early investigations demonstrated the involvement of dopaminergic and serotonergic pathways in DM1 (Ono et al., 1998a, b), and more recent work has shown alterations in the glutamatergic system (Takado et al., 2015; Sicot et al., 2017). Thus, to gain deeper insight into the neurochemical alterations in DM1, we performed studies investigating possible changes in several neurotransmitter systems.

In constitutive KO mice, we focused on the DA and NA system, and on the expression of genes related to plasticity since they play an important role in cognitive processes and depression (Seamans and Yang, 2004; Roozendaal and Hermans, 2017). We first determined the basal extracellular levels of DA using *in vivo* microdialysis. Our results showed an increase in extracellular DA levels of KO mice compared to WT littermates. In contrast to these results, previous studies in DMSXL mice reported a significant reduction in DA in the frontal cortex. The inconsistencies between both studies could probably be due to the use of different DM1 mouse models. Hence, DMSXL mice display a high content of RNA foci in the dopaminergic brain centres (such as substantia nigra) that could contribute to the reduction of DA release (Hernandez-Hernandez et al., 2013). In addition, we revealed enhanced gene expression of the DA transporter (*Dat)* and of D1 and D2 DA receptors in the mPFC, while no changes were observed in the NA transporter (*Net)* or its precursor (*Dbh)*. The cognitive impairment observed in constitutive KO mice could be associated with an excessive activation of these receptors due to excessive DA release (Druzin et al., 2000; Vijayraghavan et al., 2007; Robbins and Arnsten, 2009). In the HPC, no major changes were found, except for an exacerbated increase in *Dat* levels of KO mice. In addition, our electrophysiological studies showed

an abnormal neuronal network between mPFC and HPC in KO mice that is in line with abnormal activity reported in patients with DM1 (Romeo et al., 2010). More specifically, we found a decrease in spiking activity of the mPFC of KO mice, probably due to excessive stimulation of D1 receptors (Zahrt et al., 1997; Vijayraghavan et al., 2007). In the analysis of mRNA levels related to plasticity, we found a decrease of brain derived neurothrophic factor (BDNF) in the mPFC and HPC of KO mice. This downregulation could be associated with the cognitive and depressive-like alterations observed in KO mice since this gene has been implicated in major depression and synaptic plasticity disorders (Krishnan and Nestler, 2008).

In the tissue-specific KO mouse model, we also investigated possible changes in the DA and NA system using the targeted metabolomics approach in brain tissue homogenates. Unfortunately, DA production in mPFC and HPC were undetectable. However, the gene expression level of the α_{2A}-adrenergic receptor was significantly decreased in the mPFC and NA production was also reduced in this brain area. NA has also shown to play a powerful role in working memory and attention in the mPFC through its actions at α_{2A}-adrenergic receptors (Ramos and Arnsten, 2007). Thus, their downregulation could be associated to the cognitive impairment observed in KO mice.

Next, we measured 5-HT production in brain homogenates of tissue-specific KO mice. Our results showed a significant decrease in 5-HT production in the HPC of KO mice. Accordingly, loss of 5-HT neurons has been reported in DM1 patients (Ono et al., 1998b), and a significant decrease of its main metabolite, 5-hydroxyindolacetic acid (5-HIAA) was detected in the brainstem of DMSXL mice (Hernandez-Hernandez et al., 2013). Several gene expression changes were observed for 5-HT receptors.

Particularly, 5-HT_{1A} was significantly reduced in the mPFC and HPC of KO mice. This receptor is highly expressed in the granule cells of the DG and is involved in the regulation of depression, anxiety and memory processes (Verdurand and Zimmer, 2017). Altered levels of this receptor have been found in patients with depression (Štrac et al., 2016), and could be related to the neuronal loss found in the HPC. However, no major changes were found in the transcriptional levels of 5-HT_{2A}, 5-HT_{2C} and 5-HT_3 in the mPFC and HPC. Therefore, reduced 5-HT production and decreased gene expression of $5HT_{1A}$ receptors may have contributed to depressive-like behaviour observed in tissue-specific KO mice.

Moreover, we found a lower production of Glu in the mPFC and HPC of KO mice. A deterioration of the glutamatergic system has been suggested in patients with DM1. In particular, high levels of Gln in the frontal cortex, and reduced levels of Glu in white matter were observed (Takado et al., 2015). In addition, the Glu transporter GLT1, is decreased in DMSXL mice, and in post-mortem brains of DM1 patients (Sicot et al., 2017). Glutamate transporters protect against exacerbated extracellular concentrations and protect neurons from excitotoxicity (Kanai and Hediger, 2004; Bellamy, 2006). Moreover, the excitatory Glu neurons in the HPC are modulated by inhibitory gamma-aminobutyric acid (GABA)-releasing interneurons, and adenosine is one of the most important neuromodulators in HPC, regulating neuronal excitability and synaptic transmission in the CNS (Sperlágh and Vizi, 2011). In this respect, we found a significant reduction of adenosine levels in the HPC of KO mice, but with no alterations in the GABAergic system. GABA is the primary inhibitory neurotransmitter known to counterbalance the action of Glu and plays a role in learning and memory (Heaney and Kinney, 2016). Whilst inhibitory A_1 adenosine receptors are highly expressed in the neocortex, HPC, cerebellum and brain stem, excitatory A_{2A} adenosine receptors are restricted to the striatum and

olfactory bulb (Sebastiao and Ribeiro, 2009). It is generally accepted that adenosine acting on A_1 receptors reduces excitatory transmission and is neuroprotective in brain injuries associated with neuroinflammation or in epileptic seizures, highlighting this compound as a potential target for the treatment of neurodegenerative diseases (Ribeiro et al., 2003). Hence, interactions between GABAergic and adenosinergic systems in regulating neuronal excitability in the HPC are of crucial importance, particularly under cell-damaging conditions (Saransaari and Oja, 2005).

Given that the H_1 receptor antagonist, hydroxyzine reduces inflammatory responses and anxiety (Wiley et al., 2015); we measured the gene expression of H_1 receptor in the mPFC and HPC. The transcriptional levels of H_1 receptor were significantly higher in the HPC of KO mice. Thus, the exacerbated expression of H_1 receptors may be associated with microglia activation observed in the DG of the HPC, since these receptors contribute to the effects of histamine on microglial phagocytosis and neural death (Rocha et al., 2016). Brain histamine is crucial for motivation (Torrealba et al., 2012), and regulates cognition, attention and inflammation through different histamine receptors (Haas et al., 2008). Unfortunately, we could not validate the methodology to correctly quantify histamine production or its metabolites using the LC-MS/MS technique. Although the metabolomics approach is not as accurate as microdialysis, it allowed us to examine more comprehensively changes in several neurotransmitters at the same time in the same sample.

5. Pharmacological interventions

The second goal of this thesis was to find novel therapeutic approaches to alleviate the DM1 symptomatology related to the CNS. For this purpose, we selected two compounds: the psychostimulant methylphenidate, and the atypical antidepressant mirtazapine, since they showed anti-DM1 activity in screening assays using a *Drosophila* model of DM1 (Articles 1 and 2), and are relevant for DM1 patients, acting on DA and 5-HT neurotransmission, which is deregulated in this population. However, these compounds induce their effects through different mechanisms of action. The psychostimulant methylphenidate blocks DA and NA transporters, inhibiting presynaptic reuptake resulting in an increased concentration of both neurotransmitter levels at synapses (Volkow et al., 1998; Hannestad et al., 2010). On the other hand, mirtazapine has a more complex mechanism of action. It is an atypical antidepressant known as a NA and 5-HT specific antidepressant (NaSSA). It enhances noradrenergic transmission via blockade of α_{2A}-adrenergic receptors, antagonises the 5-HT_{2A}, 5-HT_{2C} and 5-HT_3 serotonergic receptors, as well as the H_1 histamine receptor, and activates the 5-HT_{1A} receptor (Anttila and Leinonen, 2001).

Both of these drugs were administered chronically in mice, with one daily treatment during 21 days. The doses of methylphenidate (1 and 3 mg/kg) and mirtazapine (3 and 10 mg/kg) used in our work were carefully chosen in pilot studies so that they did not produce detrimental locomotor effects. Indeed, methylphenidate induces hyperlocomotion in rodents (Gaytan et al., 1997), and mirtazapine has sedative effects in humans (Anttila and Leinoen, 2001). Thus, our behavioural studies demonstrated that low doses of methylphenidate in the constitutive *Mbnl2* KO model and mirtazapine in the tissue-specific Mbnl2$^{NEX\text{-}CRE}$ KO model reversed the cognitive impairment and depression-like behaviour, without affecting the locomotor

response or anxiety behaviours. Preclinical studies have reported contradicting results with long-term administration of methylphenidate on cognition with some showing improvement (Veetil and Mukkadan, 2011; Carmack et al., 2014a, b; Haleem et al., 2015; Salman et al., 2019), while others show impairments in memory (Scherer et al., 2010). Our data are in line with evidence of the efficacy of methylphenidate in the treatment of depressive symptoms, fatigue and apathy in humans (Hardy, 2009; Lavretsky et al., 2015; Mclntyre et al., 2017; Delsalle et al., 2018). Hence, methylphenidate is widely used in the clinic to treat attention/hyperactivity disorder (ADHD) (Greydanus et al., 2007; Takon et al., 2011; Simon et al., 2015), and as an off-label adjunct in major depression (Candy et al., 2008; Ravindran et al., 2008). The use of stimulants to treat daytime sleepiness in DM1 patients has already been described with methylphenidate (Miyamoto et al., 2002; Puymirat et al., 2012), and modafinil (MacDonald et al., 2002; Orlikowski et al., 2009), but there are no available data on the effects of methylphenidate on cognition or depression in these patients. On the other hand, our data agree with the findings that mirtazapine is effective in patients with moderate to severe depression (Gorman, 1999; Alam et al., 2013). Moreover, preclinical studies suggest that mirtazapine may possess specific pro-cognitive properties (Nowakowska et al., 1999; Borkowska et al., 2007; Rogóz, 2013), and it is used as an adjuvant therapy for cognitive impairment in schizophrenic patients (Cho et al., 2011; Stenberg et al., 2013). However, no studies have been conducted in DM1 patients with this compound.

The behavioural impairment observed in our models of MBNL2 deficiency were associated with increased microgliosis in the mPFC, HPC and striatum in the case of a constitutive deletion, and microgliosis, neuronal loss and decreased neurogenesis in the DG in the case of the tissue-specific deletion. Increasing data indicate that enhanced oxidative stress and chronic

neuroinflammation are common pathological mechanisms underlying neurodegenerative diseases (Mosley et al., 2006; Agostinho et al., 2010; Buendia et al., 2016), and increased serum levels of IL-6 have been found in patients with DM1 (Johansson et al., 2000). In constitutive KO mice, methylphenidate attenuated microglia overexpression and the pro-inflammatory cytokine IL-1β in the mPFC of KO mice. Consistent with these data, chronic methylphenidate administration decreases serum levels of other pro-inflammatory cytokines in a rat model with depression and cognitive deficits (Aga-Mizrachi et al., 2014). Similarly, in conditional KO mice mirtazapine reduced the pro-inflammatory microglial exacerbation in the HPC, reducing the transcriptional levels of IL-1β. In agreement, chronic treatment with mirtazapine has been shown to reduce plasma levels of pro-inflammatory markers in patients with major depressive disorder (Tulner et al., 2011). In addition, the beneficial effects of mirtazapine on inflammatory response, where accompanied by increased neurogenesis and reduced neuronal loss in the DG of the HPC. In accordance, hippocampal plasticity induced by chronic antidepressant treatment occurs through an increase in adult hippocampal neurogenesis (Malberg et al., 2000), and it has been reported that these cellular adaptations play an important role in the behavioural effects of antidepressants (Santarelli et al., 2003).

Taken together, our results reveal that both methylphenidate and mirtazapine may restore neuroglial communications in the mPFC and HPC that have been compromised by the loss of MBNL2, and that this effect attenuates the depressive-like state and cognitive deficits observed in KO mice.

To gain deeper insight into de mechanisms of action of these two compounds, we performed neurochemical and gene expression studies in the constitutive *Mbnl2* KO model and in the tissue-specific *Mbnl2*$^{NEX\text{-}CRE}$ KO model. Our findings indicated that chronic treatment with methylphenidate

increased D1 receptor gene expression in the mPFC, while it attenuated the enhancement in *Dat* and D2 receptors in constitutive KO mice. Accordingly, some studies have associated the therapeutic effects of methylphenidate in ADHD with the stimulation of prefrontal D1 receptors (Arnsten and Dudley, 2005). In the HPC, *Dat* transcription levels were attenuated in KO mice, while chronic methylphenidate had subtle effects on D1 and D2 receptors. More recent evidence has revealed that methylphenidate increases PFC cell activity through D2 receptors (de la Peña et al., 2018). It is known that D2 receptors play a critical role in the behavioural effects of psychostimulants (Self and Nestler, 1995; Volkow et al., 2007). In addition, we found that the downregulation of Brain-derived neurotrophic factor (*Bdnf)* gene expression observed in constitutive KO mice in the mPFC and HPC was reversed. Our findings are consistent with previous results showing that methylphenidate increased hippocampal BDNF protein and reversed spatial memory deficits in a rat model of ADHD (Jichao et al., 2017). BDNF in serum is considered a good marker for lesions in the CNS since they can easily cross the blood brain barrier. Indeed, lower levels of BDNF expression may contribute to the pathogenesis of several disorders related to cognitive deficits and depression such as Huntington's disease or Alzheime's disease (Lu et al., 2014). Notably, methylphenidate increased nuclear factor erythroid 2-related factor 2 (*Nrf2)* mRNA levels specifically in the HPC of KO mice. NRF2 is a key marker of antioxidant responses (Perez-Leal et al., 2017), and a depressive-like state has been reported in *Nrf2* KO mice (Yao et al., 2016).

Our gene expression studies using mirtazapine revealed an increase in the expression of the 5-HT_{1A} receptor and a decrease in the levels of the histamine H_1 receptor in tissue-specific KO mice. Both of these receptors are crucially involved in depression, anxiety and memory processes

(Verdurand and Zimmer, 2017). While 5-HT_{1A} receptors are critical for the behavioral and neurogenic effects of the antidepressants (Samuels et al., 2015), H_1 receptors are associated to inflammatory responses (Rocha et al., 2016). Thus, the neuroprotective and anti-inflammatory effects of mirtazapine observed in the DG of KO mice could be attributed to its action on 5-HT_{1A} receptor and H_1 receptors, respectively. We also determined the effects of chronic mirtazapine treatment on brain neurotransmitters and their metabolites related with depression and cognitive functions. Major changes were found in the HPC, where mirtazapine increased the 5-HT and NA production (Anttila and Leinonen, 2001), as expected. Interestingly, mirtazapine increased the production of GABA and adenosine neurotransmitters in the HPC of KO mice. Accordingly, previous studies have shown that mirtazapine increases GABA and reduces Glu concentrations in nucleus accumbens homogenates in a rat model of chronic mild stress (Kamal et al., 2013), suggesting a potential GABAergic target for mirtazapine in the treatment of depressive disorders. Furthermore, adenosine is one of the most important neuromodulators in HPC, regulating neuronal excitability and synaptic transmission in the CNS (Sperlágh and Vizi, 2011). While no evidence is currently available as to the effects of mirtazapine on adenosine neurotransmission, our data suggests that this compound may be increasing adenosine in the HPC to modulate neuronal and glial stability. Indeed, adenosine acting on A_1 receptors reduces excitatory transmission, and is neuroprotective in brain injuries (Ribeiro et al., 2003).

Altogether, our findings provide evidence that methylphenidate and mirtazapine could be a novel therapeutic treatment to counteract the neurodegenerative process, and the cognitive and mood alterations related to DM1 by complementary mechanisms. Thus, the beneficial effects of methylphenidate on behavioural alterations observed in mice with

constitutive MBNL2 loss-of-function may be attributed to reducing widespread microglia overexpression and antioxidant responses by increasing *Bdnf* and reducing *NRf2* levels, and by normalizing D2 receptor and increasing D1 receptor gene expression in the mPFC. On the other hand, mirtazapine attenuates the pathological behavioural manifestations due to loss of MBNL2 protein in the DG by reducing neuroinflammation and neuronal loss, and probably also by increasing neurogenesis in this brain area through $5HT_{1A}$ and H_1 receptors. Notably, we demonstrated for the first time that mirtazapine's favourable effects may involve decreasing neuronal excitation through a mechanism of action involving GABA and adenosine neurotransmission.

Despite these positive results obtained with both drugs in animal models, potential disadvantageous side effects must be highlighted and reviewed before it can be approved for use in DM1 patients. Thus, in the case of methylphenidate, it has been reported that it may increase the risk to develop a cardiac pathology in vulnerable individuals (Roose and Rutherford, 2015), and it may induce addictive properties depending on the dose and the route of administration employed (Morton and Stockton, 2000). Similarly, mirtazapine induces sedation, which would be counter beneficial for DM1 patients who suffer from excessive day time sleepiness. However, this drug has been shown to be effective and safe during long-term use (Anttila and Leinonen, 2001), and it is usually administered at night when somnolence would be less of an issue for DM1 patients.

CONCLUSIONS

CONCLUSIONS

The findings obtained in the present thesis revealed that:

1. Both mouse models of MBNL2 deficiency (constitutive *Mbnl2* KO and the *Mbnl2*$^{NEX\text{-}CRE}$ KO) resulted in cognitive deficits and depressive-like behavior, which were progressive and persisted up to 8 months of age, and they were more prominent in male than in female mice, recapitulating the neuropsychological disturbances observed in patients with DM1.

2. Behavioral alterations in constitutive *Mbnl2* KO mice were associated with a deregulated DA system in the mPFC, including increased DA levels, and increased transcriptional levels of *Dat*, and D1 and D2 receptors, and with a pro-inflammatory process in the mPFC and HPC.

3. Chronic methylphenidate treatment attenuated cognitive and depressive symptoms in constitutive *Mbnl2* KO by reducing overexpression of microglia and normalizing gene expression of *Dat* and D2 receptors in the mPFC, and by increasing plasticity and reducing oxidative stress biomarkers in HPC.

4. Behavioral abnormalities in tissue-specific *Mbnl2*$^{NEX\text{-}CRE}$ KO mice were associated with neuronal and glial alterations specifically in the DG of the HPC, as well as altered neurotransmitter levels in the HPC.

5. Chronic treatment with mirtazapine reverses the behavioral deficits in *Mbnl2*$^{NEX\text{-}CRE}$ KO mice by reducing microglia and neuronal loss in the DG of the HPC, by normalizing the gene expression of 5-HT_{1A}R and H_1R receptors in the HPC, and by modulating the balance of monoamine, GABA and adenosine levels in the HPC.

6. Methylphenidate and mirtazapine could be novel potential avenues for the treatment of the neuropsychological disturbances observed in patients with DM1.

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