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XXXII CICLO DEL DOTTORATO DI RICERCA IN

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MIRTAZAPINE FOR RETT SYNDROME: A CANDIDATE TO IMPROVE QUALITY OF LIFE

Settore scientifico-disciplinare: BIO/06 ANATOMIA COMPARATA E CITOLOGIA

DOTTORANDO / A
Javier FLORES GUTIÉRREZ

COORDINATORE
PROF. Germana MERONI

SUPERVISORE DI TESI
PROF. Enrico TONGIORGI

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To the Forgotten
and to those who taught me to not forget.

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ABSTRACT

Rett syndrome (RTT) is a progressive non degenerative neurodevelopmental disease affecting mainly females, with an incidence of 1 out 10.000 newborn girls. Its symptomatology is very variable and may include motor deficits, intellectual disability, cardiorespiratory alterations and epilepsy, among other alterations. RTT diagnosis is complex and mainly based on clinical evaluations. All RTT individuals apparently develop normally until approximately the first year of life. Then, a clear developmental stagnation occurs, followed by a regression period in which individuals lose acquired skills. In most cases, RTT is due to loss-of-function mutations in the *MECP2* gene, which encodes the methyl-CpG-binding protein 2 (MeCP2). This protein is a widespread chromatin organizer with a global role in the control of gene expression within the nervous system. Even if experiments performed in a mouse model showed a reversion of RTT-like phenotypes after the re-expression of murine Mecp2, at present there is no a cure for RTT. Pharmacological and physical therapies are the only available techniques to alleviate RTT symptomatology. Regarding drugs, all treatments used on RTT individuals are used only for specific symptoms. Since monoaminergic systems are downregulated in both RTT individuals and mouse models, antidepressants have been proposed as candidate drugs to treat this disease. Among them, mirtazapine (MTZ) has showed an excellent safety profile through years and the ability to rescue several behavioural, physiological and neuroanatomical phenotypes in a male mouse model of RTT. The present project aimed to complete and to extend these results, as well as to find some mechanisms of action of MTZ. To do this, we used *Mecp2*$^{tm1.1Bird}$ female mice, a RTT model with a verified face validity, and treated them at different ages and for different durations. This experimental paradigm allowed us to draw different conclusions. First, we verified the safety of MTZ, as we did not observe any adverse effects, even when we treated young female mice with a high dosage of MTZ for one month. Second, we found several improvements in motor, somatosensory and cognitive domains. In one case, we were able to propose a mechanism for the behavioural rescue, as MTZ normalized parvalbumin expression in a related brain area. Results in female mice were completed by the prospective analysis of a heterogeneous cohort of adult RTT patients that had been treated with MTZ for long periods. Only 2 out 11 patients showed adverse effects and were discontinued, while others showed an improvement of several clinical features in the motor domain, as well as in multiple behavioural alterations. In summary, results obtained in this thesis strongly supports MTZ as a promising treatment for RTT. They represent a robust proof-of-concept and will constitute the base of a dossier aimed to obtain the ethical authorization to initiate a randomized clinical trial. In this way, it will be possible to verify whether MTZ can effectively alleviate RTT symptomatology and, thus, improve quality of life of affected people and their families.

ABBREVIATIONS

5-HT – serotonin
AED – antiepileptic drugs
BBB – blood brain barrier
BDNF – brain-derived neurotrophic factor
***CDKL5* –** human gene encoding the cyclin-dependent kinase-like 5 protein
CSPGs – chondroitin sulfate proteoglycans
DA – dopamine
DT – Dowel test
E/I – excitation-inhibition [balance]
ECM – extracellular matrix
EPM – Elevated plus maze
***FOXG1* –** human gene encoding the forkhead box protein G1 protein
GABA – gamma-aminobutyric acid
IGF-1– insulin-like growth factor 1
HB – Horizontal bars test
HET-MTZ – heterozygous mice treated with mirtazapine
HET-VEH – heterozygous mice treated with vehicle
i.p. – intraperitoneal [injection]
LQTS – long QT syndrome familial
MBAS – Motor-Behaviour Assessment Scale
MECP2 – human gene encoding the MeCP2 protein
MeCP2 – human methyl-CpG binding protein 2
- **TRD –** transcriptional repressor domain (also known as **NID,** NCoR-interacting domain)
- **NCoR** – nuclear receptor co-repressor 1
- **MBD** – methyl-CpG-binding domain
- **NLS** – specific nuclear signal localization
- **NTD** – N-terminal domain
- **ID** – intervening domain

Mecp2 – murine gene encoding the Mecp2 protein
Mecp2 – murine methyl-CpG binding protein 2
MTZ – mirtazapine
NA – noradrenaline
NB – Nest building test
NaSSA – noradrenergic and specific serotoninergic antidepressant
OF – Open field
PNNs – perineuronal nets
PR – Patch removal test
PSV – Preserved speech RTT variant
PV – parvalbumin
PV+ – parvalbumin-positive [cells]
RCSS – Rett syndrome Clinical Severity Scale
Rt – Accelerating rotarod
RTT – Rett syndrome
RW – Rod walk test
SOM – somatostatin
SOM+ – somatostatin-positive [cells]
SSRIs – selective serotonin reuptake inhibitors
WT-MTZ – wild-type mice treated with mirtazapine
WT-VEH – wild-type mice treated with vehicle
XCI – X chromosome inactivation
Xi – "inactive" X chromosome

INTRODUCTION

1. A historical overview on RTT research

In 1966, the Austrian neurologist Andreas Rett made an apparently trivial observation. Two girls, sitting on their mothers' laps in the waiting room of his clinic, were realizing very similar stereotypical hand movements. He started to look for patients with similar symptoms, and finally published a clinical report describing twenty-two cases of young girls sharing some clinical features. All the individuals evaluated had developed typically until approximately nine months of age and, besides the distinctive stereotypical hand movements, they shared other symptoms: absent speech, reduced degree of facial expression (hypomimia), generalized spastic tone, gait abnormalities, cardiac alterations and intellectual disability (Rett, 1966). Initially, Rett proposed a metabolic origin for this clinical picture, as first blood analyses revealed hyperammonaemia in most of the affected girls. This idea was later abandoned when some laboratory errors were found.

The Austrian neurologist filmed the unique clinical presentation of his young patients, trying to raise awareness on the syndrome and, on this way, find more similar cases. His aim was achieved slowly, mainly because of two reasons: the relative rarity of the condition and the fact that Rett published almost exclusively in German – indeed, the English version of his 1966 article was published only fifty years later (Rett, 2016). In addition, many skeptical clinicians raised some doubts about the identification of the clinical picture described by Rett as a distinct syndrome. Seventeen years after first Rett's report, Bengt Hagberg and his colleagues completed the description of thirty-five clinical cases of girls, encountered over the course of more than twenty years in three different European countries. In all cases, patients presented a uniform progressive encephalopathy, whose onset had been preceded by a typical development up to the age of 7 to 18 months (Hagberg et al., 1983). They noticed that this condition was virtually the same than that previously described by Rett and, because of this, decided to attribute the name of the Austrian neurologist to the syndrome. Hagberg and his collaborators described accurately many of the typical symptoms of Rett syndrome (RTT, OMIM #312750) and managed to make it to truly enter the international medical consciousness. They were not able to hypothesize a cause for RTT but, as it had been found only in females so far, they suggested that a dominant mutation on one X chromosome – resulting in affected girls and nonviable male hemizygous embryos or foetuses – could underlie RTT.

Within the following ten years, consensus diagnostic criteria were established in different countries. Vienna criteria represented the first clinical criteria for RTT (Hagberg et al., 1985),

followed by the consensus reached in the United States of America (The Rett Syndrome Diagnostic Criteria Work Group, 1988). These internationally recognised criteria facilitated the creation of the first databases quantifying and categorizing RTT cases, such as the Australian Rett Syndrome Database (Leonard et al., 1997). Together, these different attempts to systematize RTT diagnosis allowed to better evaluate the incidence of the syndrome, which nowadays is estimated in 1 out 10.000 female live births (Fehr et al., 2011).

In the meanwhile, different research groups had started to investigate the potential genetic causes for RTT, based, among other evidence, on several cases of identical twin sisters almost concordant in all clinical signs (Tariverdian et al., 1987; Partington et al., 1988). The small number of familial cases – that is to say, heredity-dependent – facilitated the publication of two exclusion mapping studies that narrowed down the area of interest on Xq28, a band situated at the tip of the X chromosome (Schanen and Francke, 1998; Sirianni et al., 1998). Based on these findings, Amir and collaborators looked for mutations in nearly hundred candidate genes located in the Xq28 region. They found seven RTT individuals carrying different types of mutations in the *MECP2* gene, which encodes the methyl-CpG binding protein 2 (MeCP2, Amir et al., 1999). These findings were confirmed later in many other studies, which have concluded that most of the typical RTT cases are due to mutations in the *MECP2* gene (*see below*).

A direct consequence of the finding of a molecular basis for most RTT cases was the explosion of genetic testing, which currently includes both direct sequencing and alternative techniques such as multiplex ligation-dependent probe amplification (MLPA). This has led to an earlier diagnosis of the syndrome, now being 2,7 years on average (Tarquinio et al., 2015a). However, availability of these tools is still almost limited to European countries with equitable public funding systems and to appropriately insured patients in the USA. Genetic testing remains therefore inaccessible to patients in most countries, which has not only obvious social implications but also scientific, as the largest portion of the human population remains unscreened.

When the study of Amir and collaborators was published in 1999, MeCP2 was an already well-known protein in the epigenetics field (*see below*), but the finding that mutations in its encoding gene were the main responsible for RTT provoked the outbreak of many related research lines. Within the last twenty years, we have largely increased our knowledge on RTT and its underlying mechanisms (see Fig. 1). This has allowed to develop therapies that have increased life expectancy of RTT individuals and improved their quality of life. However, both people affected

by RTT and their families still face many obstacles every day and, therefore, much scientific work must be done to find better and more efficient ways to help them.

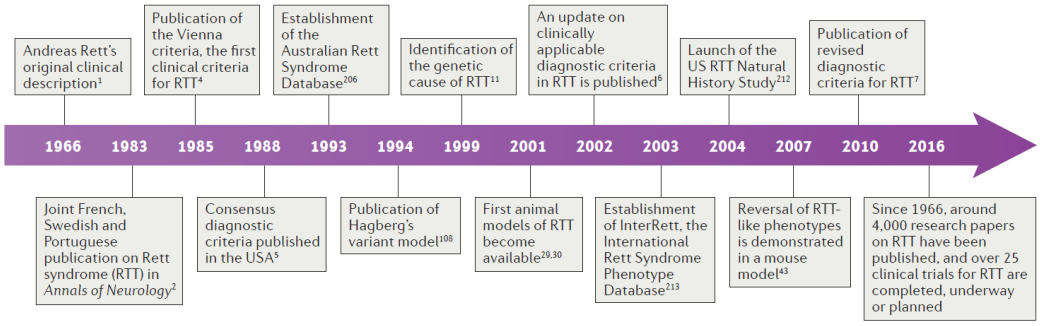


Figure 1 - Timeline of key events and discoveries in Rett syndrome (*from Leonard et al., 2017*).

2. RTT symptomatology

2.1 Typical RTT

Since the first description of the syndrome by Rett, much related clinical information has been accumulated. The resulting picture is not easy to understand, because of the large variability regarding both penetrance of specific symptoms and their severity. The considerable confusion in the context of RTT diagnosis has been partially counteracted by establishing consensus clinical criteria (*see above*), which have been modified slightly over time to reflect increased understanding of the disease features (Hagberg et al., 2002). Nowadays, RTT cases are divided in two main groups according to their clinical features. Besides typical (or classical) RTT, some "atypical variants" have been recognized (*see below*).

The girl with typical RTT achieves appropriate milestones (e.g. ability to walk, sometimes saying a few words), even if the age of acquisition is often delayed beyond the typical period. However, it has been suggested that neurological phenotypes of RTT individuals could be the cumulative result of different adverse events, which are already present at stages when no obvious signs of the pathology are evident (Bedogni et al., 2016; Cobolli Gigli et al., 2018).

After this stage of apparently typical development, clearer differences in expected skills emerge after six months of age (Neul et al., 2014). Even if progression of the disease is highly variable among RTT individuals, some stages can be generalized (see Fig. 2):

1. The first phase of the disease starts at around 6-18 months of age and is characterized by a **developmental stagnation** which includes: general growth retardation, weight loss and a weak posture brought on by muscle hypotonia. Post-natal deceleration in head growth has been eliminated as necessary criteria for RTT diagnosis because of its low sensitivity –

meaning that it is not found in all individuals –, but it remains a specific, early indicator extremely useful in alerting clinicians to a potential RTT diagnosis (Neul et al., 2010).

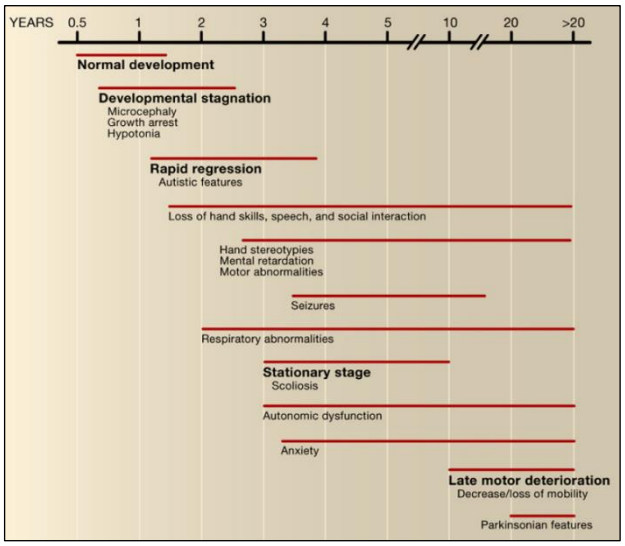


Figure 2 - Clinical progression of typical RTT (*from Charhour and Zoghbi, 2007*). After a period of apparently normal development, a healthy-looking baby girl falls into developmental stagnation, followed by rapid deterioration, loss of acquired speech, and the replacement of purposeful use of the hands with incessant stereotypies, a characteristic of the syndrome. Patients also develop social behaviour abnormalities. The condition worsens with loss of motor skills and profound cognitive impairment. In addition, patients suffer from anxiety, seizures, and a host of autonomic abnormalities.

2. Developmental stagnation is followed by a **rapid regression** stage at around two years of age. In this phase, girls with RTT lose previously learned skills, on a background of autistic behaviors such as loss of language, irritability and self-abuse (Nomura, 2005). Other autistic features also manifest at this stage, including hypomimia, hypersensitivity to sound, lack of eye-to-eye contact, indifference to the surrounding environment and unresponsiveness to social cues (Chahrour and Zoghbi, 2007). Nevertheless, since 2013 RTT is not listed as an Autism Spectrum Disorder (ASD) in the Diagnostic and Statistical Manual of Mental Disorders (DSMM) as "[The] disruption of social interaction may be observed during the regressive phase of RTT (…) However, after this period, most individuals with RTT improve their social communications skills, and autistic features are no longer a major area of concern." (Ip et al., 2018). As the regression stage progresses, RTT individuals lose purposeful use of their hands and instead develop stereotypic hand wringing or washing movements and, in some cases, clapping, flapping and mouthing of their hands (Chahrour and Zoghbi, 2007). Also within this phase, first alterations of the autonomous nervous system may appear, such as respiratory dysregulation: hyperventilation during wakefulness, breath-holding, aerophagia and apnea (Weese-Mayer et al., 2006). Another feature emerging at this phase is epilepsy, being the occurrence of seizures one of the symptoms affecting more the quality of life of RTT individuals and their families. Seizures range from easily controlled to intractable epilepsy, with the most common types being focal and tonic-clonic seizures with alteration of consciousness (Jian et al., 2006). Seizures severity usually decreases after adolescence and tend to disappear within the adulthood.

3. At around three years of age, regression is followed by a stage of **stabilization**, often named stationary, in which some individuals partially regain skills. It also includes the

appearance of new symptoms such as scoliosis and an increase of anxiety (Chahrour and Zoghbi, 2007).

4. As time goes by, a **late motor deterioration** usually occurs, often leading to parkinsonism after the adolescence period (Roze et al., 2007). The clinical condition reaches then a plateau and some RTT individuals may survive up to the sixth or seventh decade of life in a severely debilitated physical condition. Nowadays, the cause of death is generally a cardiorespiratory compromise (Tarquinio et al., 2015b).

The complexity of RTT symptomatology makes very challenging its diagnosis and, therefore, its study by scientists too. Because of this, the RettSearch Consortium, an international network of clinically-oriented RTT researchers established in 2006, conducts a continuous process of reviewing the diagnostic criteria for RTT. The most recently revised diagnostic criteria (Neul et al., 2010) has simplified the diagnosis of typical RTT by limiting the necessary criteria to the presence of developmental regression plus four main symptoms: partial or complete loss of acquired purposeful hand skills, partial or complete loss of acquired spoken language, gait abnormalities and specific stereotypic hands movements (*see Table 1 in suppl. figures*). Many other symptoms, including breathing disturbances when awake, impaired sleep pattern or growth retardation, are nowadays considered as "supportive criteria", this meaning that they are frequent but not common to most RTT individuals.

Finally, it is important to distinguish between the biological severity and the social severity, which do not always correlate. Some mildly affected girls may create complicated situations within the family context which more severely affected ones do not. Some girls are more mobile, restless and can exhibit aggressive behaviour. This is an issue to be considered in terms of management of the disease from a social point of view.

2.2 RTT atypical variants

One of the reasons why describing precisely RTT is so difficult is the existence of many cases that do not share some of the features present in most RTT individuals (the so-called "necessary criteria", *see Table 1 in suppl. figures*). These "variants" have been recognized in only a small number of cases and, therefore, it is difficult to make any clear statement concerning their defining clinical features. However, clinicians have managed to well described three variant forms of RTT: preserved speech variant, early seizure variant and congenital variant (Hagberg and Skjeldal, 1994; Neul et al., 2010). All of them share an important feature with typical RTT cases: the presence of a period of regression followed by stabilization. From a clinical point of view, this fact is very relevant, as it allows to clearly distinguish any RTT case from relentless

degenerative conditions. The different variants have been defined by their characteristic clinical features:

- The **preserved speech variant or Zappella variant** is the most common one and its distinctive feature is the recovery of language after regression, along with the rarity of epilepsy.
- The **early seizure variant or Hanefeld variant** is characterized by an early onset of seizures, more specifically infantile spasms and refractory myoclonic epilepsy, before disease regression. Some cases of this variant have been associated with mutations in the *CDKL5* gene (*see below*).
- The **congenital variant** is characterized by hypotonia and developmental delay occurring earlier than in classical RTT. It has been associated with mutations in the *FOXG1* (*see below*).

2.3 RTT in males

Although initially recognized only in girls and familial cases suggested an X-linked gene with male lethality, the first case of a boy meeting the criteria for typical RTT was described long time ago (Gillberg, 1989). Nowadays it is accepted that the lower number of males affected by RTT is related to the specific mechanisms that generate RTT-related mutations (*see below*) and not to a higher rate of spontaneous abortions (Girard et al., 2001). Regarding clinical description, a review including 57 cases of RTT in males all over the world reported that the most common symptoms within this population are: abnormal gait, loss of acquired spoken language, decelerated head growth and epilepsy (Reichow et al., 2015).

3. Molecular bases of RTT

3.1 MeCP2: a widespread chromatin organizer

The first study showing that mutations in the *MECP2* gene, which encodes the methyl-CpG binding protein 2 (MeCP2), are responsible for clinical manifestations in most typical RTT cases (Amir et al., 1999) paved the way for the study of RTT pathogenesis. Since then, hundreds of publications characterizing in detail structure and functions of MeCP2 have been published.

MeCP2 was first identified and characterized as a protein binding specifically to methylated DNA and, because of this, it was included in the methyl-CpG-binding domain (MBD) protein family, a complex group of proteins that bind to methylated cytosines in the cell nucleus (Lewis et al., 1992; Meehan et al., 1992). MeCP2 is characterized by several domains, as schematized in Fig. 3. The transcriptional repressor domain (TRD, also known as NCoR-interacting domain, NID)

regulates, together with the MBD domain, the binding of MeCP2 to methylated DNA and the consequent transcriptional repression (Guy et al., 2011). Furthermore, the TRD domain establishes a link between DNA methylation and higher-order chromatin structures (Nan et al., 1998). The specific nuclear signal localization (NLS) targets MeCP2 to the cell nucleus, even if it has been recently demonstrated that an intact MBD domain is enough to fulfil this function, this proving a redundancy between both domains (Lyst et al., 2018). Finally, MeCP2 contains an N-terminal domain (NTD), an intervening domain (ID) and a C-terminal portion, which contains in turn two DNA binding regions and one specific chromatin binding region (Ghosh et al., 2010). Human MeCP2 has been purified and its structure has been characterized accurately (Adams et al., 2007).

Figure 3 - Schematic representation of the functional domains of methyl CpG binding protein 2 (*from Gulmez Karaca et al., 2019*). MeCP2 contains an N-terminal domain (NTD), a methyl-binding domain (MBD), an intervening domain (ID), an NCoR-interacting domain (NID), and α and β C-terminal domains (CTDα and CTDβ, respectively).

Initially, MeCP2 was thought to be a mere global transcriptional repressor (Nan et al., 1998; Klose and Bird, 2003), but further studies suggested an additional role of transcriptional activator (Chahrour et al., 2008). This duplicity of roles of MeCP2 is possible thanks to the recruiting of different co-factors (Della Ragione et al., 2016). Moreover, it has been recently proposed that MeCP2 acts primarily as a context-dependent global organizer of chromatin architecture (Connolly and Zhou, 2019). According to this model, MeCP2 binds to specific methylated DNA sequences across the genome and, through interactions with other proteins such as NCoR, facilitates a specific three-dimensional chromatin state that allows in turn a precise and coordinated gene expression at a genome-wide level. Because of this, loss of MeCP2 results in a subtle perturbation of the global chromatin architectural state and a decrease in cell nuclear size (see Fig. 4). This leads to subtle but relevant changes in the expression of many different genes.

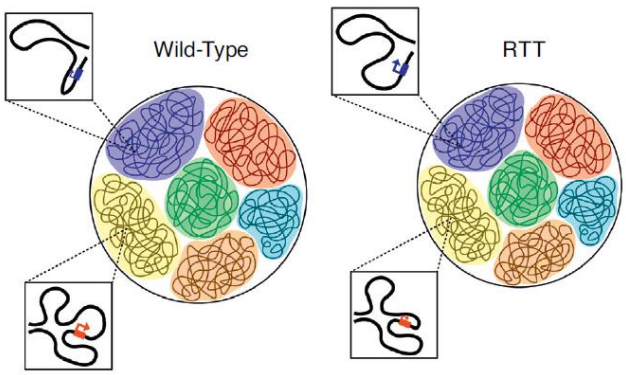


Figure 4 - Effects of MeCP2 as a global organizer of chromatine architecture within the nucleus (*from Connolly and Zhou, 2019*). According to this model, loss of MeCP2 in RTT (right) results in a subtle perturbation of the global chromatin architectural state and a decrease in nuclear size compared to wild-type cells (left). This leads to subtle changes in the expression of many different genes, directly increasing transcription of some genes (blue color, top insets) but sterically hindering the transcription of other genes (red color, bottom insets) based on their genomic positions and physical locations in three-dimensional space.

MeCP2 is ubiquitously expressed, but present at higher levels in lung, spleen and brain, with the highest levels in the latter (LaSalle, 2001; Shahbazian et al., 2002) and, more precisely, in neurons (Kishi and Macklis, 2004). In humans, MeCP2 expression starts in mid gestation an continues increasing until ten years of age, especially during neuronal differentiation (Shahbazian et al., 2002). However, it has been demonstrated that MeCP2 is critical for maturation and maintenance of neurons, rather than for cell fate decision (Kishi and Macklis, 2004). Indeed, increasing levels of MeCP2 coincides with neuronal development-related phenomena such as dendritic growth, dendritic branching and spine morphogenesis (Kishi et al., 2005). These trophic processes take place in the later stages of neurodevelopment and are fundamental steps within the development of neuronal circuits. Expression of MeCP2 starts at early stages of development and continues through adulthood, regulating all stages of neurodevelopment, being especially relevant during the periods of high sensitivity to the environment (Picard and Fagiolini, 2019) and for adult brain function (see Fig. 5). The timing of MeCP2 expression follows a caudal-rostral gradient, as it becomes apparent first in the most ontogenetically ancient parts of the brain (e.g. brainstem and thalamic regions) and then progressively in more rostral structures along development (LaSalle et al., 2001; Shahbazian, 2002).

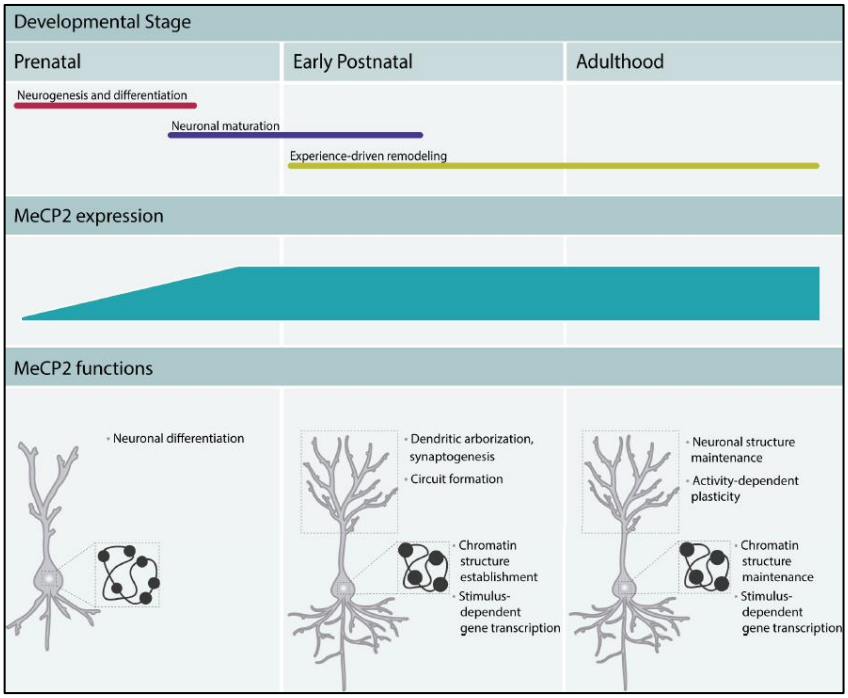


Figure 5 - Mecp2 expression and roles from embryonic to adult stages (*from Gulmez Karaca et al., 2019*). MeCP2 regulates brain development and maintains the function of mature neurons throughout adulthood. It regulates neuronal differentiation in early embryonic development, neuronal maturation, and circuit formation. MeCP2 promotes chromocenter clustering during differentiation and maturation, and thus participates in the establishment of the typical chromatin structure of mature neurons (mature neurons present fewer and denser chromocenters, represented in the Figure as black dots in the nucleus). In adulthood, MeCP2 is a critical factor for maintenance of the neuronal function. It maintains the chromatin structure and regulates the neuronal transcriptomic profile. Moreover, MeCP2 appears to maintain a permissive state for stimulus-dependent gene transcription.

The human *MECP2* gene (HGNC:6990), located at the q28 locus within the X-chromosome, contains four exons which generate 21 transcripts, even if only two of them encode for the Mecp2 protein (Ehrhart et al., 2016). This presents two different isoforms: MeCP2_e1 and MeCP2_e2. They are produced by alternative splicing and differ only at their N-terminal regions, as both include the two main domains, MBD and TRD. The corresponding transcripts are almost ubiquitously expressed but show different tissue expression patterns (Reichwald et al., 2000). The MeCP2_e2 is barely detectable in brain and lung, but very abundant in heart, skeletal muscle and spleen, which leaves MeCP2_e1 isoform as the main responsible for MeCP2 function within the nervous system.

3.2 Genetic mutations underlying RTT

Following first evidence showing that mutations in the *MECP2* gene provoke RTT (Amir et al., 1999), several studies aiming to delve into this issue found that just over three-quarters of analysed RTT individuals presented a mutation in the *MECP2* gene (Amir et al., 2000; Cheadle, 2000; Huppke, 2000; Nielsen et al., 2001; Auranen et al., 2001; Huppke et al., 2002). More recently, a battery of modern detection assays have allowed to identify *MECP2* mutations in 95-97% of individuals with typical RTT (Neul et al., 2008). Importantly, even using highly sensitive methodologies, in 3-5% of individuals undoubtedly meeting clinical criteria for typical RTT no mutation in *MECP2* has been found, this indicating that such a mutation is not required to develop typical RTT symptomatology (Neul et al., 2010). Regarding RTT variants (*see above*), only 58% of cases reported in the large North America Database have been linked to a mutation in the *MECP2* gene (Percy et al., 2007). In addition, mutations in *FOXG1* (HGNC: 3811; Ariani et al., 2008) and *CDKL5* (HGNC: 11411; Mari et al., 2005) genes have been associated with congenital and early seizure variant cases, respectively. Finally, *MECP2* mutations have been found also in people without RTT symptomatology. Aiming to create a useful framework integrating all this diversity, it has been proposed to use the expression "*MECP2*-related disorders" to refer to individuals with clinical disorders and associated *MECP2* mutations (Neul et al., 2010). Within this paradigm, RTT represents the most frequent among all the possible clinical pictures derived from *MECP2* mutations.

Just like many other disorders, RTT is associated with a much higher number of affected females (Thomas, 1996). For a long time, this discrepancy was attributed to gestational lethality in males, even if this hypothesis has not been systematically investigated for most disorders until recently. In the case of RTT, it is currently well established that mutations in the *MECP2* gene generally occur sporadically in most cases, while less than 1% are inherited from a carrier parent (Neul et al., 2008). Moreover, it has been observed that around 70% of *de novo MECP2* mutations has

a paternal origin (Amir et al., 2000; Girard et al., 2001; Zhu et al., 2010). Altogether, these observations have derived in a model according to which the preponderance of sporadic female RTT individuals is mainly due to a "protection" of males against RTT, since only females inherit the paternal X chromosome. Moreover, the finding of *MECP2* mutations in males (Villard, 2007) has led to discard definitively male lethality as a contributing factor for unequal sex ratio in RTT. Nevertheless, the reason why *MECP2* mutations occur more frequently during spermatogenesis is not yet fully understood. A possible mechanism contributing to this phenomenon may be the different activation state of the X chromosome in the oocytes and spermatocytes, as it is undermethylated in the first and hypermethylated in the latter (Zhu et al., 2010). A greater methylation state may facilitate the spontaneous conversion of a cytosine into a thymine, which represents the most common *MECP2* mutation (RettSyndrome.org).

So far, 929 mutations in the *MECP2* gene have been identified: point mutations, insertions, duplications, small or large deletions in almost all parts of the gene (RettSyndrome.org). Among them, approximately 800 mutations have been associated with RTT (Ehrhart et al., 2017). Around 70% of these are linked to eight single-nucleotide polymorphism hotspots as missense and nonsense mutations: T158M; R255X; R168X; R306C; R294X; R270X; R133C (Kyle et al., 2018; see Fig. 6). The same *MECP2* mutations found in females have been reported in approximately 56% of male cases (Reichow et al., 2015). In some of them, an extra X chromosome (XXY, a condition known as Klinefelter syndrome) allows to reproduce the RTT phenotype. In addition, in some males presenting a standard chromosome dosage it has been observed that they appear to have a mixed cellular population of wild-type and mutated *MECP2* alleles, this mimicking effects of X chromosome inactivation (XCI, *see below*). All these observations, although less relevant in terms of frequencies compared to female RTT cases, have also contributed to better understand genetic and cellular mechanisms of RTT.

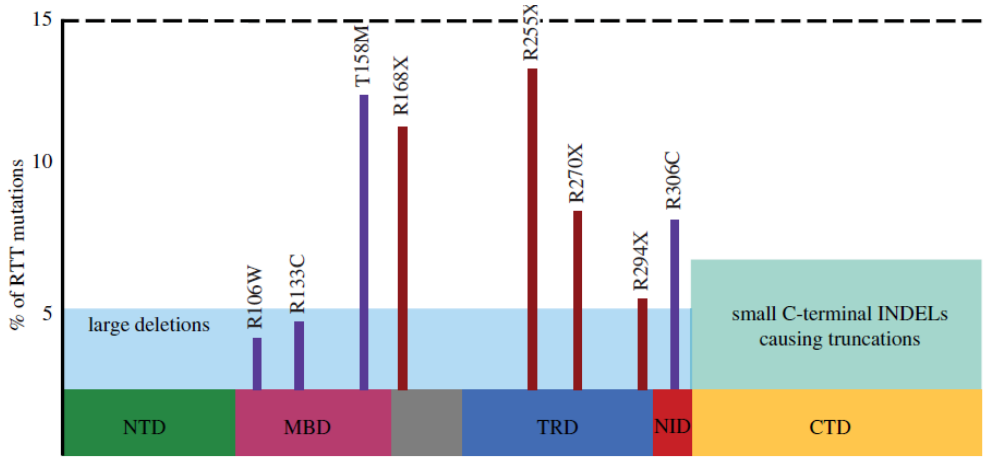


Figure 6 - Mutations in the multifunctional protein MeCP2 cause RTT (*from Kyle et al., 2018*). Coloured boxes indicate different encoded functional domains: light orange, N-terminus of MeCP2_e1; dark orange, N-terminus of MeCP2_e2; green, N-terminal domain (NTD) which has identical amino acid sequences between the two isoforms; pink, methyl-binding domain (MBD); blue, transcriptional repression domain (TRD); red, nuclear coreceptor co-repressor (NCoR) interaction domain (NID); yellow, C-terminal domain (CTD). Common damaging MECP2 mutations are shown. Y-axis represents percentage of RTT patients with indicated mutation. Missense mutations are in purple and nonsense mutations are in red. Combined, these point mutations make up approximately 70% of all RTT-causing mutations.

3.3 Genotype-phenotype correlations

Within the last years, it has been demonstrated that the large symptomatic variability observed in RTT individuals is partially due to the wide spectrum of *MECP2* mutations. For example, particular mutations such as R133C (p.Arg133Cys), R294X (p.Arg294X) and R306C (p.Arg306Cys) have been associated with more severe symptoms compared to other mutations (Cuddapah et al., 2014). In a recent study, large deletions in the *MECP2* gene were correlated with the development of several specific symptoms (microcephaly, loss of hand use, loss of language and onset of stereotypies) before thirty-six months of age (Vidal et al., 2019). However, not all RTT phenotypes are so genotype-dependent. For example, a multicentre study aiming to correlate RTT genotypes with cardiorespiratory symptomatology obtained very limited positive results (Halbach et al., 2016). Furthermore, analyses comparing mutations in *MECP2* with those occurred in other RTT-related genes have shown differences within the related frequencies of some clinical features. For example, presence of regression and gait dyspraxia are statistically more frequent in *MECP2*-mutated individuals, while epilepsy and reduction in eye-pointing capability are statistically more frequent in *CDKL5*-mutated individuals and the large majority of *FOXG1*-mutated individuals have never learned to walk, sit and speak (Frullanti et al., 2019). Phenotype-genotype correlation is an essential issue in terms of management of RTT individuals, as it may allow clinicians to partially anticipate disease progression and so to improve counselling to families.

3.4 X chromosome inactivation (XCI)

Just like most genes of the mammalian X chromosome, *MECP2* is subject to XCI (also known as lyonization; D'Esposito et al., 1996). This phenomenon consists in the random epigenetic silencing of one of the two X chromosomes, which becomes the "inactive" X chromosome (Xi). The XCI occurs in an early stage of embryonic development and acts as a dosage compensation mechanism (Lyon, 1971). As a result, females with typical RTT are heterozygous for *MECP2* mutations, presenting a mixture of cells that express either the wild-type or the mutated allele of the gene. Thus, XCI may act as a protection against RTT. First evidence of this situation emerged when, during the study of a familial RTT case, both parents of two affected girls were

found to be completely asymptomatic. Further analysis of the peripheral blood demonstrated that the mother carried a mutant X chromosome, but also that she presented an extremely skewed XCI ratio (higher than 95%), which had prevented her from developing RTT symptomatology (Sirianni et al., 1998). Within the following years, several extreme cases of "protection" against effects of *MECP2* mutations by non-random patterns of XCI were reported (Wan et al., 1999; Bienvenu et al., 2000; Villard et al., 2000). Other similar studies managed to link mild phenotypes (Amir et al., 2000) and cases of atypical RTT (Hoffbuhr et al., 2001; Zappella, 2001) to less skewed but non-random XCI ratios. A limitation of these studies is that they evaluated the methylation in only one locus, which may not reflect the general methylation of the X chromosome. In fact, a recent study analysing whether the preferentially inactivated X chromosome carried the mutant or the wild-type allele in 174 RTT individuals did not find a clear correlation between XCI and the clinical presentation (Xiol et al., 2019). Regarding molecular mechanisms of "protection" by the XCI, Xiol and her collaborators suggested that deletions and nonsense mutations may provoke the progressive selection of cells that have inactivated the X chromosome harbouring the mutation. On this way, XCI represents a factor contributing to variability within RTT symptomatology, even if the correlation is not so linear as previously thought.

4. Treating RTT

4.1 The long and uncertain road to a cure

In contrast to other neurological diseases, it does make sense to speak about a "cure" for RTT, that is to say, a technique or ensemble of techniques which, once applied to a patient, make the clinical condition to completely disappear. This hope is based on experiments demonstrating that re-expression of murine Mecp2 in mice knockout for *Mecp2* gene is sufficient to halt and reverse – at least partially – several RTT-like phenotypes (Guy et al., 2007). The fact that neurons do not die, but are just dysfunctional within the RTT brain (Zoghbi, 2016) also contributes to the idea of disease reversibility. Experiments performed by Guy and her collaborators did not provide a clear route to develop human therapies, but they did provide with an enormous motivator. Following their encouraging results, several research groups have given priority to work aimed at a cure. It is important to note that a side effect of this has been that less resources are currently dedicated to therapy-based approaches trying to improve quality of life of affected individuals and their families (Clarke and Abdala Sheikh, 2018).

Results from Guy and collaborators in 2007 gave two important indications for the development of any treatment against RTT. First, that its potential benefits could be significant no matter the

patient's age, as mice used in their experiments were fully adult. Second, and specifically for gene therapy, that the delivery of a working *MECP2* gene would need to continue throughout the person's life or, in other words, that it will not be enough to deliver it just during the child's development. This underlines the functional relevance of MECP2 after development and also that RTT is not a pure neurodevelopmental disease (Leonard et al., 2017). On the other hand, those experiments also showed the potential severe side effects of a not efficient gene therapy, as nine out of the seventeen "cured" mice died soon after treatment.

Within the last years, multiple studies have independently explored the therapeutic potential of adeno-associated viral (AAV) vector-mediated *MECP2* gene transfer in mouse models of RTT. Despite some variability in safety and efficacy, all *MECP2* gene therapy treatment paradigms used in these studies have been united by a single feat: they extended the survival of RTT mice, regardless of injection route, treatment age, or viral genome design (Sinnett and Gray, 2017). However, the general health of mice (evaluated through the 12-point scale designed by Guy et al., 2007) was improved only when vectors were injected intracranially (3 out 12 studies) and core symptoms such as respiratory abnormalities were improved only in one case. Furthermore, in several cases hepatic toxicity represented a major side effect of gene therapy.

One of the greatest known challenges for develop a cure for RTT is the so-called "Goldilocks's principle". According to this, the amount of protein needs to be precisely right in each brain cell: too much MeCP2 can be as bad as too little, as the existence of the *MECP2* duplication syndrome demonstrates (OMIM #300260; Moretti and Zoghbi, 2006). In addition, it has been observed that even more subtle increases in MeCP2 expression are able to provoke neurological disturbances (Reardon et al., 2010; Chao and Zoghbi, 2012). In other words, an effective gene therapy would need to deliver the right levels of *MECP2* to nearly all brain knockout cells and, simultaneously, to avoid delivering additional copies of the gene to cells already expressing the healthy copy. As most of studies on RTT gene therapy have been performed on male mice, few information on how to satisfy Goldilocks's principle is available.

To my knowledge, only one study has demonstrated the efficacy of a gene therapy in a female mouse model of RTT (Garg et al., 2013). Authors observed a partial rescue of survival rate, the stabilization of the general health score and improvement in some motor and respiratory features. However, the number of mice used for behavioural testing in this study was quite low and therefore results should be confirmed by future studies. Finally, authors admitted that several technical features (such as vector size, mode of delivery and promoter efficiency) have to be further improved.

Approaches aiming to restore normal gene dosage alternative to AVV are also being investigated. For example, it has been shown the potential of techniques such as the X-chromosome reactivation (Carrette et al., 2018; Przanowski et al., 2018) and gene editing through CRISPR/Cas9 technology (no specific publications available). In addition, just like announced by the Gene Therapy Consortium, a randomized clinical trial on RTT patients was launched early in 2019, but any results has been published yet, so effective applications in patients are probably far to be obtained (Clarke and Abdala Sheikh, 2018).

On the other hand, even if the challenge derived from "Goldilocks's principle" was overcome, we should expect important side effects of gene therapies. For example, the re-expression of MeCP2 in RTT people is likely to increase the volume of their brains, which would be a major problem to solve, as we know that RTT individuals have smaller head circumference on average compared to neurotypical individuals (Hagberg et al., 2001). We should also expect unwanted sensory and psychological effects derived from any treatment changing deeply the brain neurobiology. Some brain functions that will reawaken might be unpleasant, for instance pain, which is often reduced in RTT individuals (Downs et al., 2010). The effects on behaviour of a partially effective therapy have also to be considered. Affected individuals could become physically fitter and abler after treatment while still having cognitive and communication deficits, which may generate psychological distress and confusion.

In summary, research on a cure for RTT has made great steps within the last years, but effective applications in RTT individuals are far to be achieved and, even if this will ever happen, they could present several major side effects. In this context, it seems reasonable to dedicate consistent resources to short-term approaches aiming to improve quality of life of RTT people, even if they will not eliminate the disease.

4.2 Physical therapies

With the objective of improving motor, cognitive and social abilities of RTT people, specialists working daily with them have developed several effective approaches which have a great relevancy in their lives. Because of the nature of this type of work, literature on these topics is scarce, but some of the accumulated knowledge has been reported in scientific articles. Within the last decade, research on physical activity and sedentary behaviour in individuals with a range of child-onset disabilities have exponentially increased (Mcphee and Gorter, 2017). One apparently trivial but important issue that has emerged from this systematic study is that physical activity must not be prescribed to people with chronic physical disability following the

guidelines used for healthy people. In fact, it has been observed that benefits on health in people with chronic physical disability such as RTT individuals can be achieved at remarkably low levels of activity. In any case, promoting physical activity across the lifespan is vital in RTT people and particularly during the transition from adolescence to adulthood, as positive habits formed at this time may continue throughout life (Downs et al., 2017).

In developing a physical therapy program for a RTT patient, it is important not only to evaluate the present state but also anticipate future problems, considering what it is known about the usual course of the disease (*see above*). Due to the large clinical variability of RTT, a rehabilitation program must be individually tailored, based on a careful analysis of which factors are interfering with functional movements or causing other disturbances. Therapies should be more frequent during periods of loss of transitional skills and the basic goals will normally include: develop or maintain ambulation, develop or maintain transitional skills, alleviate discomfort and irritability and, in general, improve personal independence (Hanks, 1990).

Compared to other approaches, such as pharmacological treatments, physical therapies are less subjected to specificities, and physical therapists often use techniques on RTT individuals that have demonstrated to be effective in other types of disorders. In any case, these therapies remain an indispensable element within the daily care of RTT people, even when more efficient drug-based therapies will be available.

4.3 Current pharmacological treatments

Presently, no specific drug is approved for the treatment of RTT. Therefore, as in others neurodevelopmental diseases, the pharmacological management of RTT individuals is very variable and limited to the treatment of single symptoms, without tackling any root cause, even if aiming to improve quality of life. Life expectancy of RTT people has increased within the last years, in a way that survival into the fifth decade is quite typical, and death due to extreme frailty has become rare. Instead, the leading cause of death in people affected by RTT remains cardiorespiratory compromise (Tarquinio et al., 2015b).

As our knowledge on RTT has increased, it has become evident the need of a accurate planning for long-term care of these people, also from a pharmacological point of view. In particular, seven clinical domains are the main focus of pharmacological therapies in RTT patients (Chapleau et al., 2013):

- ***Sleep disturbances.*** RTT individuals may have difficulty falling to sleep or suffer from frequent awakenings during the middle of the night (Piazza et al., 1990). This may be

due to different disease states such as otitis, airway obstructions due to respiratory alterations or gastrointestinal dysfunctions, but also to neurological alterations within related brain networks. Several medications are effective to treat sleep disturbances in the RTT population: trazodone and chloral hydrate, which modulate serotonergic and GABAergic transmission respectively, have been shown to be safe and to aid sleep induction. Melatonin, an endogenously produced hormone which is secreted by the pineal gland and has an essential role in maintenance of circadian cycles, has also been proposed as a candidate to treat sleep disturbances in RTT. Finally, the L-carnitine, an amino acid derivate of methionine and lysine that is required for energy metabolism, has been shown to be beneficial in sleep maintenance in a subset of RTT patients.

- ***Cardiac dysfunction.*** The most relevant consideration regarding this domain is the greater incidence of sudden death within the RTT population as compared to non-RTT population (Acampa, 2006). Two main underlying mechanisms has been proposed for this: respiratory dysfunction (often characterized by hyperventilation and breath holding in RTT individuals) and irregularities in cardiac contractility (often leading to prolonged QT intervals and a delayed timing between ventricle depolarization and repolarization – conditions that are related to a higher risk of fatal arrhythmias). Considering these features, it is necessary to avoid medication that lowers respiratory rate in order to prevent deadly adverse drug responses. It is also important to avoid drugs or combination of drugs able to slow the QT interval, such as macrolide antibacterial agents.
- ***Bone mass and fractures.*** Osteopenia, a condition characterized by reduced bone mineralization and increased likelihood of fractures, is a concern in the RTT population and the cause that RTT individuals are at a greater risk of sustaining a fracture than the general population (Downs et al., 2008). Main hypotheses for this phenomenon are the lack of ambulation and an inadequate diet, in a way that in many cases a supplementation with vitamin D, an essential molecule for bone formation, can be beneficial. Drug-diseases interactions must be considered also regarding this domain. For example, some antiepileptic drugs (AEDs) alter the metabolism of bone, this increasing the risk of fractures. Thus, the use of AEDs in the RTT population needs to be closely monitored for alterations in bone structure.
- ***Behavioural alterations.*** Many RTT individuals show anxious and fearful behaviours, as well as general mood disturbances, particularly those carrying certain *MECP2* mutations (R133C, R294X and R306C; Mount et al., 2002; Robertson et al., 2006). Increased anxiety in RTT include difficulties maintaining sitting and standing posture, as well as irregularities in breathing patterns. From a pharmacological point of view, the most

successful management of anxiety and mood behaviour is the use of selective serotonin (5-HT) reuptake inhibitors (SSRIs). Among other mechanisms, these drugs are thought to modulate the hypothalamus-pituitary-adrenal axis, which is upregulated in RTT in association with increased anxiety (McGill et al., 2006). Moreover, 5-HT transmission is downregulated in RTT (*see below*) and so adjusting levels of this neurotransmitter might improve mood alterations in RTT individuals.

- ***Gastrointestinal dysfunctions.*** Basic steps in the digestive process, such as chewing or bowel motility, are often impaired in RTT individuals, this leading to dysphagia and constipation, among other disturbances. Gastroesophageal reflux due to irregularities in the motility of the gastric region and esophageal tract is also common within RTT population. All these features make daily intake a difficult task. Simethicone is used to prevent and alleviate the feeling of gas and can help in the symptoms of reflux. Regarding constipation, polyethylene glycol seems to be the best tolerated among drugs alleviating this symptom. Gastroesophageal reflux is an important issue for RTT patient and several non-pharmacological and pharmacological methods to treat it have been developed.
- ***Epilepsy and seizures disorders.*** It has been reported that approximately 60% of RTT individuals suffer from seizure activity during first years of life and some genotype-phenotype correlations to this regard have been identified (Glaze et al., 2010). Currently, there are many pharmacological options to alleviate seizures in RTT, being the most effective: valproate, lamotrigine, carbamazepine and oxcarbamazepine (Krajnc et al., 2011). When these therapies fail, levetiracetam has shown to be effective in reducing seizure frequency (Specchio et al., 2010). Since AEDs are associated with many different adverse drug events, it is critical to determine whether it is worth to initiate a pharmacological treatment and, if it is, which one is the most adequate.
- ***Breathing irregularities.*** In RTT population, issues such as hyperventilation, apnea, breath holding and air swallowing (occurring generally when awake) are quite common. Successful management of breathing irregularities in RTT remains elusive. Given the paucity of alternative treatments, it has been proposed that sarizotan, an agonist of serotonergic 5-HT_{1A} receptor, could be used to improve respiratory arrhythmias in some RTT individuals (Cheng et al., 2019). However, a close monitoring of electrocardiogram is advised, and its use must be avoided in patients with existing long QT syndrome (LQTS), a hereditary cardiac disease, or in combination with other QT prolonging medication.

4.4 Clinical trials

Data from multiples studies performed within the last years indicate that RTT can be considered a synapthopathy, as alterations in the maturation and function of synapses are a key element within the RTT physiopathology (Gadalla et al., 2011). These physiological alterations are an intermediate phenomenon between mutations in RTT-related genes (*see above*) and RTT symptoms. As gene therapy and other approaches aiming to cure RTT present many obstacles (*see above*), restoring the normal activity of neurotransmitter systems at the synaptic level could not only alleviate general RTT symptomatology, but also slow down its progression.

Based on this principle, most research on pharmacological approaches for RTT targets downstream factors of MeCP2 function such as neurotrophines and neurotransmitter systems (glutamatergic, cholinergic, GABAergic and monoaminergic systems). Among neurotrophines, the brain-derived neurotrophic factor (BDNF), a neurotrophin with important roles in brain development and plasticity, present lowered levels in RTT brain. In addition, pre-clinical data indicate that delivery of BDNF could be an effective treatment for RTT (Li and Pozzo-Miller, 2015). However, BDNF does not cross the blood brain barrier (BBB), and therefore it has no immediate therapeutic application for RTT patients. Because of this, the insulin-like growth factor 1 (IGF-1), which crosses the BBB and triggers cascades that resemble those of the TrkB receptor (one of the main targets of BDNF), has been proposed to treat RTT (Pini et al., 2012). Regarding neurotransmitter systems, many studies in mouse models indicate them as a very relevant target for treating RTT (*see below*).

According to ClinicalTrials.gov, a registry of publicly and privately supported clinical studies conducted around the world, 34 interventional (clinical) trials related to RTT has been conducted so far. Among these, only 17 have been registered as "completed": 10 trials aimed to evaluate the safety and efficacy of 8 different drugs, one tested a dietary supplement and the others studied different aspects ("uptime" activities, new genes involved in RTT, osteoporosis, metabolic alterations and natural history). The eight drugs tested in these clinical studies have completed phase II efficacy trials, meaning that their safety has been quite well verified and that some benefits have been proved (Mahan, 2014):

- **Vatiquinone (a.k.a EPI-743; Hayek, 2015).** Mitochondrial dysfunction, with resulting redox imbalance and increased oxidative stress, has been implicated in RTT pathogenesis (Filosa et al., 2015). Based on this, vatiquinone has been tested in a trial involving twenty-four RTT patients aged 2 to 8 years, and a significant increase in head circumference was observed. In addition, a subgroup of children with the greatest

degree of head growth showed improvements in oxygenation, hand function and disease biomarkers.

- **Glatiramer acetate (a.k.a copaxone; Djukic et al., 2016).** This molecule is a synthetic polypeptide containing four naturally occurring amino acids (L-glutamic acid, L-lysine, L-alanine and L-tyrosine) and being similar to the myelin basic protein. Ten 10-year-old ambulatory girls carrying different *MECP2* mutations participated in the trial. Results showed improvements in gait velocity, memory and breath holding index, but the trial was halted because of potential life-threatening side effects (Nissenkorn et al., 2017).
- **Dextromethorphan (Smith-Hicks et al., 2017).** This potent non-competitive NMDA antagonist and σ-1 receptor agonist was tested on twenty-five girls with RTT from 3 to 15 years of age to assess its safety and potential to normalize electrographic spikes, clinical seizures and behavioural and cognitive functions. Results indicated that it is a safe drug over a period of six months. In addition, significant dose-dependent improvements were seen in clinical seizures, receptive language and behavioural hyperactivity, while no improvement in global clinical severity was observed.
- **Trofinetide (a.k.a. NNZ-2566; Glaze et al., 2017)**. This drug is a synthetic analogue of the amino-terminal tripeptide of IGF-1 and was proved to be safe in 56 adolescent and adult females, with few adverse events observed. In addition, some improvement was showed in the RTT Motor-Behaviour Assessment Scale (MBAS) and other secondary scorings.
- **Recombinant human IGF-1 (a.k.a mecasermin; O'Leary et al., 2018).** This trophic factor was tested in 30 girls with typical RTT and a relatively high safety was observed, even if it did not reveal any significant improvement and some of the assessed parameters worsened.
- **Desipramine (Mancini et al., 2018).** This noradrenaline (NA) reuptake inhibitor has been successfully tested in RTT mouse models (Roux et al., 2007; Zanella et al., 2008). However, it did not show clinical efficacy in a II phase trial including 36 RTT girls, even if authors considered that their study provided with relevant reasons to test in other ways the noradrenergic pathway.
- **Lovastatin.** Statins, also known as HMG-CoA reductase inhibitors, are a class of lipid-lowering medications normally used to treat different cardiovascular diseases. Although the classification of RTT as a pure metabolic disease was abandoned after Rett associated erroneously the syndrome to hyperammonaemia (*see above*), within the last years the importance of cholesterol homeostasis for RTT physiopathology has been demonstrated (Mouro et al., 2019). Based on this, two preclinical studies have tested lovastatin, a well-known statin medication, in two different male mouse models of RTT

(Buchovecky, 2013; Villani et al., 2016). Only the first study obtained some positive results. However, a II phase clinical trial has been completed this year, showing a good safety profile and some beneficial effects, even if results are uncompleted.

- **Fingolimod (a.k.a FTY720)**. This sphingosine-1 phosphate receptor was first approved for immunotherapy on multiple sclerosis patients (Cohen et al., 2019) and then was tested in a mouse model of RTT, showing promising results (Deogracias et al., 2012). A phase II clinical trial was completed last year, but results are not available yet.

Taken together, results from these clinical trials are promising and will be useful to better manage RTT symptomatology. However, number of involved patients must be increased, and in any case no drug has proved to produce a general improvement in quality of life so far. In addition, different organizations are moving away from funding these types of studies, mainly because of high costs of drug development (Clarke and Abdala Sheikh, 2018). In this context, drug repositioning appears as a viable approach to develop pharmacological interventions able to enhance the quality of life of RTT individuals (Gogliotti and Niswender, 2019).

5. Modelling RTT in experimental animals

5.1 General homologies between mice and humans

The house mouse (*Mus musculus*) has long served as a model of human biology and disease, mainly because of their phylogenetic relatedness and physiological similarity to humans. In addition, they are easy to maintain and to breed in the laboratory, and there are currently many available inbred strains (Morse, 2007). Evidences of striking homologies between murine and human genomes (Mouse Genome Sequencing Consortium, 2002; Brown and Hancock, 2006) initiated the creation of transgenic, knockout and knockin mice, which have become powerful tools for research. Studies on mice have greatly contributed to our understanding of human biology (Fox et al., 2006) but, too often, mice respond to experimental interventions in ways that differ markedly from humans (Perlman, 2016). Therefore, each biological feature must be carefully evaluated to see whether it can be modelized in mice in a consistent way. Those features rooted in natural adaptations derived from evolutionary pressures are more prone to be conserved among species, while artificial genes, diets and environment imposed to experimental rodents are more likely to present great differences when compared to humans (Buffenstein et al., 2014).

Genes homologous to human *MECP2* are not found in non-vertebrate genetic model organisms such as the fruit fly and some worms (Hendrich and Tweedie, 2003). Instead, they are found in all vertebrates and DNA sequence is particularly highly conserved in human *MECP2* and murine

Mecp2 (Reichwald et al., 2000). Furthermore, amino acid sequence of resulting proteins (human MeCP2 and murine Mecp2) are 95% identical (Shah and Bird, 2017). These similarities suggest that molecular consequences of *MECP2*/*Mecp2* mutations are roughly comparable between the two species, this having supported the development of several mouse models of RTT by introducing different mutations in the *Mecp2* gene (Katz et al., 2012). Considering that not all RTT cases are due to *MECP2* mutations (*see above*), data obtained from experimentation with these animals are applicable to most cases of RTT, but not all, and because of this more specific RTT mouse models have been also generated.

5.2 The *Mecp2*$^{tm1.1Bird}$ mouse model

Mecp2 deficiency in mice generates several phenotypes which are very similar to specific symptoms observed in most RTT individuals. In general, the motor domain is the best modelized in mouse models, meaning that neurological defects observed in mice carrying mutations in the *MeCP2* gene are similar to those observed in RTT individuals (Ricceri et al., 2013). Even if milder, some cardiorespiratory abnormalities have been also been found in RTT mouse models, as well as alterations in the anxiety behaviour and the social-cognitive domain.

The *Mecp2*$^{tm1.1Bird}$ mouse line is one of the most widely used RTT mouse models. It was generated by eliminating *Mecp2* exons 3 and 4 through a Cre-Lox recombination system (Guy et al., 2001). On this way, the production of Mecp2 protein is completely abolished at the cellular level, in a way that hemizygous *Mecp2*$^{tm1.1Bird}$ male mice completely lacks the protein, while heterozygous female littermates present a mosaic of wild-type and knockout cells due to the XCI patterns (*see above*). Contrary to what observed prior to the publication of Guy and colleagues, this model demonstrates that general absence of Mecp2 does not cause embryonic lethality in male mice.

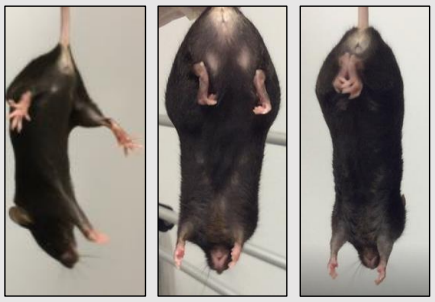


Figure 7 - Hindlimb clasping evaluation (*according to Guy et al., 2007*). Differences in reflex of hindlimbs can be observed between wild-type and symptomatic hemizygous or heterozygous *Mecp2*$^{tm1.1Bird}$ mice when they are suspended by the tail. This clinical sign is exclusive of mutant mice, as it is never observed in wild-type littermates.

In the original paper, Guy and collaborators described the most conspicuous phenotypes. Comparably to what happens in RTT individuals, both male and female mice showed no initial phenotype, but both developed a stiff, uncoordinated gait and reduced spontaneous movement between 3 and 8 weeks of age. Authors also observed that most animals subsequently developed hindlimb clasping, which represents a key phenotypic sign of *Mecp2*$^{tm1.1Bird}$ mice and that somehow mimic RTT individuals' hands stereotypies (see Fig. 7). Guy and her collaborators did not detect neither

motor nor sensory defects in mutant mice and did not observed obvious histological abnormalities in a range of organs, including the brain.

Characterization of *Mecp2*$^{tm1.1Bird}$ mice phenotypes has been improved through years, as this model has been used in multiple studies. Thus, respiratory abnormalities (Abdala et al., 2014), as well as several motor deficits (Horiuchi et al., 2017) have been confirmed in male mice. Most of studies using the Bird model have been conducted on Null male mice, because of three reasons. First, they present an earlier disease onset (some days after birth); second, the phenotypic variability is quite low and, finally, every male mouse becomes symptomatic before eight weeks of age (Guy et al., 2001). Instead, heterozygous female *Mecp2*$^{tm1.1Bird}$ mice present a later onset of the disease and a larger phenotypic variability mainly due to XCI. However, several deficits, mainly in the motor domain, have been verified in female *Mecp2*$^{tm1.1Bird}$ mice (Samaco et al., 2013; Vogel Ciernia et al., 2017). Performing experiments with female mice is more challenging, but their use is mandatory for any pre-clinical study aiming to have a significant translational value, as most RTT individuals are also females (*see above*).

5.3 Other animal models

While the Bird model allows to evaluate effects of the completely lack of Mecp2 protein in knockout cells, other animal models have been created to study different physiological scenarios. For example, the Jaenisch model (Chen et al., 2001) expresses small MeCP2 protein fragments. Despite molecular differences, *Mecp2*$^{tm1.1Jae}$ mice are phenotypically very similar to *Mecp2*$^{tm1.1Bird}$ mice. They also develop normally until 4 weeks of age and then start to develop RTT-like phenotypes: hindlimb clasping, tremors, breathing irregularities, loss of muscle tone and hypoactivity. Heterozygous female *Mecp2*$^{tm1.1Jae}$ mice develop same features at 4-6 months of age and typically live a normal lifespan. Because of these reasons, the Jaenisch model has been used extensively to study underlying pathophysiological mechanisms and potential treatments for RTT.

Despite the good face validity of these two models, they do not represent all human cases molecularly, since many RTT individuals carry missense mutations that result in a less efficient or unstable MECP2 protein rather than a complete loss of it. Because of this, several mouse lines with point mutations and deletions in MECP2 have been engineered to recapitulate clinically relevant mutations observed in RTT individuals (Vashi and Justice, 2019).

Although mice have long been the preferred species in the modelling of RTT, relatively recent advances in the ability to manipulate the rat genome has allowed to develop RTT rat models

that present similar RTT-like phenotypes compared to mice (Patterson et al., 2016). The increased size of the rat, and subsequently the size of all its anatomical structures, offers obvious advantages in a surgical setting aiming to perform region-specific studies of the central nervous system.

6. Neurotransmission alterations in RTT

6.1 Monoaminergic systems

Since the RTT entered the international medical consciousness, different lines of research have aimed to discover its underlying pathophysiological mechanisms. Among these, alterations in monoaminergic systems have been largely investigated. A first post-mortem single-case study showed a severe reduction of NA, 5-HT and dopamine (DA) in a RTT patient compared to a control (Brücke et al., 1987). At the same time, two other studies revealed that NA, 5-HT and DA metabolites were lowered in the cerebrospinal fluid of young RTT individuals compared to controls (Zoghbi et al., 1985; Zoghbi et al., 1989). These results were not confirmed in a following study analysing endogenous DA levels in several brain regions (Wenk, 1996) and neither in a study including cerebrospinal fluid and urine analyses of DA and 5-HT metabolites (Lekman et al., 2008). However, two newer studies support first observations by Zoghbi, as showed reduced levels of 5-HT and other monoamines in the plasma of young RTT individuals (Guideri et al., 2004; Ormazabal et al., 2005). Serotoninergic deficiency is also coherent with sleep alterations observed through analysis of sleep-awake rhythm and all-night polysomnography (Nomura, 2005). Two more recent studies found decreased levels of aminergic neurotransmitters metabolites in the cerebrospinal fluid of relatively big groups of typical RTT individuals (25 and 64, respectively) compared to control subjects (Temudo et al., 2009; Samaco et al., 2009). Finally, a recent study using both RNA sequencing and proteomics approaches showed altered monoamine metabolism in heterozygous *Mecp2*$^{tm1.1Jae}$ female mice, as two key enzymes were found downregulated (Pacheco et al., 2017). Taking together, these results indicate that alterations in monoaminergic systems are a relevant phenomenon in most RTT cases.

Regarding conflicting results, a hypothesis is that alterations of monoaminergic systems are brain area-dependent and, therefore, not detectable through too wide analysis. Supporting this, a study found different alterations on NA, DA and 5-HT systems only in some brain areas in male *Mecp2*$^{tm1.1Bird}$ mice (Santos et al., 2010). For instance, authors found decreased 5-HT levels in the prefrontal and motor cortices, the hippocampus and the cerebellum, while reduced DA levels were observed only in the cerebellum. These results indicate that negative peripheric measurements of monoamines metabolites cannot be interpreted as definitive, as they sensibility is not optimal.

It has been proposed that monoaminergic deficiency is partially due to a specific decrease in the number of medullary neurons expressing tyrosine hydroxylase, a rate-limiting enzyme of catecholamines (DA, NA and adrenaline) synthesis (Roux et al., 2007). In the same study, authors tested desipramine, an antidepressant specifically inhibiting NA reuptake, and found an improved respiratory phenotype in *Mecp2*$^{tm1.1Bird}$ male mice. Even if the clinical trial based on this study did not confirm desipramine therapeutic potential in patients, results from Roux and collaborators confirmed the relevance of monoaminergic alterations in RTT pathophysiology. On a similar way, sarizotan, an agonist of serotonergic 5-HT$_{1A}$ receptor, has shown a certain efficacy in improving respiratory arrhythmias in some RTT patients (*see above*).

On the other hand, mirtazapine (MTZ) is a noradrenergic and specific serotoninergic antidepressant (NaSSA) with an excellent safety profile (Szegedi and Schwertfeger, 2005), as it lacks anticholinergic (Burrows and Kremer, 1997) and cardiorespiratory side effects (Hartmann, 1999). When tested in male *Mecp2*$^{tm1.1Bird}$ mice, MTZ rescued several behavioural, physiological and neuromorphological phenotypes (Bittolo et al., 2016), demonstrating to be a good candidate to treat RTT. MTZ is currently used primarily for the treatment of major depressive disorders, but it has also sedative, antiemetic, anxiolytic and appetite-stimulating effects. Because of this, it is used off-label in a series of conditions, such as insomnia, panic disorder, post-traumatic stress disorder, obsessive-compulsive disorder, generalized anxiety disorder, social anxiety disorder, headaches and migraines (Jilani and Saadabadi, 2019). As an antidepressant, it has a unique action profile, characterized by a relatively rapid onset of action, high response and remission rates, a favourable side-effect profile and several unique therapeutic benefits over other antidepressants (Alam et al., 2013). So far, 18 receptors have been identified as targets of MTZ in the brain (see Table 2), but effects exerted on nervous system are just partially known. MTZ shows its highest affinity for histaminergic H1 receptor, which is responsible for sedation when used at low doses (Fawcett and Barkin, 1998).

Regarding monoaminergic systems, MTZ enhances 5-HT and NA transmission through different mechanisms. At the presynaptic level, it antagonizes α-2 auto and hetero adrenoreceptors, this leading to an increasing of NA release, while at the postsynaptic level it antagonizes 5-HT$_2$ and 5-HT$_3$ receptors, this indirectly enhancing 5-HT$_{1A}$-mediated serotoninergic transmission (Anttila and Leinonen, 2006). In addition, NA released in the raphe nuclei further stimulates postsynaptic α-1 receptors, causing 5-HT release from downstream axon terminals such as those in the cortex (Nakayama et al., 2004; Sthal, 2010).

NEUROTRANSMITTER	RECEPTOR	MTZ AFFINITY
Histamine	H1	9.3
Serotonine	5-HT_{2A}	8.2
Serotonine	5-HT_3	8.1
Noradrenaline	α-2 heteroreceptor	8.0
Serotonine	5-HT_{2C}	7.9
Noradrenaline	α-2 autoreceptor	7.7
Noradrenaline	Postsynaptic α-2	7.3
Noradrenaline	Presynaptic α-2	6.8
Serotonine	$5HT_{2B}$	6.7
Noradrenaline	α-1 adrenoreceptor	6.5
Acetilcoline	muscarinic	6.2
Dopamine	D1	5.8
Dopamine	D2	5.6
Serotonine	$5HT_{1A}$ - $5HT_{1D}$	5.3
Serotonine	$5HT_{1B}$	4.9

Table 2 - Affinity of MTZ for different neurotransmitter receptors expressed as pA_2 or pK_i (*modified from Anttila and Leinonen, 2001*).

6.2 Excitation-inhibition imbalance

Gamma-Aminobutyric acid (GABA)-ergic and glutamatergic systems are the main responsible for the maintaining of the fine excitation-inhibition (E/I) balance during neuronal maturation and in the mature brain. Regarding RTT, several studies have indicated that *Mecp2* loss-of-function alters E/I balance, this creating too "noisy" networks which are not optimally functional. Electrophysiological studies using mice constitutively lacking Mecp2 showed weaker and fewer glutamatergic connections in both isolated neurons and brain slices (Chao et al., 2007; Dani and Nelson, 2009), this indicating a shift of the E/I balance in favour of inhibition. Supporting this, transgenic mice lacking Mecp2 only in a subset of forebrain GABAergic neurons recapitulate many RTT features (Chao et al., 2010). In the same study, authors observed that Mecp2-deficient GABAergic neurons show reduced inhibitory quantal size, together with a consistent presynaptic reduction in glutamic acid decarboxylase-1 and -2 levels and GABA immunoreactivity.

However, E/I imbalance is not shifted in the same way in all brain areas and in all developmental time points. Thus, the ratio E/I is increased in several brainstem areas (Medrihan et al., 2008; Taneja et al., 2009; Kline et al., 2010; Kron et al., 2012) and in the hippocampus (Calfa et al., 2011; L. Zhang et al., 2008) of Mecp2 mutant mice, while a hypoexcitability generally characterizes cortical regions in RTT mouse models (Dani et al., 2005; Wood et al., 2009; Wood and Shepherd, 2010; Durand et al., 2012; Kron et al., 2012b; Kang et al., 2014).

Finally, a recent study has revealed that Mecp2 differently regulates the expression of several genes in excitatory and inhibitory neurons in postnatal tissue (Johnson et al., 2017), which provides with a molecular mechanism for the E/I imbalance. Some of these genes, coding for

glutamatergic receptors and ion channels, are dysregulated as early as at embryonic day fifteen (Bedogni et al., 2016).

6.3 Parvalbuminergic neurons

The overwhelming majority of long-distance connections in the brain cortex originates from glutamatergic neurons, while GABAergic cells are mostly interneurons (the "short axon cells" described by Ramón y Cajal). The spectacular diversity of GABAergic interneurons (see Fig. 8) enables an elaborate division of inhibitory control through many different mechanisms that shape highly nuanced spatiotemporal dynamics of cortical circuit computation. Hundreds of studies on GABAergic interneurons have revealed their stunning specificity in terms of synaptic connectivity, physiological characteristics and functional properties in circuit operations (Huang and Paul, 2019). Major GABAergic subclasses have been defined by classical anatomical, physiological, molecular and developmental studies (Fig. 8).

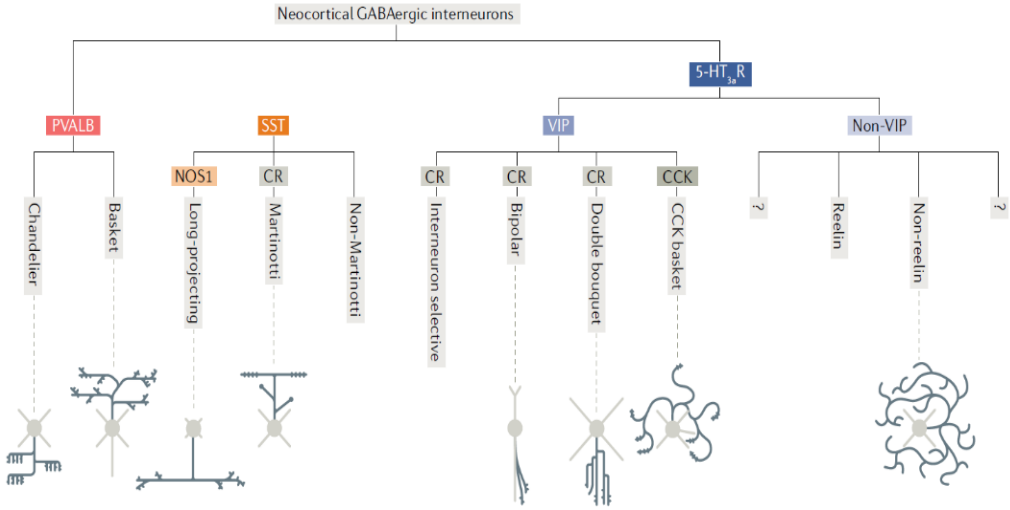


Figure 8 - Uncompleted taxonomy of transcriptomic neuron types of the cortical GABAergic system (*from Huang and Paul, 2019*). Schematics of the characteristic morphology of major GABAergic subclasses are shown. Light grey lines represent dendrites, while dark grey lines represent axons. 5-HT3AR, serotonin 3A receptor; CCK, cholecystokinin; CR, calretinin; NOS1, nitric oxide 1; PVALB, parvalbumin; SST, somatostatin; VIP, vasoactive intestinal peptide.

Besides to parvalbumin (PV) and somatostatin (SOM), two well-known non-overlapping biomarkers that covers around the 80% of all interneurons, the ionotropic serotonergic receptor 5-HT_{3A} has been identified as a third major biomarker in this domain (Lee et al., 2010). Together, these three subpopulations account for nearly 100% of GABAergic neurons in the somatosensory cortex of mice (Rudy et al., 2011)

In the context of RTT research, PV-positive (PV+) neurons have emerged as a highly interesting GABAergic subpopulation. Density of PV+ neurons is increased in Mecp2 knockout mice already at early post-natal developmental stages, while SOM+ neurons are unaffected (Tomassy et al.,

2014). PV expression is also altered in RTT, as protein levels are reduced in both primary somatosensory and primary motor cortices of *Mecp2*$^{tm1.1/Jae}$ male mice (Morello et al., 2018). In addition, mice lacking Mecp2 only in PV+ neurons have been observed to develop several RTT-like motor, sensory, social and cognitive phenotypes (Ito-Ishida et al., 2015). Taking together, these results demonstrate that, despite its broad expression, *Mecp2* mutations selectively affects neocortical GABAergic interneurons subtypes, being PV+ neurons the mainly affected population. Indeed, a pre-clinical study has demonstrated that ketamine administered to *Mecp2* deficient mice exerts some of its beneficial effects by modulating activity of *N*-methyl-D-aspartate (NMDA) receptors onto PV neurons (Patrizi et al., 2016).

PV+ cells are fast-firing GABAergic cells which accounts for the 40% of all interneurons in the neocortex of rodents (Jiang et al., 2016). PV is a member of the EF-hand family of Ca^{2+}-binding proteins and serves as a buffer for free Ca^{2+} after income following action potentials (Permyakov et al., 2017). In the brain cortex, they are responsible for the maintenance of E/I balance in pyramidal neurons (Xue et al., 2014). General loss of *Mecp2* induces an early maturation of PV+ cells, which leads to increased inhibitory innervations onto pyramidal cells and reduced neuronal activity in the visual cortex (Durand et al., 2012).

6.4 Perineuronal nets (PNNs)

Approximately 10-20% of the brain volume is occupied by the extracellular matrix (ECM), which is a dense network of proteins and glycans having essential roles in brain function. Within the ECM, three compartments can be distinguished: the basement membrane, the interstitial matrix and PNNs. The latter surround some somata and dendrites, leaving free space for synapses to come into contact. On this way, PNNs plays very important roles for synaptic homeostasis (Bosiacki et al., 2019). Although PNNs were described meticulously for the first time by Camillo Golgi in 1893, they fell into oblivion few years later due to the heated debate raged between reticularists and neuronists (Spreafico et al., 1999). It was not until 1960-80's that new histological procedures demonstrated not only the real existence of PNNs but also provided some insights into their possible functional significance.

During postnatal development, chondroitin sulfate proteoglycans (CSPGs) progressively accumulate around somatic and dendritic synapses of certain neurons, contributing to the formation of PNNs (Celio et al., 1998). CSPGs are secreted by different cells, but there are evidences that neurons alone are able to construct PNN (Miyata et al., 2005), even if a role of glial cells in this process in brain has not been discarded. A detailed description of PNNs chemical composition is displayed in Fig. 9.

The best-known histological technique to detect the presence of PNN is using lectin-agglutinin isolated from *Wisteria floribunda* (WFA; Apte, 2020). Histological staining with this molecule has revealed the presence of PNNs throughout the entire brain and spinal cord in mammals, but with different cell-type specificity. In the brain, most of neurons surrounded by PNNs are inhibitory neurons, specially PV interneurons (Miyata et al., 2018) even if some pyramidal cells in the deeper cortical layers are also surrounded by PNNs (Matthews et al., 2002). In subcortical areas such as the hippocampal CA2 region and the amygdala, the PNNs surround both excitatory and inhibitory neurons (Morikawa et al., 2017), while in the deep cerebellar nuclei only large excitatory neurons were surrounded (Carulli et al., 2006). It is important to note that there are also WFA-negative PNNs, including cortical output neurons which are instead recognized by aggrecan antibodies (Matthews et al., 2002).

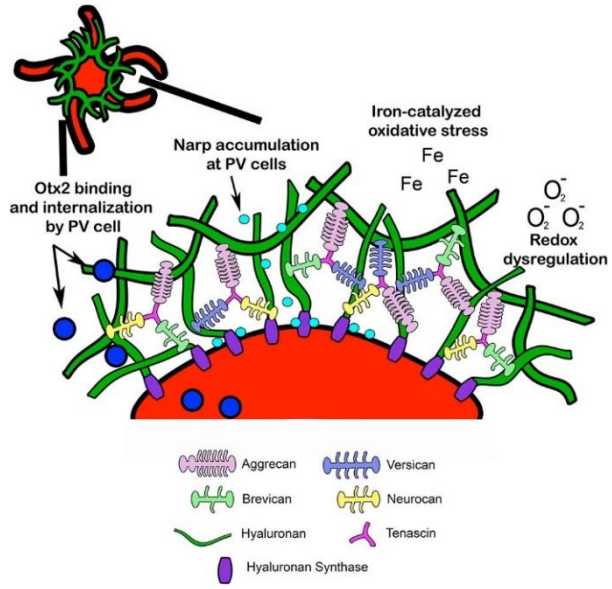


Figure 9 - Structural composition of PNN *(from Wen et al., 2018)*. Lecticans are attached to a hyaluronan backbone via link proteins such as cartilage link protein and hyaluronan link proteins (*not pictured*). Hyaluronan synthase contributes to hyaluronan synthesis and anchors hyaluronan to the cell membrane. Tenascin promotes crosslinking of lecticans and helps maintain PNN structural integrity. Narp, neuronal activity-regulated pentraxin; Otx2, orthodenticle homeoprotein 2.

An accelerated maturation of GABAergic system and, more specifically, of the PV network has been shown to trigger an early onset of the plasticity critical period in the visual cortex (Huang et al., 1999; Di Cristo et al., 2007). The maturation of PV networks is crucial for experience-dependent formation of synaptic connections and wiring of functionally related neuronal pathways (Hensch, 2005) and its schedule is in part defined by molecular markers such as PNNs (Nowicka et al., 2009). The visual cortex, which has been explored in these studies, exemplifies the importance of PNN-related critical periods within brain plasticity processes and this might be generalized across systems. For example, PNNs have been also demonstrated to underlie the restricting synaptic plasticity in hippocampal area CA2 (Carstens et al., 2016). It has been proposed that PNN modulates neural plasticity via three different mechanisms:

- Acting as a physical barrier to the formation of new neuronal contacts (Corvetti and Rossi, 2005; Barritt et al., 2006).
- Offering a scaffold for chemorepulsive axon guidance proteins such as semaphorin3A (Vo et al., 2013).

- Limiting lateral mobility of synaptic surface receptors, in particular glutamatergic AMPA receptors (Frischknecht et al., 2009)

Additionally, it has been observed than, in physiological conditions, PNNs protect neurons from redox dysregulation and oxidative stressors such as superoxide free radicals (O_2^-) and metal ions (Cabungcal et al., 2013).

Overexpression of PNNs in RTT was shown long time ago (Belichenko et al., 1997). In this first study, authors analysed post-mortem several brains coming from typical RTT individuals and observed that PNN expression was increased in several cortical areas. These results have been recently confirmed in male knockout mice of the *Mecp2*$^{tm1.1Bird}$ mouse model (Patrizi et al., 2019), which underlines the importance of PNNs within RTT neuropathology.

7. Neuroanatomical alterations

Opposite to most common neurological clinical conditions such as Parkinson's or Alzheimer's diseases, RTT is not characterized by neuronal loss (Zoghbi, 2016). The general phenomenon underlying RTT is the failure of neuronal networks, this opening the road for many therapeutic interventions (*see above*). This functional failure is associated with a general atrophy of brain. A study evaluating twenty-three girls with *MECP2* mutations revealed reductions of dorsal parietal gray matter and the anterior frontal lobe, which correspond to brain regions underlying key functional deficits observed in RTT (Carter et al., 2008). In a more recent publication, authors performed a similar analysis in both *Mecp2*$^{tm1.1Bird}$ and *Mecp2*^{tm2Bird} lines and found that many cortical and subcortical regions are reduced in volume in mutant mice (Allemang-Grand et al., 2017). Macroanatomical atrophy is due to a decrease in neuronal soma size and dendritic arborization, as several studies in RTT individuals have reported (Armstrong et al., 1995; Kaufmann and Moser, 2000; Armstrong, 2001). Regarding RTT mouse models, generalized neuronal atrophy has been confirmed through both *in vivo* (Belichenko et al., 2009; Bittolo et al., 2016) and *in vitro* approaches (Baj, Patrizio et al., 2014).

AIMS AND EXPERIMENTAL PARADIGM

The main goal of my PhD project was to provide with evidences that MTZ is a good candidate to alleviate in a general way RTT symptomatology and eventually slow down its progression. In addition, data coming from this project should give some specific indications about how to use MTZ for the mentioned purpose. Finally, the project aimed to deepen into mechanisms through which MTZ exerts its beneficial effects on the central nervous system.

In order to achieve these aims, an *in vivo* approach was used, as it is currently the best way to preclinically test a drug. Specifically, we treated mice carrying a *Mecp2*-mutation and showing RTT-like phenotypes with MTZ at a high dose, and evaluated its effects in several behavioural domains and at a histological level. Moreover, MTZ was tested at different ages and with different treatment durations in order to contextualize main results.

MATERIALS AND METHODS

1. Experimental design

Timelines of the three different experimental designs used can been seen in Fig. 10. We started by testing female *Mecp2*$^{tm1.1Bird}$ mice at 6 months of age as literature indicated that almost most of them are symptomatic at this developmental point (Guy et al., 2001). After performing a phenotypic evaluation in younger female mice (*see Results*), we decided to treat mice also at 9 weeks of age, which corresponds to a late developmental state. On this way we were able to assess effects of MTZ in a context where more plasticity processes are still active, compared to adult ages. The specific behavioural tests were selected considering the existing literature and according to the age and sex of mice. In all cases, we tried to cover different domains (general health, motor performance, anxiety state, short-term memory), as MTZ could theoretically exert beneficial effects in all of them. All measurements were performed blind to the genotype and treatment of the animals, and all control animals were wild-type (WT) age-matched littermates of heterozygous (HET) *Mecp2*$^{tm1.1Bird}$ mice.

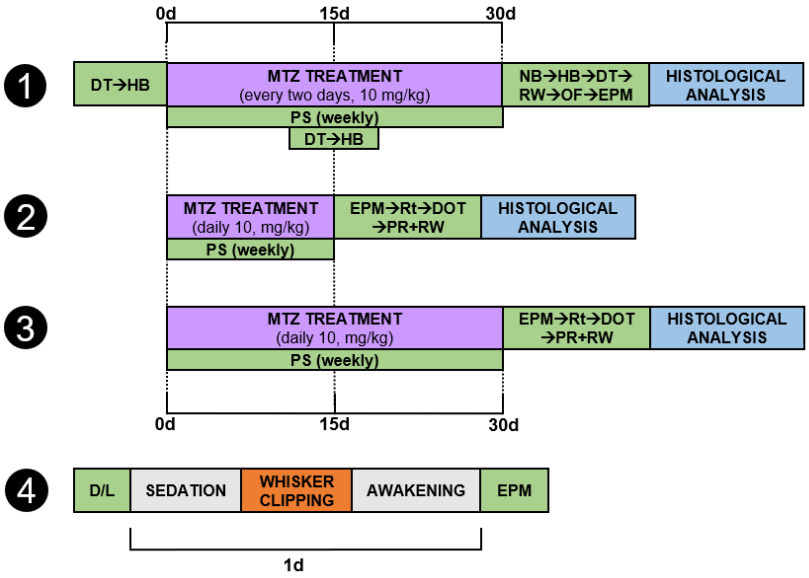


Figure 10 - Experimental timelines of experimental designs. The four different general protocols are shown. MTZ treatment was initiated when mice were either 6 months (protocol 1) or 9 weeks old (protocols 2 and 3). Only in the first protocol some behavioural tests were performed before the treatment (DT, Dowel test; HB, horizontal bars). DT and HB were also performed in the middle of the treatment within the first protocol. In all cases, general health was evaluated weekly, beginning at the first day of treatment. MTZ dosage administered in each single injection was not changed among protocols. However, MTZ was injected every two days in the first protocol and every day in the second and the third ones. After the treatment, different tests were used in each protocol: NB, nest building; RW, rod walk; OF, open field; EPM, elevated plus maze; Rt, accelerating rotarod; DOT, 4-different objects test; PR, patch removal test. Beginning from the day after the last injection, one test was performed per day. At the end of every protocol, mice brains were dissected and prepared for different histological analysis. Protocol 4 was additionally used to investigate phenotype of 6-month-old mice at the EPM. Untreated mice were first tested at the Dark-Light exploration test (D/L). After that, they were sedated ad then their whiskers were shortened. Mice were left in their home cages overnight and the day after they were tested at the EPM.

2. Mouse model

2.1 Experimental animals

Mice were treated according to the institutional guidelines, in compliance with the European Community Council Directive 2010/63/UE for care and use of experimental animals. Authorization for animal experimentation was obtained from the Italian Ministry of Health (Nr. 124/2018-PR), in compliance with the Italian law D. Lgs.116/92 and the L. 96/2013, art. 13. All efforts were made to minimize animal suffering and to reduce the number of animals used. For production of experimental adult mice, *Mecp2* HET females (*Mecp2*$^{+/-}$, B6.129P2(C)-*Mecp2*$^{tm1.1Bird/J}$, Stock No: 003890, The Jackson Laboratory, USA; Guy et al., 2001) were bred with wild-type C57/BL6J male mice (The Jackson Laboratory, USA). For production of young females, we generated a new mouse line by crossbreeding *Mecp2*$^{tm1.1Bird/J}$ female mice with male carrying the *EGFP* gene under the control of Thy-1 promoter (Thy1-GFP line M, Tg(Thy1-EGFP)MJrs/J, Stock No: 007788; The Jackson Laboratory, USA). This will facilitate the analysis of the neuronal morphology in the future. After weaning, all mice were housed in ventilated cages under 12h light/dark cycle with food and water ad libitum. No environmental enrichment elements were added to the cages. For both adult and young mice evaluations, we used female HET mice because they represent the most reliable model of RTT and because MTZ have been already tested on hemizygous *Mecp2*$^{tm1.1Bird/J}$ male mice (Bittolo et al., 2016).

2.2 Mice genotyping

Biopsies from ear punches were incubated with 250 µL of DNA extraction buffer (TRIS 10 mM pH 7,5, EDTA 5 mM pH 8, SDS 0,2%, NaCl 10 mM, proteinase K 0,5 mg/mL) and left overnight at 55 °C. The day after, samples were centrifuged (12000 rpm, 20 min, RT), then 100 µL of the supernatant were mixed with isopropanol (1:1) and precipitated DNA was centrifuged again (12000 rpm, 30 min, RT). Supernatant was then discarded and three washes with cold 70% ethanol with subsequent centrifugations (12000 rpm, 5 min, RT) were realized. Once ethanol had evaporated, DNA pellets were homogenously dissolved in milli-Q water. Genotypes were assessed by PCR on ear genomic DNA using specific primers (forward common primer 9875 5' – AAATTGGGTTACACCGCTGA–3', reverse mutant primer oIMR9877 5'–CCACCTAGCCTGCCTGTACT – 3', reverse WT primer oIMR7172 5' – CTGTATCCTTGGGTCAAGCTG – 3'). PCR reactions were performed with 1U GoTaq polymerase (Promega, Madison, USA), 1X green GoTaq buffer, 0,2 mM dNTPs each, 2,5 mM $MgCl_2$, 0,5 µM of each primer and 10 ng/µL of genomic DNA, as follows: 95 °C, 3' > 30 cycles: 95 °C, 20"; 58 °C, 20"; 72 °C, 20" > 72 °C, 2'. This PCR generates a 400-bp product for WT allele and an additional 416-bp product for heterozygous mice (Bittolo et al., 2016).

3. Drugs

3.1 Experimental treatment with MTZ

Beginning from either 5 months or 9 weeks of age (see Fig. 10), HET females and WT littermates were intraperitoneally (i.p.) injected with vehicle (VEH = 0,9% aqueous solution of NaCl and 5% ethanol) or MTZ (10 mg/kg, ab120068, Abcam, Cambridge, UK). Adult mice were treated for 30 days on alternate days, based on literature showing that half-life of MTZ in mice is higher than 48 hours. We arrived to this conclusion because MTZ has a half-life of 20-40 hours in humans (Timmer et al., 2000) and this value is between 33 and 300% higher in mice (Bachmann et al., 2007).

Young mice were treated every day (in order to mimic normal posology in patients) for either 15 or 30 days. The MTZ dosage (10 mg/kg) was unchanged in all protocols, as it is equivalent to 45 mg/day, the maximum dose admitted in patients for the treatment of depression (Nair and Jacob, 2016). In all cases, the same homogeneous experimental groups were created: wild-type mice treated with vehicle (WT-VEH), wild-type mice treated with mirtazapine (WT-MTZ), heterozygous mice treated with vehicle (HET-VEH) and heterozygous mice treated with mirtazapine (HET-MTZ). Comparison of MTZ effects between WT-VEH and either WT-MTZ or HET-VEH allowed us to evaluate MTZ toxicity and RTT-like phenotypes, respectively. In addition, comparison between HET-VEH and HET-MTZ mice allowed us to detect eventual phenotypical rescues by MTZ.

3.2 Sedation for whiskers clipping

This procedure was applied only to a group of untreated 6-month-old WT and HET mice. Prior to the whisker clipping, mice were tested at the L/D test (see Fig. 10). After that, they were injected with 100 µg/kg medetomidine (Domitor®, Vetoquinol, Magny-Vernois, France), an α-2 adrenergic agonist with hypnotic and sedative effects, to prevent potentially risky movements. Mice were then left into an incubator at 37 °C and when they were completely sedated, we proceeded to shorten all whiskers up to few millimetres from the skin (leaving untouched whiskers bulbs). Immediately after that, unwhiskered mice were injected i.p. with 1 mg/kg atipamezole (Antisedan®, Zoetis, New Jersey, USA), a specific antagonist of α-2 adrenergic receptors. Mice were then kept for some hours in an incubator at 30 °C. When they were completely awake, we put them back in their home cages and left there overnight. The day after, unwhiskered WT and HET mice were tested at the EPM.

4. Assessment of behavioural phenotypes

General scheduling of different MTZ treatments and behavioural testing is represented in Fig. 10. Specific tests used at each age was adapted to previous data on behavioural phenotypes (e.g. when we performed analysis of adult mice, no information about cognitive phenotypes had been reported on our mouse model). In addition, we realized a separated behavioural assessment of untreated mice whose whiskers had been clipped.

The following domains were assessed through behavioural tests: general health, motor performance, anxiety and short-term memory. As RTT is a progressive disorder with an evolution over time which is specific for each patient and this is similar in HET *Mecp2*$^{tm1.1Bird}$ mice, we decided to over time effects of MTZ on some behavioural phenotypes. Thus, we selected two motor tests that can be repeated without alterations (DT and HB) and performed them not only after the treatment, but also before and during, in 6-month-old *Mecp2*$^{tm1.1Bird}$ mice (see Fig. 10). This was not repeated in younger mice as they do not show any phenotype when subjected to these behavioural tests.

4.1 General health

Six-month-old mice were removed from their home cages and place onto a laboratory bench for observation at the same time of the day. Body weight was measured, and phenotype severity was evaluated through a semiquantiative scoring system modified from Guy et al. (2007). The main evaluation comprehends the following features: mobility, gait, hindlimb clasping, tremor, breathing and state of fur and eyes. Scoring was as follows: 0 = absent or as observed in WT; 1 = moderate phenotype; 2 = severe phenotype. By summing these values, we obtained an average value for each mouse. In addition, we also evaluated qualitatively the following features during observation: reactive vocalizations, eyes aperture, hierarchy inside the cage (based on loss of hair in the back due to barbering behaviour), involuntary (clonic and tonic) movements and grooming behaviour.

In younger mice, only the hindlimb clasping and the body weight were evaluated, as all the other clinical signs were almost never observed. However, all mice were carefully monitored every day in order to detect any unexpected event.

4.2 Motor performance

· **Dowel test (DT).** Following a modified protocol based on Deacon (2013), we positioned the mouse with its four paws on the free edge of a striped 60-centimeter wooden dowel and

measured the latency to fall (endpoint = 120 s). The test was consecutively performed on two dowels with decreasing diameters (12 and 10 mm) and repeated before, during and after the treatment. For each trial, we calculated the averaged latency to fall from the two dowels. This test was performed only in 6-month-old mice (see Fig. 10).

· **Horizontal bars test (HB).** Following a modified protocol based on Deacon (2013), we let the mouse hang, with his forelimbs only, to a horizontal bar and then we measured the latency to fall (endpoint = 30 s). An individual score was assigned to each mouse: 1-5 s = 1 point; 6-10 s = 2 points; 11-20 s = 3 points; 21-30 s = 4 points; no fall = 5 points; traveling to the end of the bar = 6 points. The test was consecutively performed on bars with decreasing diameters (4 and 2 mm). Corresponding scores from both bars were summed, obtaining an individual score for each mouse. This test was performed only in 6-month-old mice (see Fig. 10).

· **Nest building test (NB).** Following a protocol that takes advantage of natural trend of mice to create a nest with different materials (Deacon, 2006), we put each mouse in an individual cage one hour before the beginning of the dark phase. We kept them in the cage for 16 h, in presence of a 3-gram nestlet (Ancare, New York, USA) but without any other environmental enrichment. The day after, mice were returned to their home cages and nests were visually assessed and unshredded material was weighed. This test was performed only in 6-month-old mice (see Fig. 10).

· **Rod walk test (RW).** Following a modified protocol based on Luong et al. (2011), we positioned the mouse at one edge of a 60-centimeter wood dowel (standing 50 cm above the floor), where a repulsing stimulus (strong light) was present. On the other side of the dowel, we positioned an attracting stimulus (dark cage with nesting material from the mouse's home cage). In adult mice, transition time was measured in two consecutive trials, performed with striped dowels of 12 and 10 mm of diameters. In younger mice, dowels of 10 mm and 8 mm of diameter were used instead, as their smaller size make too easy the transition through that of 12 mm.

· **Open field (OF).** Each mouse was individually placed in the centre of the open field (40 x 40 x 40 cm) and left to freely explore it for 20 min. Movements of mice were recorded from above to avoid interference of the experimenter and videos were later analysed with ANY-maze software (Stoelting, New Jersey, USA). Analysis was performed as previously described (Bittolo et al., 2016). In short, we divided the open field area in central, middle and border zones and measured automatically or manually the following parameters: entries and time spent in each zone, total travelled distance, mean speed, immobility episodes, grooming levels, vertical activity and hopping behaviour. This test was performed only in 6-month-old mice (see Fig. 10).

· **Accelerating rotarod (Rt).** This test was performed three times a day (with one inter-trial hour) for three consecutive days. In each trial, we placed mice on the rotarod apparatus (Ugo Basile, Varese, Italy) and initiated a specific program (increasing speed from 5 to 40 rpm for a maximum

time of 5 min), according to previous literature (Vogel Ciernia et al., 2017). For each mouse, we measured the latency to fall. Each individual trial was considered over when the animal fell or hanged to the rod, without moving, for three loops. This test was performed only in 11-week-old mice (see Fig. 10).

· **Patch removal test (PR; modified from Bouet et al., 2009).** The three days before the trial, mice were habituated to individual cages for 15 minutes. For testing, we first immobilized the mouse and placed one adhesive tape strip (0,5 x 0,5 cm) on each forepaw. We then replaced the mouse into its individual experimental cage and measured time to remove both tape strips and the number of contacts forepaw-mouth realized to achieve this goal.

4.3 Anxiety

· **Elevated plus maze (EPM).** Each mouse was individually placed in the centre area of a black Plexiglass elevated plus-maze for a 5-min test session (Vogel Ciernia et al., 2017). Mice movements were recorded and later analysed with the ANY-maze software (Stoelting, New Jersey, USA). We semiautomatically measured the following parameters: entries per arm, time spent in each arm and number of fallings. This test was performed in 6-month-old and younger mice after MTZ treatment (see Fig. 10). In addition, it was also performed in unwhiskered untreated 6-month-old mice.

· **Dark-light exploration test (D/L).** This test was performed as previously described (Vogel Ciernia et al., 2017). In short, the apparatus was made of two Plexiglass compartments separated by a partition with a small opening. One compartment was transparent and illuminated, while the other was opaque and closed on top. Each mouse was individually placed into the centre of the light compartment and allowed to freely explore the area for 10 min. The number of transitions between light and dark sides, as well as time spent in light compartment were recorded and later analysed with ANY-maze software (Stoelting, New Jersey, USA). This test was performed in untreated 6-month-old mice before whiskers clipping (see Fig. 10).

4.4 Short-term memory

Four-different object recognition (4-DOT). We adapted the protocol described in (Sannino et al., 2012). We first familiarized animals to the experimenter and to the presence of small objects (different and smaller from those of experiment), as well as to individual cages. To do this, the three days before the trial, we used the following sequence every day: habituation to small objects in the home cage (5 min), habituation to the isolation cage (15 min) and habituation to small objects in the home cage (5 min). The trial day we first positioned each mouse in the isolation cage and left there for 15 min. For habituation phase (T1), each mouse was positioned in an open field arena (35 x 47 x 60 cm) and left to freely explore it for 10 min. Then the mouse

was put back in its isolation cage and leave there for 1 min. In the meanwhile, we put four different objects in a symmetrical way in the open field. Then the mouse was put again into the open field and left to explore objects (studying phase, T2). After 10 min, the mouse was put back in the isolation cage for 1 min and, in the meanwhile, we put away all objects. Three of them were substituted by identical objects and just one was substituted for a completely different object (the novel object). Position of novel object was changed across animals in a random order. Each mouse was left to explore the four new objects for 5 min (test phase, T3). We then analysed registered video and measured the time of exploration (sniffing) in the second five minutes of T2 and in T3. We then calculated a re-exploration index for each object and each mouse (exploration time in T2 – exploration time in T3). A negative re-exploration index indicates that the mouse recognizes the specific object as already known, considering that mice are neophile animals. On the contrary, a positive re-exploration index indicates a higher interest for the object in T3, which in WT mice corresponds to the normal exploration of a novel object. Only young mice were tested through this task (see Fig. 10).

5. Histological analyses

5.1 Brain preparation

All mice were sacrificed within one week after the end of the treatment (see Fig. 10) and dissected brains were fixed on PFA 4% solution (24 h, 4 °C). They were then washed and cryoprotected in increasingly concentrated sucrose solution (20% and 30%) and stored at 4 °C. We produced 20-micron transversal sections with a cryostat (Leica, Wetzlar, Germany) from approximately Bregma +1,54 to Bregma -1,58 (Paxinos and Franklin, 2008). This segment of the brain includes four regions of interest: primary motor cortex, primary somatosensory-barrel cortex, basolateral amygdala and dorsal hippocampus (*see Fig. 11 in suppl. figures*). Slices were alternatively mounted on gelatine-coated microscope glasses (for Nissl staining) or kept free-floating in PBS (for immunofluorescence).

5.2 Nissl staining for cortical thickness analyses

Slices coming from MTZ-treated 6-month-old mice brains were attached to the glass and then left to dry. About one hour later, slices they were incubated in 0,2% cresyl violet solution (cresyl violet, Sigma, 0,5% glacial acetic acid and 0,01 M sodium acetate) for 20 min at 37 °C. Slices were then sequentially dehydrated with rapid washes in: 70% ethanol, 95% ethanol, 100% ethanol, methanol, 1:1 methanol-xylene solutions. Finally, glasses were covered by using Eukitt (Sigma, Sant Louis, USA) and kept at 4 °C until analysis at the microscope.

5.3 Immunofluorescence techniques

Free-floating brain slices from 6-month-old and younger mice were first permeabilized (1% Triton X-100) for 1 h at RT, incubated in blocking solution (0,1% Triton X-100, 2% BSA) for 1 h at RT and then incubated in fresh blocking solution containing primary antibodies overnight at 4 °C. The day after, we washed slices and incubated them in PBS containing Alexa Fluor secondary antibodies. Slices were finally incubated in 1:1000 Hoechst solution (33342, Sigma).

The following primary antibodies were used in separated incubations: rabbit anti-PV (1:5000, PV235, Swant) and rabbit anti-Mecp2 (1:500, 3456, Cell Signaling). In both cases, the day after slices were washed and incubated in donkey anti-rabbit antibodies Alexa Fluor 568 conjugate (1:250, A-10042, Life Technologies). In addition, lectin from *Wisteria floribundia* (1:250, L1516, Sigma) and streptavidin Alexa Fluor 647 conjugate (1:250, S21374, Life Technologies) were used together with anti-PV antibodies to stain PNNs only in younger mice.

5.4 Image acquisition and analysis

- ***Cortical thickness.*** Pictures of Nissl-stained brain sections were acquired with a Nikon AMX1200 digital camera on a Nikon E800 Microscope (10X magnification, NA = 0.30). Bregma position was assigned to each slice following Franklin and Paxinos (2008) and then FIJI software was used to measure cortical thickness (Bittolo et al., 2018). In short, we first produced a densitometrical plot profile from a line spanning the cortex perpendicularly from the pial surface to the white matter underlying cortical layer VI. We then identified transitions among layers as variations in the densitometrical plot profile, which reflect cell density changes between cortical layers, and measured their lengths in the plot. This approach allowed us to measure total cortical thickness and thickness of cortical layers I, II/III-IV and V-VI in the primary somatosensory-barrel cortex but only the total cortical thickness in the primary motor cortex. Eight mice per group and 10-15 slices per mice were analysed.
- ***Analysis of PV+ cells.*** For the analysis of adult brains, images of slices from immunofluorescence with PV antibodies were acquired with a Nikon Eclipse Ti-E epifluorescence microscope equipped with a Nikon DS-Qi2 camera. We first used a 4X magnification to identify the Bregma position of each slice and then took a large picture with a 10X magnification (NA=0.3, 2 x 2 fields, total area = 1,31 cm^2) of the correspondent regions of interest (see Fig. 10). Acquisition and analyses were performed by using NIS-Elements software (v4.60, Nikon). We generated digital boxes (width = 230,34 µm) spanning from the pial surface to the corpus callosum that were superimposed at the level of the barrel and primary motor cortices on each brain slice (Tomassy et al., 2014). PV+ and Hoechst-positive cells were counted automatically after having applied an intensity threshold specific for each picture. From 5 to 10 slices for each mouse, and 4-5 mice per group were analysed. We used FIJI software to measure intensity of

PV+ cells. In short, we manually created specific ROIs for PV+ cells and then automatically measured their mean pixel intensity. Around twenty neurons and three additional ROIs for background assessment were measured in each slice. Averaged background value was later subtracted to the mean intensity.

For the analysis of young brains, we followed analyses performed by Patrizio et al. (2019). Thus, images of the correspondent regions of interest were taken from immunofluorescence with PV and PNN staining with a 20X magnification (NA=0,50, field area = 2,1 cm^2) at a laser-scanning spectral confocal microscope (Nikon Eclipse C1). Z-stack option was used in order to cover the whole thickness of the slice (z-step = 3,5 µm). Acquisition was performed by using EZ-C1 software (v3, Nikon), while for image analysis we used FIJI software (Schindelin et al., 2012). In short, we selected the z-project/maximum intensity option to create a 2D image and then manually counted all PV neurons of each image. We finally measured automatically pixel intensity of the PV-specific channel by using a homemade macro implemented in FIJI.

- ***Analysis of PNNs.*** It was performed together with PV analysis only in the younger brains. We manually counted the number of PNNs in each image and automatically measured pixel intensity of the PNN-channel as described for PV.
- ***Analysis of Mecp2 expression mosaicism.*** It was performed on slices coming from immunofluorescence with Mecp2 antibodies. Large pictures of the motor cortex were taken with a 10X magnification (NA=0,30, field area = 16 cm^2) by using a standard fluorescence microscope. We then manually counted all Hoechst-positive nuclei and all double Hoechst-Mecp2 positive nuclei. Cells positive only for Mecp2 were never observed. We then calculated the percentage of the double positive cells (WT for *Mecp2*) out the total number of cells (Hoechst-positive). The resulting value was considered as an individual quantification of the Mecp2 cellular mosaicism derived from XCI (*see above*).

5.5 Collection and analysis of clinical data

· ***Patients.*** We retrospectively identified patients with typical RTT (and one with the preserved speech variant, PSV), all aged more than 16 years, who had received MTZ as adjunctive therapy to stabilize mood, anxiety and sleep between years 2012 and 2017 in the Child Neuropsychiatry Unit, Le Scotte Hospital (Azienda Ospedaliera Universitaria Senese, Siena, Italy). For comparative purposes, age-matched patients with typical RTT who had not received MTZ were considered as a control group. All RTT patients were identified with a *MECP2* mutation and clinically evaluated according to the revised diagnostic criteria by Neul et al. (2008). Parents of all patients signed written informed consent to participate to the study, and the project was conducted according to the Ethic Guidelines of the institute and the recommendations of the declaration of Helsinki and the Italian DL n° 675 of the 31-12-1996.

· ***Collected data.*** Patients data regarding demographic, type of mutation, main therapy regimen, dosage and duration of mirtazapine treatment were documented from the medical records (*see Table 3 and 4 in suppl. figures*). We also extracted data of the MBA change index, a clinician rated scale that focuses on the subset of RTT phenotypic item of MBA, that had been collected by the clinician at each visit at the clinic. The MBA change index is comprised of a subset of 17 items (each ranging 0-4, the sum ranging from 0 to 68). The total score is directly proportional to the clinical severity. Beneficial effects are considered when the treatment is able to lower the MBA change index of at least 2 points vs baseline. We also extracted data of the Rett syndrome Clinical Severity Scale (RCSS), an RTT-specific, validated scale that had been used by the same clinician at each visit to assess the severity of key symptoms. RCSS consists of 13 items which rate core RTT symptoms on a Likert scale of either 0-4 or 0-5 with a maximum total score of 58. Tolerability to MTZ was assessed via the documentation of possible adverse effects during treatment. Information regarding adverse events was recorded according to reports from the patients themselves or their caregivers.

6. Statistical analysis

All statistical analysis and representation of data coming from experiments with mice were performed with GraphPad Prism 8 software (GraphPad, La Jolla, California, USA). In all cases, normality of data was assessed through the Shapiro-Wilk test (Ghasemi and Zahediasl, 2012). According to this analysis, we alternatively used Student's t-test or Mann-Whitney test, when comparing two groups, and either one-way ANOVA (followed by Dunnett's post-hoc test) or Kruskal-Wallis test (followed by Dunn's post-hoc test), when comparing more than two groups. To compare the combined effect of two independent variables, we used two-way ANOVA (followed by Dunnett's post-hoc test). When comparing two different groups and control groups were all identical, we used the Wilcoxon signed-rank test. Outliers detection was performed by using Grubb's test. In figure graphs, data are represented either as mean ± SEM (for parametric data) or as median and interquartile range (for non-parametric data).

Inferential statistical tests were performed by using SPSS (v21, SPSS, Chicago, USA). Quantitative variables were represented as either mean plus standard deviation (for parametric data) or median (por non-parametric data), whereas qualitative variables were represented as percentages. One-way ANOVA was used to evaluated between-group differences of continuous variables and the Chi-squared test was used for categorical variables.

RESULTS

1. General health in adult WT and HET mice is not affected by a short-term treatment with MTZ

To determine whether a short-term treatment with MTZ (10 mg/kg i.p injections on alternate days for 30 days) was able to improve general health in fully adult HET female mice, we performed a semiquantitative phenotypic analysis at the end of each week of treatment, including a pre-treatment time point (see Fig. 10). We used a modified protocol from Guy et al. (2007) and confirmed that general health of vehicle-treated HET (HET-VEH) mice was significantly reduced compared to vehicle-treated WT (WT-VEH) mice already before the treatment and that it remained mostly unchanged along the analysed period (see Fig. 12A). No significant improvement was observed in HET mice treated with MTZ (HET-MTZ) compared to HET-VEH mice neither within single time points nor in delta values (see Fig. 12A-B). However, when considering the evolution of single mice along the treatment with improved, unchanged and worsened scoring, beneficial effects of MTZ can be observed in a small subset of HET-MTZ mice (Fig. 12C). Regarding the possible side effects of MTZ at 10 mg/kg, a dose equivalent to the maximum dosage used in patients (45 mg/day), we verified the complete safety of the treatment, as general health of WT-MTZ was unaffected compared to WT-VEH mice (Fig. 12A-C).

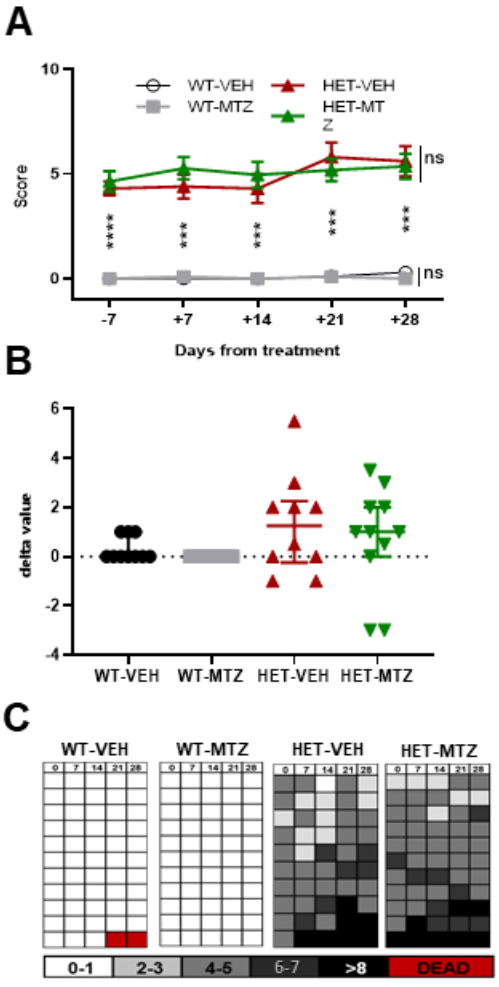
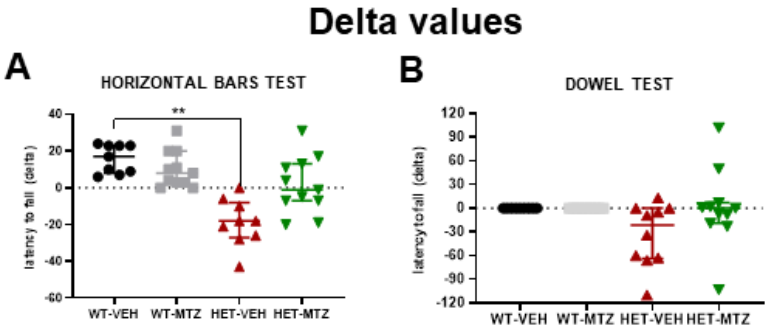


Figure 12 - General health evaluation of 6-month-old WT and HET female mice before, during and after treatment with MTZ (10 mg/kg). (**A**) Main evaluation of the phenotypic scoring along the experimental timeline. (**B**) Delta values for the main evaluation of the phenotypic scoring, calculated as the difference between the first evaluation (pre-treatment) and the last one (post-treatment). (**C**) Grid representing time course of phenotypic scoring for single mice. Within each experimental group, each row represents a different mouse. All data are expressed as mean ± SEM, n = 10-11 mice per group. According to results of Shapiro-Wilk test, we performed: one-way ANOVA, followed by Tukey's post-hoc test. Multiple selected comparisons comprehended: WT-VEH vs WT-MTZ, WT-VEH vs HET-VEH and HET-VEH vs HET-MTZ. ns: not shown; p> 0,05; *: p ≤ 0,05; **: p ≤ 0,01; ***: p ≤ 0,001.

2. Motor deficits present in adult HET mice are just partially rescued by a short-term MTZ treatment

Motor deficits are one key feature of RTT symptomatology, as they are very frequent in RTT individuals and have been largely described in several murine RTT models, including female *Mecp2*$^{tm1.1Bird}$ mice (Samaco et al., 2013; Vogel Ciernia et al., 2017). In this context, we evaluated whether MTZ was able to rescue different motor phenotypes in adult HET mice by testing motor performance in three different time points: before, during and after the treatment (see Fig. 10). We performed Dowel test (DT) and Horizontal bars test (HB) prior to the treatment to verify whether mice were already symptomatic in motor domain, and in the middle of the treatment to detect possible rapid effects of MTZ (see Fig. 10). We found that in the HB and in the DT, both WT-VEH and WT-MTZ mice showed a better performance at the end with respect to the beginning of the testing period, calculated as the mathematical difference (delta) of the latency to fall both time points (see Fig. 13A-B).

This result likely reflects the acquisition of learned motor skills upon repetition of the tests. VEH-treated HET mice showed instead negative delta values in both tests indicating worsening of the general motor coordination during the treatment period. In contrast, MTZ-treatment prevented many HET mice from worsening, as their delta values were close to zero in both tests, and even positive in two of them. Regarding possible rapid effects of MTZ, we did not observe any effect in neither DT nor HB when performed in the middle of the treatment (see Figs. 9 and 11C-D). In the Open Field test (OF), which was performed once at the end of treatment to measure the general locomotor activity levels and anxiety, HET mice showed a significantly shorter travelled distance during the observation time (20 min) with respect to WT mice, but MTZ did not show any significant effect in either genotypes (see Fig. 12E). In the Nest building test (NB), which assesses fine motricity of the forepaws, we did not find neither a significant deficit nor any effect by MTZ (see Fig. 12F). In the DT, HB and Rod walk test (RW), we only observed a clearly motor deficit in HET-VEH mice (see. Fig. 12G-I), In conclusion, we observed a protective trend of mirtazapine on motor deficits, while no side effects on motor performance in any of the tests performed was observed.



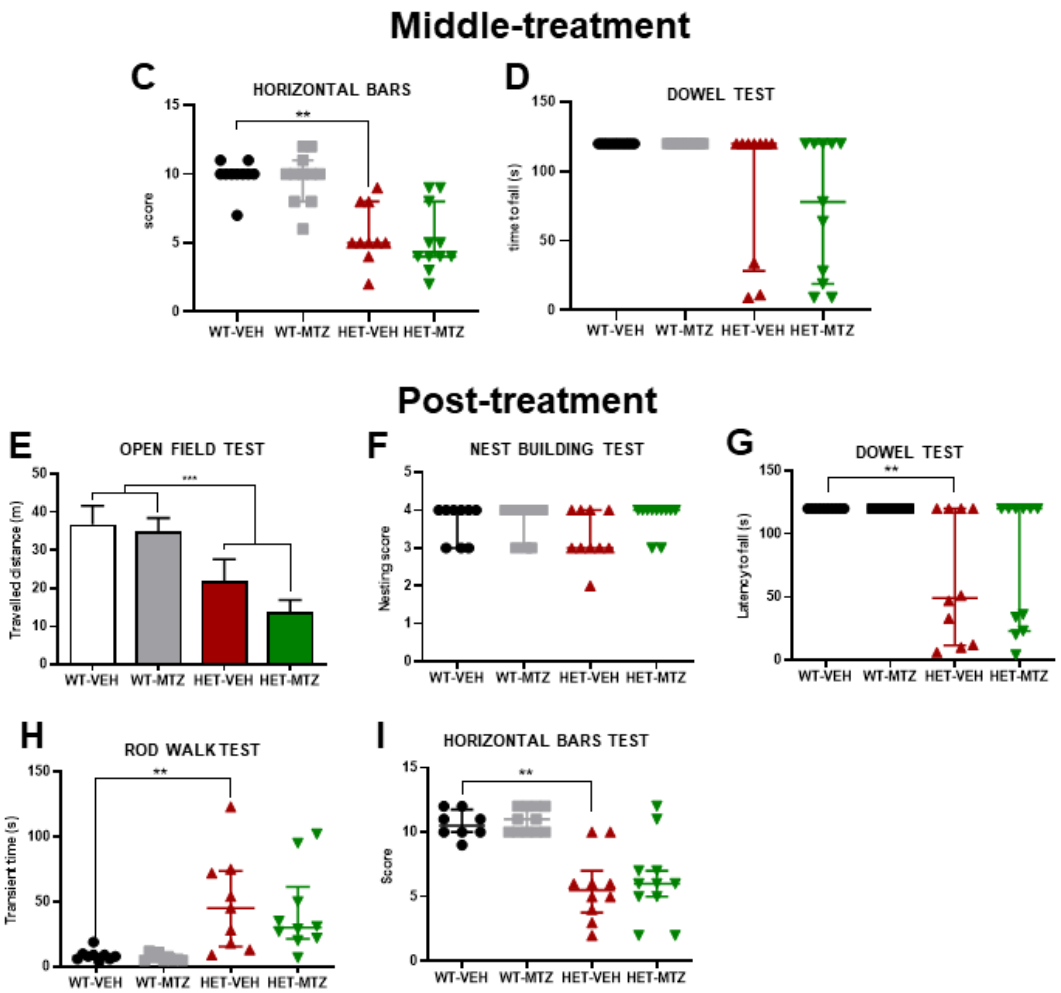


Figure 13 - Motor assessment of 6-month-old WT and HET female mice treated with MTZ (10 mg/kg) at different time points of the treatment. (**A-B**) Delta values for the latency to fall in the HB and the DT, calculated as the difference among values obtained before and immediately after the treatment. **(C-D)** Scoring obtained at the DT and the HB when performed in the middle of the treatment, to detect eventual rapid effects of MTZ. **(E-I)** Assessment of several motor phenotypes at the end of the MTZ treatment. All data are expressed as mean ± SEM, n = 10-11 mice per each group. According to results of Saphiro-Wilk test, we performed Kruskal-Wallis test, followed by Dunn's post-hoc test. Multiple selected comparisons comprehended: WT-VEH vs WT-MTZ, WT-VEH vs HET-VEH and HET-VEH vs HET-MTZ. In order to determine an eventual genotype effect, 2-way ANOVA was performed in some cases. ns: p> 0,05 (not shown), *: p ≤ 0,05, **: p ≤ 0,01, ***: p ≤ 0,001.

3. MTZ rescues the phenotype of adult HET mice at the Elevated plus maze

Behavioural abnormalities of RTT individuals usually include anxiety episodes elicited by distressful external events (Mount et al., 2001). In RTT mouse models, anxiety-related behaviours have been typically assessed through the Elevated plus maze (EPM) and a characteristic phenotype have been clearly described in both male (Samaco et al., 2013; Bittolo et al., 2016) and young female (Vogel Ciernia et al., 2017) *Mecp2*$^{tm1.1Bird}$ mice. Specifically, HET mice tend to stay longer in the open arms compared to their WT littermates. In the present study, we tested 6-month-old female mice at the EPM after a short-term treatment with MTZ (see Fig. 10). Our results confirmed the presence of the phenotype and, moreover, a total recovery by MTZ, as HET-MTZ mice explored the maze following the same pattern than WT-VEH mice (see Fig.

14A). These results are perfectly in agreement with those obtained by Bittolo et al. (2016) in male mice.

4. EPM-related phenotype in adult HET Mecp2$^{tm1.1Bird}$ *mice is likely due to enhanced sensitivity of whiskers*

According to the standard interpretation, the Elevated plus maze (EPM)-related phenotype confirmed in adult mice (see. Fig. 14A) reflect a state of lower anxiety or a high risk-taking behaviour. However, this is in contrast with the typical symptomatology found in RTT individuals (*see above*). We then hypothesized that behaviour of HET mice at the EPM might be due to an avoidance of the closed arms, rather than a preference for the open ones. Taking inspiration from the study performed by Flanigan et al. (2014) on a mouse model of Alzheimer's disease, we hypothesized that HET mice may present a whiskers hypersensitivity, which would make the narrow, closed arms of the EPM too disturbing for them. To verify this hypothesis, we clipped all the whiskers of untreated 6-month-old HET mice (HET-UNTw-) and tested them at the EPM the day after. We observed that the open arms-preference phenotype described before was no longer present in HET-UNTw- mice (see Fig. 14A). This finding strongly supports the hypothesis that HET mice present an avoidance of closed arms, most likely because of a hyperstimulation of whiskers by the maze walls. To confirm that there is no actually an anxiety-related phenotype in adult female *Mecp2*$^{tm1.1Bird}$ mice, we tested them at the D/L, a classical anxiety test that is performed in a large platform which can be explored without almost whiskers stimulation. As expected, this test did not show any phenotype in untreated HET mice (see Fig. 14B). Finally, we analysed anxiety-related parameters of the OF test and confirmed the absence of a specific phenotype (see Fig. 14C). Altogether, these findings support the view that whiskers hypersensitivity, rather than other phenomena, underlies the preference for the open arms at the EPM observed in *Mecp2*$^{tm1.1Bird}$ HET and Null mice (Bittolo et al., 2016).

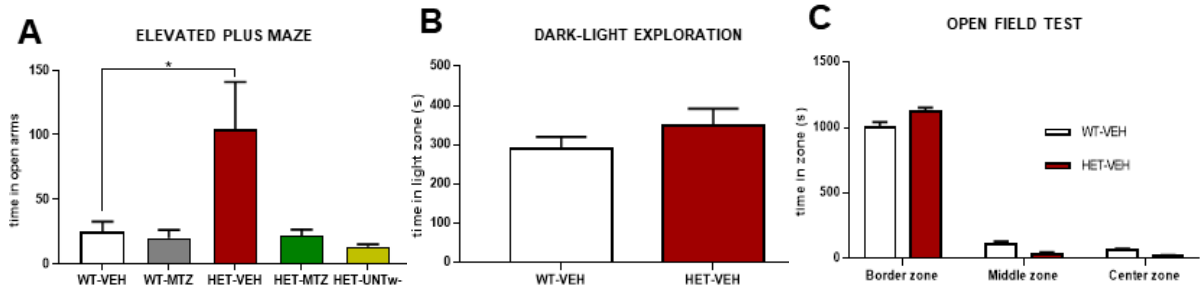


Figure 14 - Assessment of anxiety-related behaviors in 6-month-old HET female mice treated with MTZ (**A**) Time spent in open arms in the Elevated plus maze (**B**) Time spent in each zone in the Open field (**C**) Time spent in the light zone in the Dark-Light exploration test. All data are expressed as mean ± SEM, n = 7-11 mice per group. According to results of Saphiro-Wilk test, we performed: one-way ANOVA, followed by Tukey's post-hoc test, or Kruskal-Wallis test, followed by Dunn's post-hoc test. Multiple selected comparisons comprehended: WT-VEH vs WT-MTZ, WT-VEH vs HET-VEH and HET-VEH vs HET-MTZ, HET-VEH vs HET-UNTw- *****: $p \leq 0,05$; ******: $p \leq 0,01$; *******: $p \leq 0,001$.

5. Alterations in PV+ cells are rescued by MTZ in adult HET mice

In the study made by Flanigan et al. (2014), authors not only observed a preference for the open arms when 5xFAD transgenic mice were tested in the EPM, but also demonstrated that these mice present a decreased expression of PV in barrel cortex interneurons. Authors suggested that this alteration could imply a reduction of the inhibitory activity in the barrel cortex, causing whiskers hypersensitivity. Considering the relevance of PV+ cells in GABAergic function and its role in RTT physiopathology (*see above*), we hypothesized that some of the phenotypes observed in adult HET female mice could be associated, at least partially, to alterations into PV

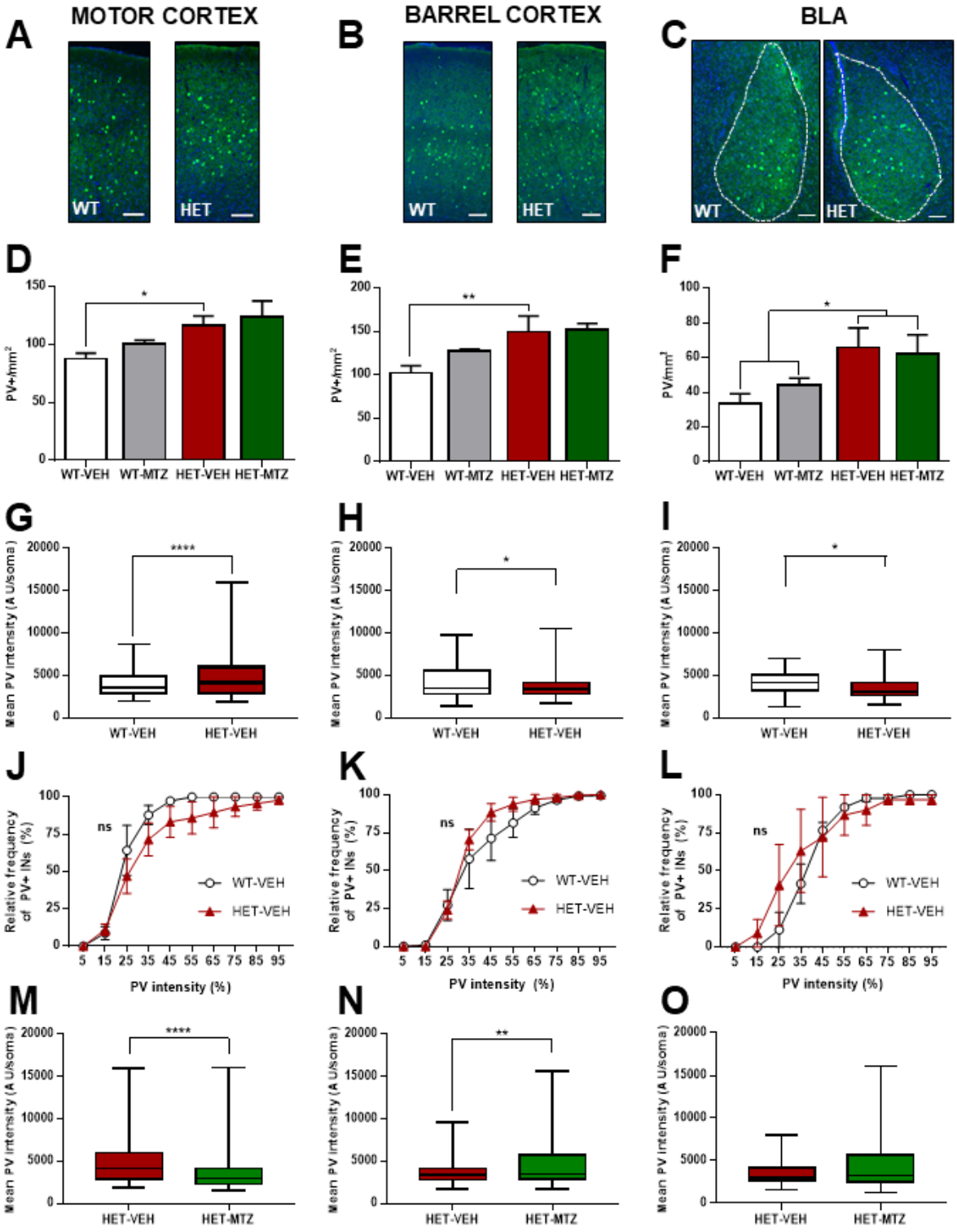


Figure 15 - Analysis of PV-positive interneurons (PV+ INs) in primary motor cortex, primary somatosensory-barrel cortex and basolateral amygdala (BLA) of 6-month-old WT and HET female mice after treatment with MTZ (**A-C**) Examples of PV and Hoechst staining (scale bar: 50 µm) of brain sections. (**D-F**) Density of PV+ INs in the three ROIs. (**G-I**) Cumulative and (**J-L**) binned PV mean fluorescence of WT-VEH and HET-VEH mice in the three ROIs. (**M-O**) Cumulative PV mean fluorescence in vehicle and mirtazapine-treated HET mice. All data are expressed as mean ± SEM, n = 4-5 mice per group. According to results of Saphiro-Wilk test, we performed Student's t-test and one-way ANOVA, followed by Tukey's post-hoc test or Mann-Whitney or Kruskal-Wallis test, followed by Dunn's post-hoc test, respectively. Multiple selected comparisons comprehended: WT-VEH vs WT-MTZ, WT-VEH vs HET-VEH and HET-VEH vs HET-MTZ. In order to determine an eventual genotype effect, 2-way ANOVA was performed in some cases. Kolmogorov-Smirnov test was used to compare relative frequencies. ns: p> 0,05 (not shown); *: p ≤ 0,05; **: p ≤ 0,01; ***: p ≤ 0,001.

networks in relevant brain areas. To test this hypothesis, we carried out immunofluorescence techniques to identify PV+ interneurons on brain slices from 6-month-old WT and HET female mice treated with MTZ (10 mg/kg). The cell density and the immunoreactivity of PV+ cells were measured in three brain areas of interest: primary somatosensory-barrel cortex, primary motor cortex and basolateral amygdala. While the general cell density was not significantly different between groups (data not shown), we found a slightly increased density of PV+ cells in HET-VEH mice in the three analysed brain areas, with no effect on MTZ in any area (see Fig. 15 D-F).

Furthermore, the immunoreactivity of PV in the somata of these neurons was significantly increased in the primary motor cortex (see Fig. 15G), while resulted to be significantly decreased in both barrel cortex (see Fig. 15H) and basolateral amygdala (see Fig. 15I). Interestingly, using PV intensity frequency plots, the major differences, even if not significant, were observed among neurons with medium PV intensity (Fig. 15J-L). Finally, we observed a complete recovery to normal PV immunoreactivity levels in the motor cortex upon MTZ treatment (see Fig. 15M). Importantly, the low PV immunoreactivity observed in the barrel cortex of HET-VEH mice were significantly increased in HET-MTZ mice, even beyond WT-VEH levels (see Fig. 15N). A similar trend could be observed in basolateral amygdala, even if the increase in PV staining in HET-MTZ mice was not statistically significant (see Fig. 15O). Taking together, these cytological results are evidence of a structural effect of MTZ on PV networks and are largely in agreement with the observed behavioural results, especially regarding the primary somatosensory-barrel cortex.

6. Brain cortex of adult HET mice do not show a clear generalized atrophyrain atrophy is a common feature to most RTT individuals (*see above*) and it has been observed also in Null *Mecp2*$^{tm1.1Bird}$ male mice (Bittolo et al., 2016). We aimed to verify whether this phenotype was also present in adult HET mice and thus we analysed cortical thickness in both primary somatosensory-barrel and primary motor cortices. We did not find any difference in cortical thickness between WT-VEH and HET-VEH mice (see Fig. 16). In the case of primary motor cortex, the absence of a group effect is due to a high variability within HET-VEH mice, as some of them

presented a clear cortical atrophy while others showed values comparable to those of WT-VEH mice (see Fig 16D).

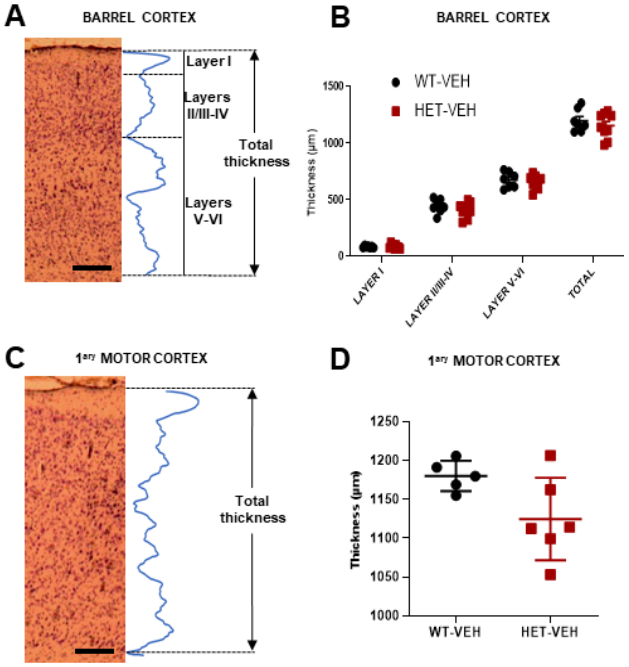


Figure 16 - Analyses of cortical thickness on 6-month-old HET female mice (**A**) Example of picture (scale bar: 200 µm) of a primary somatosensory-barrel cortex section stained with Nissl coloration. Densitometrical analysis allows to distinguish different cortical layers, **(B)** Barrel cortex thickness of layers I, II/III-IV, V-VI and total thickness. (**C**) Example of picture (scale bar: 200 µm) of a primary motor cortex section stained with Nissl coloration. In this case, densitometrical analysis allow to measure only total cortical thickness. (**D**) Motor cortex total thickness. All data are expressed as mean ± SEM, n = 8 mice per group and n = 10-15 brain sections per mouse. According to results of Saphiro-Wilk test, we performed unpaired Student's t-tests. No significative differences between WT and HET animals were revealed.

7. An heterogenous small group of adult RTT patients showed improvements in several symptoms after a chronic treatment with MTZ

We performed a prospective clinical study on a heterogeneous cohort of adult RTT patients (*see Table 3 in suppl. figures*) treated with different dosages of MTZ for a relatively long-time period (range 1-5 years; *see Table 4 in suppl.*). Mirtazapine, which is primarily used for treating major depressive disorder and other mood disorders with poor sleep quality (Karsten et al., 2017), was administered to these RTT patients to treat irritability, insomnia and mood changes. This cohort was compared with a group of untreated (UNT) RTT patients, of comparable age and disease severity, that were admitted to hospital during the same time period. Rett Clinical Severity Scale (RCSS) and Motor Behaviour Assessment Scale (MBAS) were routinely used as core measures to monitor disease progression by two well experienced clinicians (Joussef Hayek and Claudio de Felice) and were subsequently analysed to evaluate MTZ effects on patients. The treatment was well tolerated by nine patients, while two patients showed exaggerated anxiety behaviour and were discontinued (*see Table 4 in suppl. figures*).

As RTT is a progressive disorder with an evolution over time that is specific for each patient and its natural history, in order to estimate the possible efficacy of MTZ we decided to evaluate the delta variations (end of the observational time period vs. initial time point) of both clinical scores and MBAS sub-scores. MBAS is a 37-item scale categorized in three main areas: social behavior (M1, 16 items), oro-facial/respiratory (M2, 7 items), and motor/physical signs (M3, 14 items) (FitzGerald et al., 1990). We found significant differences between groups, as UNT patients

showed progressive worsening (i.e. increased values post-treatment vs. pre-treatment time points) of both total RCSS and MBAS scores (Fig. 17B and 17D, respectively) over the observational time. At the opposite, the MTZ-treated group showed either stable or even significantly improved clinical scores. In particular, MBAS scores were significantly reduced, indicating an improvement, whereas no difference was observed for RCSS, indicating protection from worsening (Fig. 17A and 17C).

Specific results for each MBAS item are shown in Fig. 18 (statistically significant results only) and Table 4 (all results). Comparing the delta values medians for each MBAS item in the MTZ-treated and UNT patient cohorts, we found statistically significant improvements in the MTZ-treated group for 8 items in the areas of social communication/responsiveness (hypomimia, unresponsiveness, apathy. Fig. 18A), irritability/aggressiveness (irritability, hyperactivity and aggressiveness; Fig. 18B) and self-aggressiveness (biting and self-aggressiveness. Fig. 18C). When considering the individual disease trajectories (see Table 4) rather than group median values, we observed that several UNT patients presented worsening symptoms for 9 items (trend to increasing delta values), no change for 18 (delta value=0) and improvement for 2 (trend to decreasing delta values). In contrast, none of the MTZ-treated patients showed worsening of any item (Table 4). Overall, no change for 23 items was observed, while we found a trend towards improvement in patients for 6 items (i.e., purposeful hand use, poor social eye contact, hand stereotypies, verbal language deficits, vasomotor disturbance and breath holding) is observed. Table 4 also suggests a protective trend of MTZ treatment as the MTZ-treated group showed no difference in the items as well as feeding difficulties, chewing difficulties, apraxia/ataxia, bradykinesia and dystonia, while a trend towards worsening could be observed in the UNT group. Although observations of individual disease trajectories have no statistical significance, they support a general positive trend of MTZ effects. Of note, for the item "vasomotor dysfunctions" a spontaneous improvement was observed in all MTZ-treated and UNT patients, indicating that this domain is not affected by MTZ. "Mouthing" was the only MBAS item showing an improvement exclusively in the UNT patient group. Finally, 14 MBAS items showed no change in all MTZ-treated and UNT patients (regression motor skills, regression of communication skills, sphincteric control deficits, masturbation, seizures, hypoalgesia, bruxism, hyperventilation, drooling, hands clumsiness, truncal rocking, oculogyric movements, scoliosis, myoclonus), likely reflecting previous, unmodifiable disease history (Table 4). Taken together, these results are evidence of a clear effect of MTZ in multiple disease domains in adult RTT patients undergoing chronic treatment.

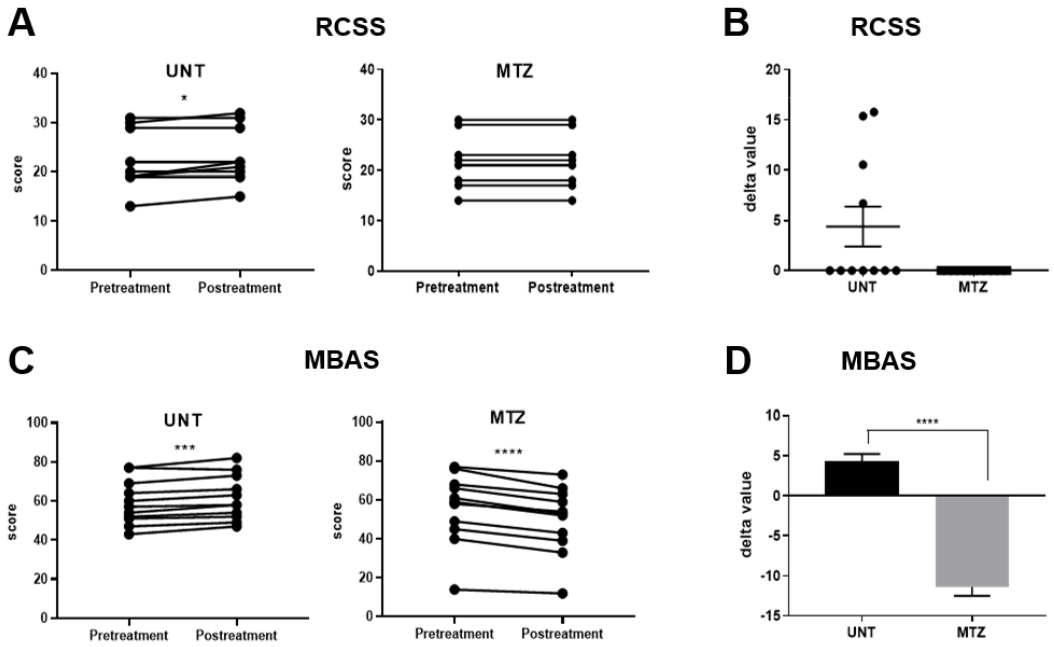
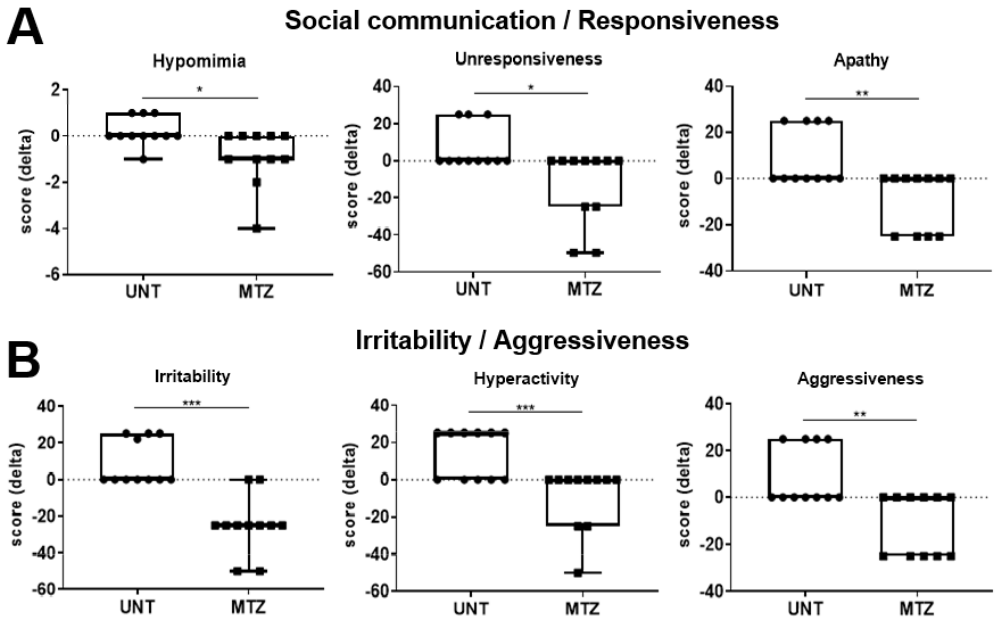


Figure 17 - Effects of MTZ on RCSS and MBAS in RTT patients (I). (A) RCSS was retrospectively analyzed in both untreated (UNT) and MTZ-treated cohorts of RTT patients. Scores before and after the treatment (or an equivalent time period when no treatment was present) for each patient are individually represented. **(B)** Comparison of RCSS delta averages between groups. A not significant but clear trend can be observed. Data are represented as mean ± SEM. **(C)** MBAS was retrospectively analyzed in both UNT and MTZ cohorts of RTT patients. Scores before and after the treatment (or an equivalent time period when no treatment was present) for each patient are individually represented. **(D)** Comparison of MBAS delta averages between groups. Data are represented as mean ± SEM. In all cases, we first realized a Shapiro-Wilk test and then corresponding parametric or non-parametric tests were used. We used paired Student's t-test or Wilcoxon signed rank test for comparisons between pre and post-treatment time points, while we used unpaired sample Student's t-test or Mann-Whitney test to compare averages between treated (n = 11) and untreated (n = 11) groups. ns: p> 0,05 (not shown); *: p ≤ 0,05; **: p ≤ 0,01; ***: p ≤ 0,001.



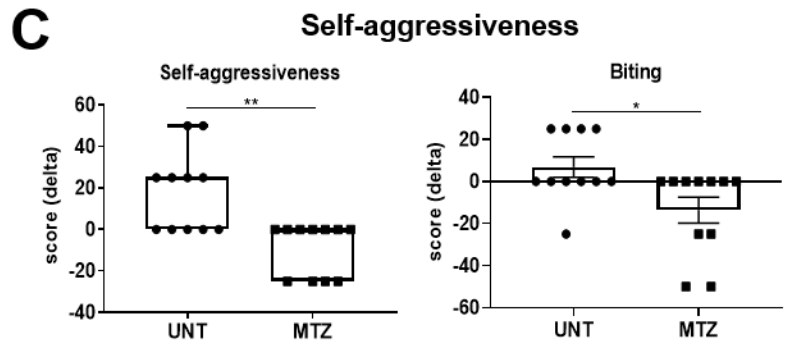


Figure 18 - Effects of MTZ treatment on several behavioral features of RTT patients. (A) Main features regarding social communication and responsiveness were retrospectively analyzed in both untreated and MTZ-treated cohorts. **(B)** Main features regarding irritability and aggressiveness were retrospectively analyzed in both UNT and MTZ cohorts. **(B)** Main features regarding self-aggressiveness were retrospectively analyzed in both UNT and MTZ cohorts. Complete results are shown in Table 3. All data are represented as boxplots displaying median, quartiles and extremes. According to results of Shapiro-Wilk test, we performed Mann-Whitney tests to compare averages between MTZ (n = 11) and UNT (n = 11) cohorts. ns: p> 0,05 (not shown); *: p ≤ 0,05; **: p ≤ 0,01; ***: p ≤ 0,001.

8. HET mice present several RTT-like phenotypes before adult age

When we treated adult *Mecp2*$^{tm1.1Bird}$ female mice with MTZ, only one clear phenotypical rescue was obtained (*see above*). For instance, scarce beneficial effects on motor phenotypes, which are one of the most common alterations in patients, were detected (see Fig. 13). We thought that one of the possible reasons for these limited results could be the low plasticity that characterizes the adult mouse brain. We therefore decided to treat younger HET mice to evaluate MTZ potential not only to rescue phenotypes but also to slow down their appearance and progression in earlier stages of development. However, few information on young HET female mice was available at that moment and thus we had first to evaluate at which age different phenotypes become evident. We decided to assess different domains in order to better characterize progression of the disease in mice. In this context, we performed a behavioural phenotyping at 6 and 11 weeks of age, which correspond to "childhood" (few days after weaning) and "late adolescence" (during sexual maturation), respectively (Dutta and Sengupta, 2016). We found that body weight was already increased in HET mice as soon as 6 weeks of age (see Fig. 19A). However, this increase was so small at both ages that it could not represent a confounding factor for motor tests, contrary to what happens with adult mice (*see above*). Hindlimb clasping was present only in some HET mice at both ages but at group level a significant difference with wild-type mice was observed (see Fig. 19B). Regarding motor phenotypes, we observed that the phenotype described in adult HET mice at the HB test is not present in 11-month-old HET mice, while both Rt and RW do show consistent phenotypes at 6 and 11 weeks of age (see Fig. 19C-E). In addition, we observed a not yet described phenotype in the PR test, which evaluates fine motricity of hindlimbs, at 11 weeks of age (see. Fig. 19F). The

phenotype observed in adult mice when tested at the EPM was confirmed in young mice at both 6 and 11 weeks of age (see Fig. 19G). Finally, we tested mice at the 4-DOT and observed a clear deficit of the short-term memory, only at 11 weeks of age (see Fig. 19H). In summary, this preliminary characterization of the *Mecp2*$^{tm1.1Bird}$ female at young ages allowed us to identify 11 weeks as an optimal window to test MTZ. Our behavioural phenotyping revealed several previously unknown alterations in different domains, making *Mecp2*$^{tm1.1Bird}$ female mice a good model to test drugs also at young ages, where plasticity processes are more active.

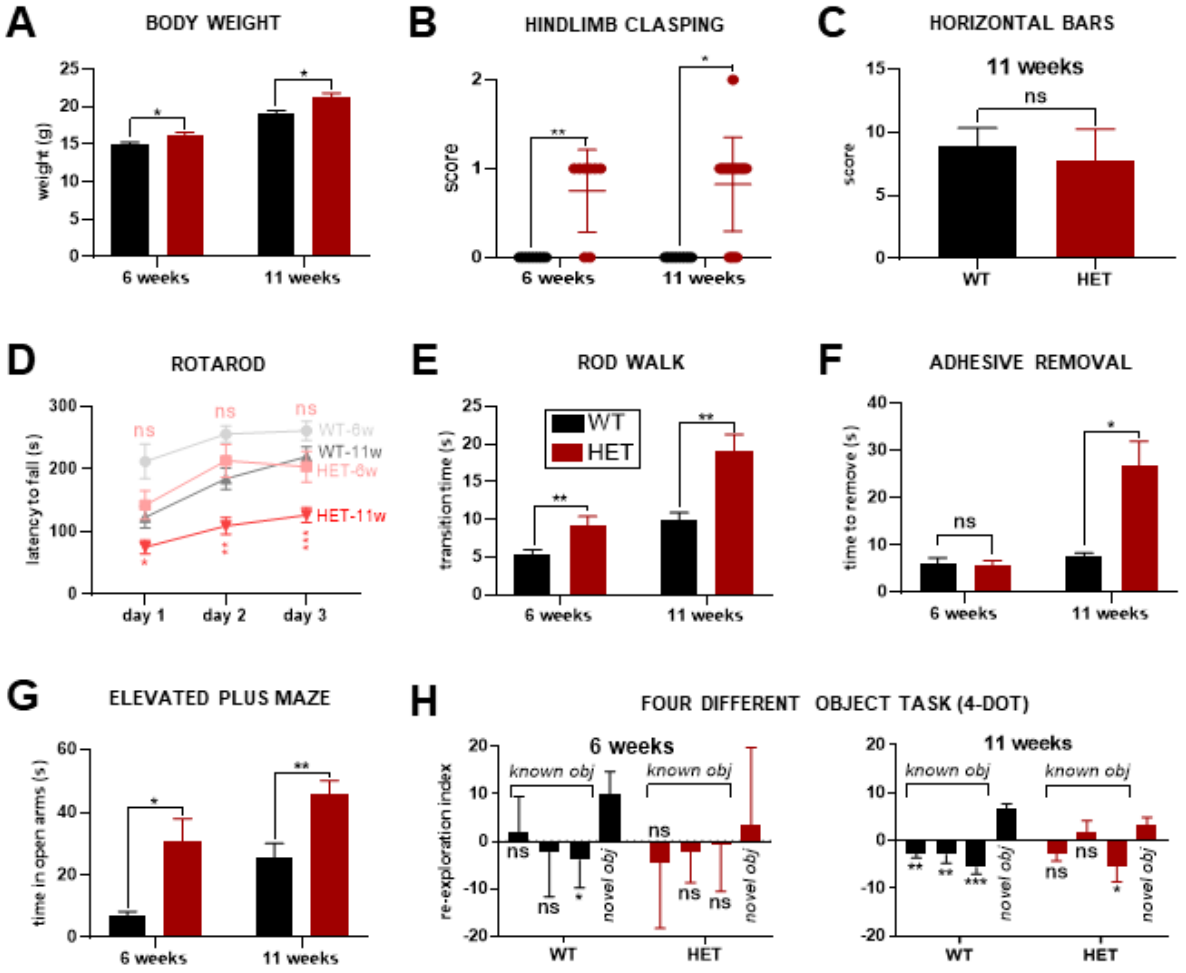


Figure 19 - Behavioral phenotypes in HET female mice at 6 and 11 weeks of age. (A) Body weight of mice is shown. Results obtained demonstrate that it is not a confounding factor for motor performance. **(B)** Hindlimb clasping was evaluated following Guy et al. (2007): 0, absent; 1, mild; 2, strong. **(C-F)** Motor tests. HB was not performed at 5 weeks of age because of absence of phenotype at 11 weeks. In the Rt, statistical significance was evaluated for differences between genotypes at a same age. **(G)** Time spent in the open arms of the EPM **(H)** Four-DOT re-exploration indices. In this case, statistical analysis was used to detect significant differences between exploration index of each known object and the correspondent novel object. All data are expressed as mean ± SEM, n = 8-17 mice per each group. According to results of Saphiro-Wilk test, we performed Student's t-test. For the 4-DOT, a two-way ANOVA with repeated measures was used to compare the re-exploration index, followed by Dunnett's multiple comparison test. ns: p> 0,05, *: p ≤ 0,05, **: p ≤ 0,01, ***: p ≤ 0,001.

9. A 15-day treatment with MTZ rescues only short-term memory in young HET mice

Considering the observed phenotypes, we decided to treat 11-month-old mice, as they were more consistent at this age (see Fig. 19). The dosage was unchanged compared to the treatment on adult mice (10 mg/kg) but we decided to treat mice daily rather than for 30 days on alternate

days as we did in the study on adult mice (see Fig. 10). All the behavioural tests that had showed a clear phenotype were included in the protocol (see Figs. 10 and 19). We confirmed that no relevant differences in body weight existed between HET and WT mice (see Fig. 20A) and that hindlimb clasping was present in most but not all HET mice, even if the group effect was statistically significant (see Fig. 20B). None of these parameters was affected by 15-day MTZ treatment. Regarding motor behaviours, we confirmed alterations in both Rt and RW, while in the PR test only a trend was observed (see Fig. 20C-E). In these tests, no effect of MTZ was observed in neither WT nor HET mice. In the EPM, HET mice showed an untypical anxious behaviour, as they explored less the open arms compared to WT mice, even if this difference was not significant (see Fig. 20F). Here, MTZ seemed to exert anxiolytic effects, as HET-MTZ tended to explore more the open arms compared to HET-VEH mice. Finally, the 4-DOT showed a deficit in HET-VEH mice and a complete rescue by MTZ when administered for 15 days, as performance of HET-MTZ mice was optimal (see Fig. 19G).

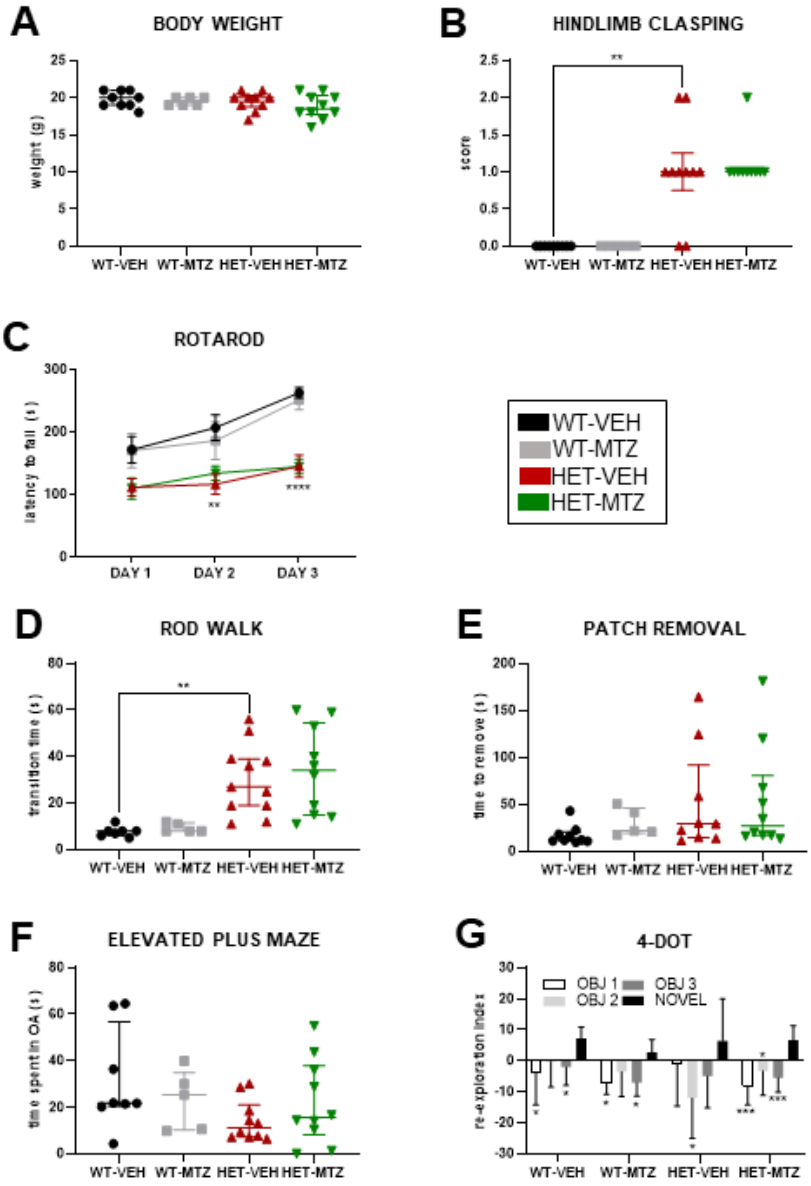
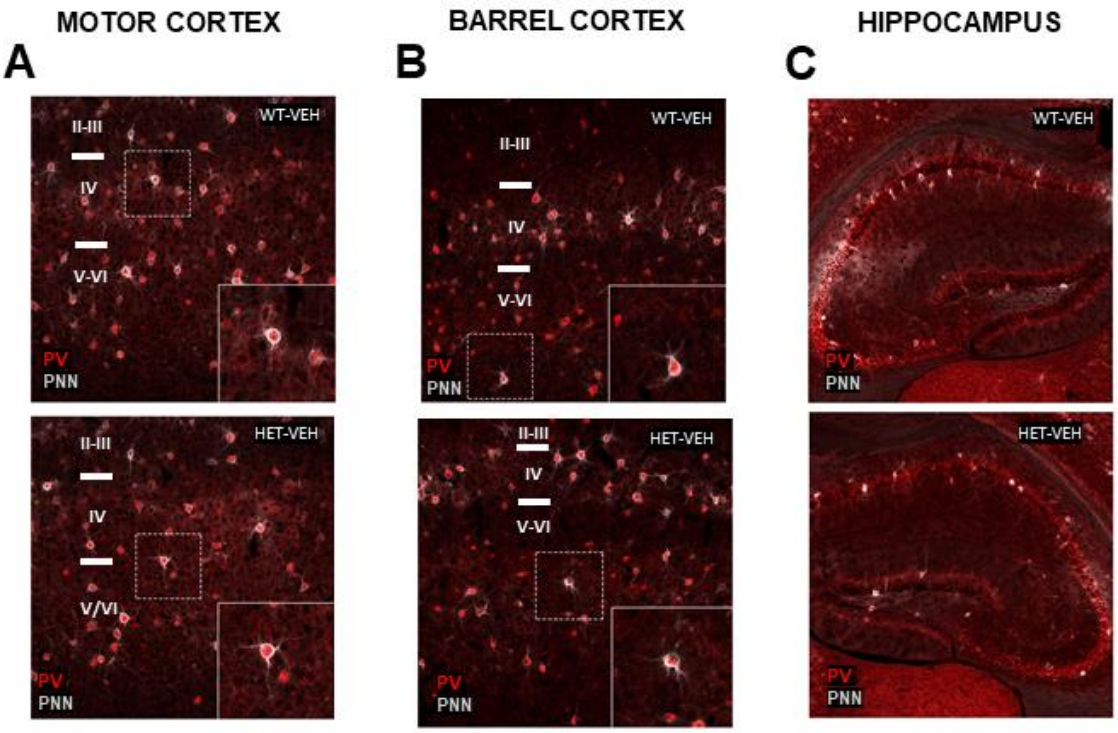


Figure 20 - Behavioral phenotypes of 11-week-old *Mecp2*$^{tm1.1Bird}$ female mice treated with MTZ for 15 days. **(A)** Body weight of mice at the end of the treatment is shown. **(B)** Hindlimb clasping was evaluated following Guy et al. (2007): 0, absent; 1, mild; 2, strong. **(C-E)** Motor tests. In the case of Rotarod, statistical analysis was performed to detect difference between groups within each test day. **(E)** Elevated plus maze **(G)** Four different object task. Statistical significance was calculated for differences between each known object and the correspondent novel object. All data are expressed as mean ± SEM, n = 6-11 mice per group. According to results of Saphiro-Wilk test, we performed Kruskal-Wallis test, followed by Dunn's post-hoc test. Multiple selected comparisons comprehended: WT-VEH vs WT-MTZ, WT-VEH vs HET-VEH and HET-VEH vs HET-

10. No evident alterations are found in PV networks of HET mice treated with MTZ for 15 days

After behavioural testing, we dissected brains from treated 11-week-old female mice and evaluated PV networks by measuring both immunoreactivity and density of PV interneurons and associated PNNs. We performed this analysis in the primary motor and the primary somatosensory-barrel cortices, as they are related to several RTT-like phenotypes in *Mecp2*$^{tm1.1Bird}$ mice. In addition, we evaluated density of both PV interneurons and PNNs in the dorsal hippocampus, which is involved in the performance of the 4-DOT (Sannino et al., 2012). No differences were observed for any of these parameters in any of the studied regions (see Figs. 21-22).



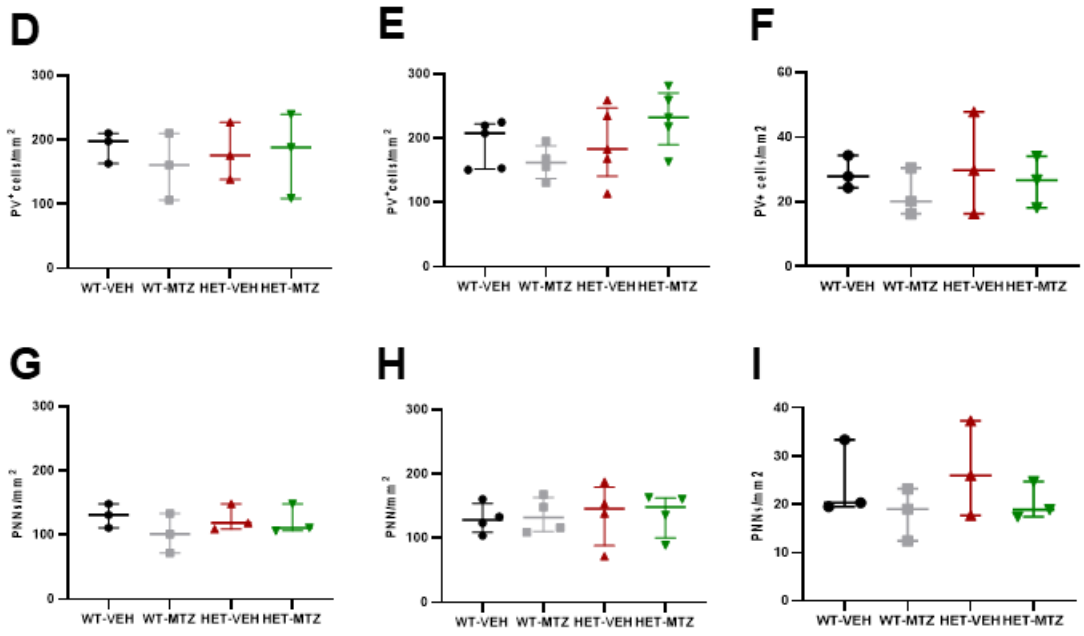


Figure 21 - Density of PV-positive interneurons and PNNs in the primary motor cortex, the primary somatosensory-barrel cortex and the dorsal hippocampus in 11-week-old HET and WT female mice treated with MTZ for 15 days (**A-C**) Examples of brain sections with PV and PNN staining. Visible brain cortical areas are indicated. (**D-F**) Density of PV+ interneurons in the three regions of interest. (**G-I**) Density of PNNs in the three regions of interest. All data are represented as median + interquartile range. We performed Kruskal-Wallis test followed by Dunn's post-hoc test. Multiple selected comparisons comprehended: WT-VEH vs WT-MTZ, WT-VEH vs HET-VEH and HET-VEH vs HET-MTZ. No significative differences were detected in any analysis. ns: p> 0,05 (not shown); *: p ≤ 0,05; **: p ≤ 0,01; ***: p ≤ 0,001.

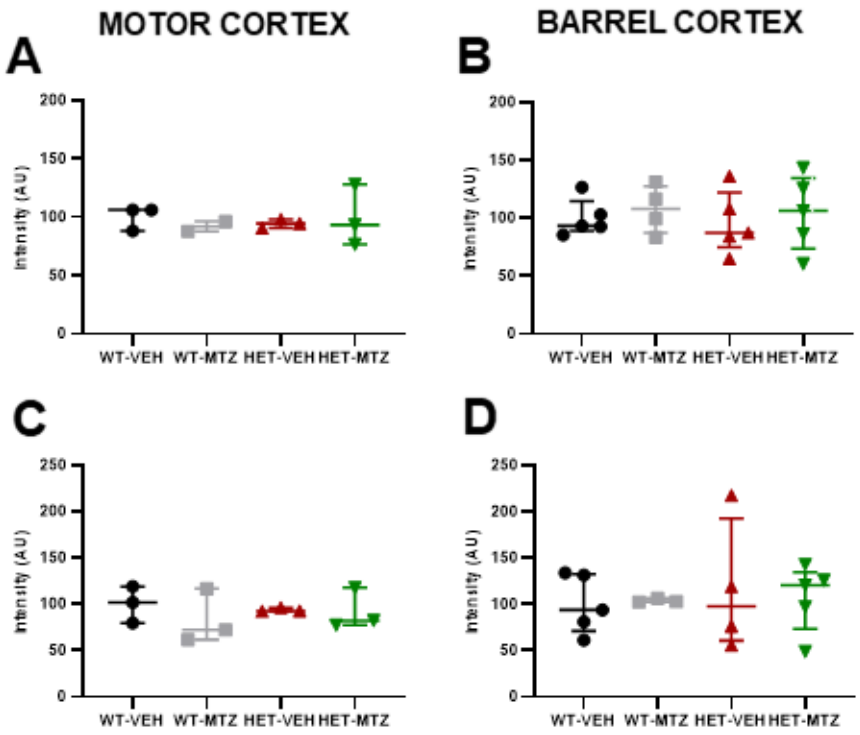


Figure 22 - PV and PNN immunoreactivity of primary motor and primary somatosensory-barrel cortices in 11-week-old *Mecp2*$^{tm1.1Bird}$ female mice after a 15-day treatment with MTZ (**A-B**) Intensity of the PV network in both ROIs. (**C-D**) Intensity of PNNs in both ROIs. According to results of Saphiro-Wilk test, we performed Kruskal-Wallis test, followed by Dunn's post-hoc test. Multiple selected comparisons comprehended: WT-VEH vs WT-MTZ, WT-VEH vs HET-VEH and HET-VEH vs HET-MTZ. ns: p> 0,05 (not shown); *: p ≤ 0,05; **: p ≤ 0,01; ***: p ≤ 0,001.

11. HET mice present very variable XCI patterns

The XCI has been identified as a factor contributing to symptomatologic variability in RTT individuals (*see above*). In most RTT cases, XCI is non-balanced, in a way that the expression of the WT allele is favoured. This phenomenon has been observed in a RTT mouse model too, together with correlations between XCI patterns and some phenotypes (Young and Zoghbi, 2004). To confirm that this phenomenon occurs in HET *Mecp2*$^{tm1.1Bird}$ mice, we evaluated the percentage of Mecp2-positive cells in brains from 11-month-old HET mice after the treatment with MTZ. As expected, all evaluated WT mice presented around 100% of Mecp2-positive cells (see Fig. 22B). In contrast, half of the analyzed HET mice showed an XCI pattern that partially favoured the WT allele expression, while the other half presented an inverse XCI pattern (see Fig. 23C).

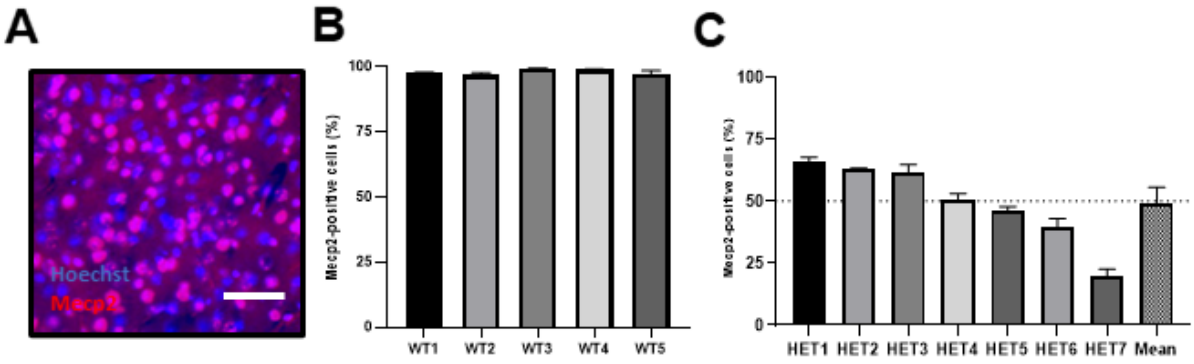


Figure 23 - Descriptive analysis of *Mecp2* mutation cellular mosaicism in 11-week-old WT and HET female mice treated with MTZ for 15 days. (**A**) Brain slice from a HET mouse with Mecp2 (red) and Hoechst (blue) staining. The pinkish coloration indicates co-localization. (**B**) A group of WT mice was evaluated to verify that the protocol worked properly. As it can be seen, the proportion of Mecp2-positive cells is close to the 100% in all cases. (**C**) Some HET mice (VEH and MTZ) were evaluated, showing different XCI patterns. For each mouse between 3 and 5 slices were analyzed. All data are represented as mean ± SEM. No statistical analysis was performed.

12. A 30-day treatment with MTZ rescues several motor phenotypes in young HET mice

Because of the limited effects observed after 15 days of daily treatment with MTZ, we considered that the duration of the treatment could represent an important issue to consider in this preclinical project. Therefore, we decided to increase the treatment period, from 15 to 30 days (see Fig. 10). Despite the low number of mice analysed so far (the experiment is still ongoing), significant effects of MTZ treatment were achieved in the motor domain. First, hindlimbs clasping was either eliminated or prevented in HET-MTZ mice, while 3 out 4 HET-VEH mice showed a worsening of this clinical sign (see Fig. 24B). Second, when tested at the Rt, HET-VEH mice showed a significant deficit, while performance of HET-MTZ mice was completely rescued, as these mice showed indices closer to that of WT mice (see Fig. 24C). A similar trend was observed in the RW test, as the deficit present in HET-VEH mice was partially rescued in HET-

MTZ mice (see Fig. 24D). In both cases, we expect that a statistical significance will be achieved when the number of tested mice will be higher (experiments are ongoing).

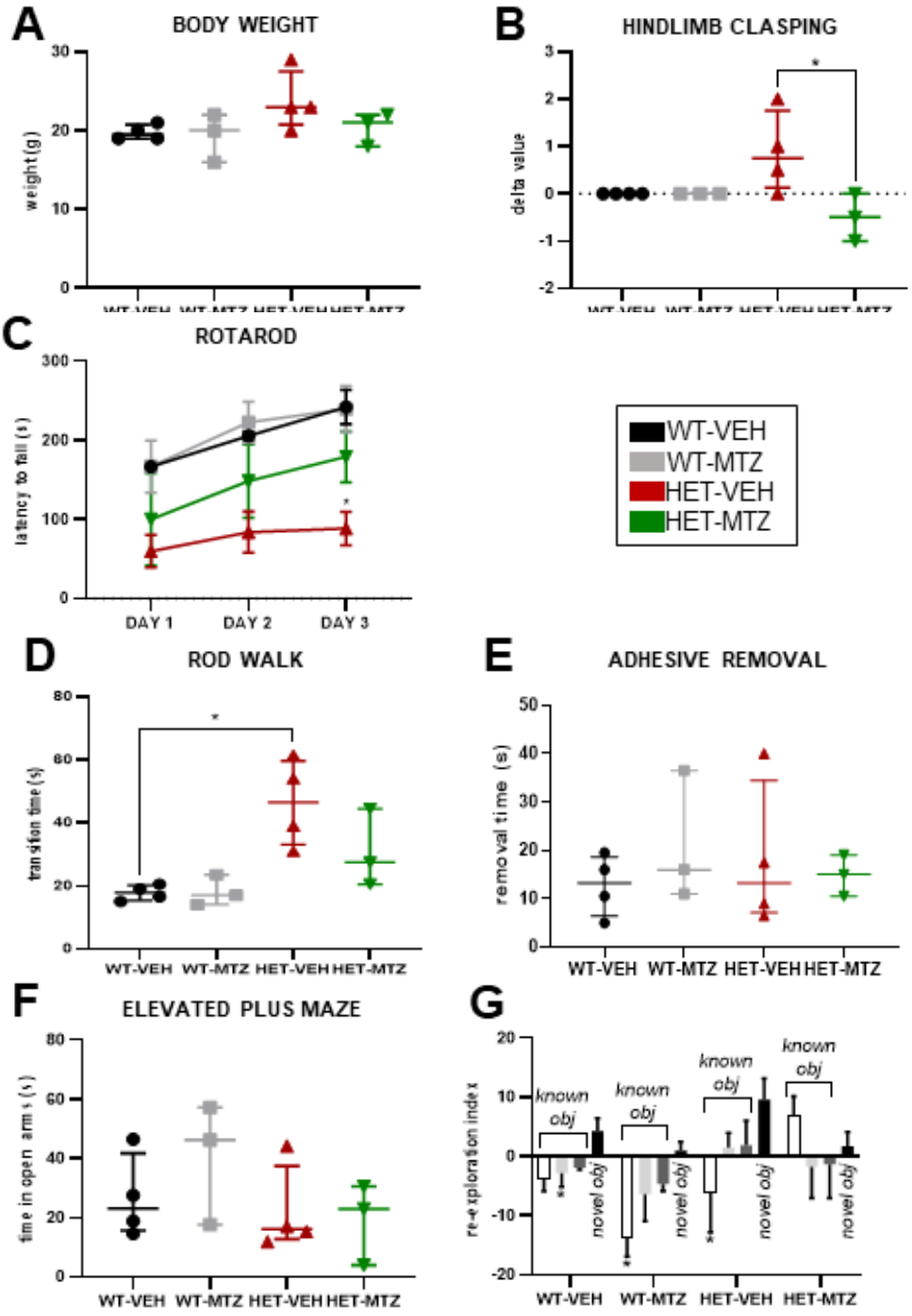


Figure 24 - Behavioral phenotypes of 11-week-old *Mecp2*$^{tm1.1Bird}$ female mice treated with MTZ for 30 days (A) Body weight of mice at the end of the treatment. **(B)** Hindlimb clasping was evaluated weekly following Guy et al. (2007): 0, absent; 1, mild; 2, strong. Delta values, calculated as the difference between the last and the first values. **(C-E)** Motor tests. In the accelerating rotarod, performance of the different groups was compared within every day of the test. A significant difference was found between WT-VEH and HET-VEH mice the third day. **(F)** Elevated plus maze. **(G)** Four different object task. Statistical significance was calculated for differences between each known object and the correspondent novel object. All data are expressed as mean ± SEM, n = 6-11 mice per group. All data are expressed as median + interquartile range, n = 3-4 mice per group. We performed Kruskal-Wallis test, followed by Dunn's post-hoc test. Multiple selected comparisons comprehended: WT-VEH vs WT-MTZ, WT-VEH vs HET-VEH and HET-VEH vs HET-MTZ. For the Four different objects task (4-DOT), a two-way ANOVA with repeated measures was used to compare the re-exploration index, followed by Dunnett's multiple comparison test. ns: p> 0,05 (not shown), *: p ≤ 0,05, **: p ≤ 0,01, ***: p ≤ 0,001.

Body weight was confirmed to be unchanged between groups (see Fig. 24A), this confirming that it is not a confounding factor for motor performance assessment in younger HET female mice. Just like after the 15-day treatment, also in this case we did not observe any phenotype in neither the adhesive removal test nor the elevated plus maze (see Fig. 24E-F). This supports the idea that injections *per se* may be a disturbing factor for these more sensitive tests. Analysis of the 4-DOT after the 30-day treatment showed a mild phenotype in HET-VEH mice, while no significant improvement was observed after treatment with MTZ (see Fig. 24G). Taken together, these results demonstrate the potential of MTZ to alleviate and even slow down motor deficits, just like we found in RTT patients (*see above*).

DISCUSSION

Nowadays, pharmacological therapies are, together with physical therapies, the only short-term strategy to improve, through the alleviation of symptomatology, the quality of life of RTT individuals and their families. However, few drugs have demonstrated this potential so far, and none of them has arrived to enter the clinic consensus to treat RTT. We started this project considering several relevant pharmacological features that strongly indicated MTZ as a good candidate. Above all, this antidepressant satisfies the most important requirement for any drug candidate to treat RTT: it is a relatively safe drug. Since its approval for the treatment of major depression in 1996, it has showed an excellent safety profile (Szegedi and Schwertfeger, 2005). Moreover, a recent study comparing twenty-one well-known antidepressants has concluded that MTZ is one of the most suitable ones to treat major depression disorders in terms of tolerability (Cipriani et al., 2018). Finally, its numerous off label uses (Jilani and Saadabadi, 2019) are also evidence of the few side effects exerted by MTZ. In this project, we have largely confirmed MTZ safety. First, we have observed no toxicity in the analysed mouse model, as WT-MTZ and HET-MTZ mice did not show any alteration compared to their correspondent VEH controls, even when we treated young mice for 30 days with a high dose of MTZ (10 mg/kg, the equivalent to the maximum dose admitted in humans, 45 mg/day; *see Fig. 24*). Second, our retrospective study on a heterogeneous cohort of RTT patients having treated with MTZ for up to five years showed relevant side effects in only 2 out 11 individuals (*see Table 4 in suppl. figures*). The high tolerability to MTZ has important implications for future research, as it will be possible to test a reasonably wide range of dosages in an eventual randomized clinical trial on RTT patients.

Through a direct comparison between mice and humans, this project extended the current knowledge on *Mecp2*$^{tm1.1Bird}$ female mice as an RTT model. We have confirmed the good face validity that the RTT mouse model developed by Guy et al. (2001) has demonstrated for years in both *in vitro* and *in vivo* experiments (Katz et al., 2012). However, most of these studies have been conducted on Null male mice, mainly because of an earlier onset of disease and a much lower variability. Despite these characteristics make easier performing studies on males, results obtained in female mice provide with a higher translational value, as the large majority of RTT individuals are also females (*see above*). In this project, we initially chose to treat HET female mice at 6 months of age because it was reported that most of them were symptomatic at that age (Guy et al., 2001). Six months represents a fully adult state, as sexual maturation in mice (which marks the end of puberty and the growth plate closure) occurs between 56 and 96 days after birth approximately (Dutta and Sengupta, 2016). It does not exist an absolute method to

compare ages between humans and mice, but some estimations can be realized to verify that a mouse model is adequate in terms of developmental stage. Following methods reviewed by Dutta and Sengupta, we calculated that six murine months are equivalent to approximately thirty human years, which corresponds approximately to the mean age of patients treated with MTZ presented in this study (27 years, *see table 3 in suppl. figures*). Thus, six months was the optimal age for comparisons between our mouse model and adult RTT individuals. Most of symptoms present in adult RTT individuals have relatively clear correspondent phenotypes in adult *Mecp2*$^{tm1.1Bird}$ female mice, such as worsened general health, shortened lifespan, sensory deficits and locomotion impairments (Guy et al., 2001). In addition, just like it happens in female RTT individuals, we verified that HET female mice present a high variable dosage of the *Mecp2* gene (*see Fig. 23*), which depends on the specific mosaicism pattern created by the XCI (*see above*). These results are consistent with previous literature (Oikawa et al., 2014) and pave the road to the study of correlations between *Mecp2* cellular mosaicism and specific phenotypes in the Bird mouse model. Despite all its advantages, we found an important limitation of adult *Mecp2*$^{tm1.1Bird}$ female mice as a RTT model. The high weight gain, which is common to all female HET mice from about the third month of life (*see Fig. 18*) and especially in adult stages (*see Fig. 11*) is inexistent in RTT individuals and results particularly troublesome for behavioural testing, as it markedly impairs the performance in motor tests.

We did not only verified safety of MTZ, but also demonstrated that it is able to alleviate or even slow down different deficits in both *Mecp2*$^{tm1.1Bird}$ mice and RTT individuals. In adult mice, we observed the complete rescue by MTZ of the phenotype typically observed in the EPM (*see Fig. 14*). Furthermore, following experiments performed by Flanigan et al. (2014), we demonstrated that this phenotype should not be interpreted in terms of anxiety (which is the issue classically studied through the EPM), but rather as a hypersensibility of the whiskers. We also proposed a mechanism for this somatosensory dysfunction, as we found that increased PV expression in the barrel cortex (which likely reflect an increased internal inhibition, Aponte et al., 2008) was completed rescued after MTZ treatment. On this way it is reasonable to think that MTZ rescue the behavioural phenotype by normalizing internal inhibition of barrel cortex.

Moreover, the phenotype observed at the EPM somehow reflect an increased irritability, since HET mice perceive as a negative stimulus what it is not for WT mice. Interestingly, the bottle-brush test, a behavioural test commonly used to evaluate irritability in rodents, is based on a specific stimulation of whiskers which generates different aggressive and defensive behaviours (Riittinen et al., 1986; Kimbrough et al., 2017). It is possible to draw a parallel between this test and EPM: walls of the closed arms play the role of the disturbing object, just like the bottle-brush

does in the homonymous behavioural test. Although human behaviour is very much complex compared to mice, these results can be assimilated to those obtained in RTT patients, as irritability scoring was clearly better in MTZ-treated ones (*see Fig. 18*). This demonstrates that MTZ is able to improve a clinical feature very relevant for the day-to-day life of RTT patients and their families.

Regarding the motor domain, which is one of the most relevant within RTT symptomatology (*see above*), we observed several beneficial effects of MTZ treatments. In adult mice, a significant worsening of motor performance through the experimental period (around 1 month) was verified in HET-VEH, as relative delta values for both HB and DT were negative (*see Fig. 13A-B*). Instead, delta values of HET-MTZ mice were much closer to zero, this indicating a clear trend of MTZ to slow down the progression of motor deficits. Furthermore, two HET-MTZ mice showed an improvement of motor performance (probably a consequence of motor learning), while any type of improvement was observed in HET-VEH mice. These differences were not statistically significant, partially due to variability within HET mice and may be due also to the low brain plasticity and the obesity that characterizes adult stages of HET mice. This second hypothesis is supported by the fact that in young mice treated for 30 days we did observe a clear rescue of motor features, even if the number of mice analysed was quite low (the correspondent experiments are still ongoing). In particular, MTZ proved to eliminate or prevent the hindlimb clasping (*see Fig. 24B*), which is a characteristic clinical sign of HET mice that can be somehow assimilable to hand stereotypical movements observed in RTT girls (*see Fig. 7*). In the Rt, which evaluates not only the general motor performance but also the motor learning, a clear trend to improvement can be observed by comparing HET-MTZ with HET-VEH mice (*see Fig. 24C*). Similar results were obtained in the RW test (*see Fig. 24D*), which evaluates general motor coordination. In both cases, it is reasonable expecting to reach the statistical significance after the analysis of all mice currently under evaluation, as the effect size is already considerable. Increasing of PV expression in the primary motor cortex has been correlated to phenotypes observed in the Rt in male *Mecp2*$^{tm1.1Bird}$ mouse (Morello et al., 2018). We confirmed this alteration in adult HET mice and also a normalization of PV levels by MTZ (*see Fig. 15*), while in young HET mice we did not detect any alteration in PV networks (*see Figs. 21-22*). In this context, it is reasonable to think that some of the beneficial effects of MTZ on the motor domain are exerted via a normalization of PV networks, even if results in young mice indicate that probably there are other involved mechanisms. In order to achieve a definitive conclusion, we should use more sensitive analyses to confirm that PV networks are not affected in young female mice. MTZ potential to improve motor deficits is also supported by results obtained in the retrospective analysis of patients.

Specifically, we observed an improvement in the MBAS, as MTZ-treated patients showed negative delta values significantly different from UNT patients (*see. Fig. 17D*).

The cognitive domain has been poorly studied in RTT mouse models, but some information is available. For instance, Vogel Ciernia and collaborators (2017) tested young *Mecp2*$^{tm1.1Bird}$ female mice at the novel object recognition but found no phenotype in HET mice. Based on these results and following the study performed by Sannino et al. (2012), we designed a more sensitive test that included four instead of two objects. This change in the protocol increased the difficulty of the test and allowed us to show a deficit of short-term memory associated with tactile and visual stimuli in 11-week-old female mice (*see Fig. 19*). This test was also performed on mice at this age after 15-day and 30-day treatments with MTZ (*see Figs. 20G and 24G*). In both cases the phenotype was reproduced, but with a lower effect size and a lower statistical significance. These differences between untreated and treated mice are probably due to the disturbing effect of daily i.p. injections (with either VEH or MTZ). Nevertheless, a relevant effect of MTZ was found in HET mice treated with MTZ for 15 days, as their performance was optimal, even better than that of control mice. These results should be further investigated in order to obtain a clear conclusion but indicate that MTZ could also be used to treat some cognitive deficits.

The retrospective analysis on adult RTT patients not only showed an improvement of general motor performance (measured by the MBAS evaluation), but also provided with robust evidence of MTZ efficacy in improving several other types of symptoms. We found that MTZ treatment can rescue a large number of altered behaviors (*see Fig. 17*), which represent a major breakthrough in the field. A recent large observational study on the natural history of RTT has identified behavioral problems as a key emerging issue in RTT (Buchanan et al., 2019). Together with sleeping disturbances, seizures and breathing problems, mood and behavioral problems are a major issue in terms of impact on the quality of life of RTT patients and their families (Corchón et al., 2018). Considering the heterogeneity and size of the studied cohort of patients, we found remarkably that MTZ treatment was able to improve or slow down most of the behavioral abnormalities listed into the natural history RTT study.

Concerning the possible mechanisms of action of MTZ, it should be noted that this antidepressant has a unique profile. It is characterized by a relatively rapid onset of action, high response and remission rates, a favourable side-effect profile and several unique therapeutic benefits over other antidepressants (Alam et al., 2013). So far, 18 receptors have been identified as targets of MTZ in the brain (Anttila and Leinonen, 2006), but effects exerted by them on nervous system are just partially known. MTZ shows its highest affinity for histaminergic H1

receptor, which is responsible for sedation when used at low doses (Fawcett and Barkin, 1998). Regarding monoaminergic systems, which are generally downregulated in RTT individuals and mouse models (Temudo et al., 2009; Samaco et al., 2009; Santos et al., 2010), MTZ enhances 5-HT and NA transmission through different mechanisms. At the presynaptic level, it antagonizes α-2 auto and hetero adrenoreceptors, leading to an increasing of NA release, while at the postsynaptic level it antagonizes 5-HT_2 and 5-HT_3 receptors, which may indirectly enhance 5-HT_{1A}-mediated serotoninergic transmission. In addition, NA released in the raphe nuclei further stimulates postsynaptic α-1 receptors, causing 5-HT release from downstream axon terminals such as those in the cortex (Nakayama et al., 2004; Sthal, 2010). Specific agonists of 5-HT_{1A} receptor have shown promising abilities to alleviate brainstem and extrapyramidal dysfunction in preclinical studies on RTT autonomic phenotypes (Abdala et al., 2014). However, some of the most prominent 5-HT_{1A} agonists have also shown to partially or totally antagonize DA receptors. This makes them not suitable for RTT, as also dopaminergic system is generally altered in RTT individuals and mouse models. For example, low spinal fluid DA levels, which are normally assumed to be a marker for central DA levels, have been found in women carrying *MECP2* mutations and meeting the clinical criteria for RTT (Samaco et al., 2009). Furthermore, the "DA hypothesis" is supported by the fact that, later in life, RTT individuals develop Parkinsonian features (Roze et al., 2007). Regarding these aspects, MTZ is an optimal candidate for RTT, as it not only enhances 5-HT transmission through 1A receptors (with minimal occurrence of serotonergic side effects), but also increases DA transmission (Nakayama et al., 2004; Masana et al., 2012). On this way, rescues observed in the motor domain after MTZ treatment in both HET mice and RTT treatments may be due to a normalization in DA levels in brain.

The present project has also showed that MTZ is able to normalize some alterations observed in PV networks. These interneurons have a main role in GABAergic inhibition and have been associated with RTT symptomatology and related phenotypes in mice (*see above*). To extend this analysis, we also evaluated PNNs, which surround mainly PV interneurons in the brain cortex and have been also associated with RTT physiopathology. Our hypothesis was that an abnormal early development of PNNs, which physiologically limit synaptic activity, could contribute to behavioural RTT-like phenotypes (Patrizi et al., 2019). In our analysis we did not find differences nor in the PNNs density nor in their immunoreactivity (*see Fig. 20-21*), this meaning that PNN are not greatly altered in 11-week-old *Mecp2*$^{tm1.1Bird}$ female mice. However, also in this case the number of mice evaluated could be increased and more specific analysis could be performed in order to detect more subtle alterations and extend on this way previous results.

We also investigated brain atrophy in adult mice. We did not observe a decrease in cortical thickness in HET mice, contrary to what had been previously showed in Null mice (Bittolo et al., 2016). However, some HET mice showed single values much lower compared to every WT mouse in the primary motor cortex. This reflects, once again, the large phenotypic variability within HET mice compared to Null mice, which makes more difficult to observe group effects. Therefore, these results cannot discard that an atrophy exist in HET brains. Further investigations in more specific features (such as single neuronal morphology) may obtain significant results that help us to better understand RTT physiopathology and MTZ mechanisms of action.

CONCLUSIONS

The present project satisfied quite well the initially purposed objectives. First, we verified that MTZ is a quite safe drug, as we observed very few side effects in both HET mice at different ages and adult RTT individuals, even when administered chronically. We also demonstrated that MTZ is able to rescue several deficits derived from *MECP2* mutations. In mice, short-term treatments with MTZ improved several domains: motor, somatosensory and cognitive. We also proposed some underlying mechanisms for these deficits, as we found alterations into the PV networks in related brain areas in adult mice. In addition, we showed the rescue of PV expression in the primary motor and the barrel cortices. Regarding the latter, we proposed that the normalization of PV cells activity, and therefore the internal inhibition in the barrel cortex, was the mechanism underlying the normalization of the behaviour at the EPM in adult mice. In younger mice these deficits were not detected, but results have to be confirmed before reaching a definitive conclusion.

We realized a behavioural phenotyping of *Mecp2*$^{tm1.1Bird}$ HET female mice at two different ages and found several previously unknown phenotypes that indicate a new time window at 11 weeks of age to test drugs. We then treated 11-week-old mice with MTZ and verified that it was more effective than in adult stages, indicating that plasticity process may be a target of this drug. Finally, in a heterogeneous cohort of RTT patients, MTZ significantly improved several features within the motor domain, as well as numerous behavioural alterations. Taken together, results from the present project strongly supports MTZ as a candidate to treat young and adult people affected by RTT. We consider that there is enough information to initiate a randomized clinical trial to verify whether MTZ is able to improve and partially prevent RTT symptomatology and, on this way, improve quality of life of affected people and their families.

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SUPPLEMENTARY FIGURES

RTT Diagnostic Criteria 2010

Consider diagnosis when postnatal deceleration of head growth observed.

Required for typical or classic RTT

1	A period of regression followed by recovery or stabilization*
2	All main criteria and all exclusion criteria
3	Supportive criteria are not required, although often present in typical RTT

Required for atypical or variant RTT

1	A period of regression followed by recovery or stabilization*
2	At least 2 out of the 4 main criteria
3	5 out of 11 supportive criteria

Main Criteria

1	Partial or complete loss of acquired purposeful hand skills.
2	Partial or complete loss of acquired spoken language**
3	Gait abnormalities: Impaired (dyspraxic) or absence of ability.
4	Stereotypic hand movements such as hand wringing/squeezing, clapping/tapping, mouthing and washing/rubbing automatisms

Exclusion Criteria for typical RTT

1	Brain injury secondary to trauma (peri- or postnatally), neurometabolic disease, or severe infection that causes neurological problems***
2	Grossly abnormal psychomotor development in first 6 months of life#

Supportive Criteria for atypical RTT##

1	Breathing disturbances when awake
2	Bruxism when awake
3	Impaired sleep pattern
4	Abnormal muscle tone
5	Peripheral vasomotor disturbances
6	Scoliosis/kyphosis
7	Growth retardation
8	Small cold hands and feet
9	Inappropriate laughing/screaming spells
10	Diminished response to pain
11	Intense eye communication - "eye pointing"

Table 1. Revised diagnostic criteria for RTT (*from Neul et al., 2010*). *Because *MECP2* mutations are now identified in some individuals prior to any clear evidence of regression, the diagnosis of "possible" RTT should be given to those individuals under 3 years old who have not lost any skills but otherwise have clinical features suggestive of RTT. These individuals should be reassessed every 6-12 months for evidence of regression. If regression, the diagnosis should then be changed to definite RTT. However, if the child does not show any evidence of regression by 5 years, the diagnosis of RTT should be questioned. **Loss of acquired of language is based on best acquired spoken language skill, not strictly on the acquisition of distinct word or higher language skills. Thus, an individual who had learned to babble but then loses this ability is considered to have a loss of acquired language.

Group	Patient	Gender	Age	MECP2 mut. type	RCSS	Clinical diagnosis
UNTREATED	#1	F	20	Del. ex1 – ex2	30	Typical RTT
	#2	F	16	R168X	22	Typical RTT
	#3	F	23	R294X	13	Typical RTT
	#4	F	26	T158M	19	Typical RTT
	#5	F	31	R294X	19	Typical RTT
	#6	F	16	T158M	31	Typical RTT
	#7	F	16	R255X	22	Typical RTT
	#8	F	42	R270X	29	Typical RTT
	#9	F	24	P152 xfs	19	Typical RTT
	#10	F	16	R133C	20	Typical RTT
	#11	F	27	R306C	22	Typical RTT
MTZ-TREATED	#1	F	16	P152R	29	Typical RTT
	#2	F	40	1159 del. 44	17	Typical RTT
	#3	F	16	R294X	21	Typical RTT
	#4	F	32	P388 xfs	21	Typical RTT
	#5	F	34	R306C	29	Typical RTT
	#6	F	40	c. 1164_1207	30	Typical RTT
	#7	F	18	L386 xfs	18	Typical RTT
	#8	F	32	877 del. G	14	Typical RTT
	#9	F	16	T158M	22	Typical RTT
	#10	F	27	R133C	23	PSV
	#11	F	25	R294X	23	Typical RTT

Table 3. Demographics and relevant features of untreated control patients and patients treated with mirtazapine for variable time periods (see table 4). All studied patients were diagnosed with typical RTT except of one, which was diagnosed with a PSV. General symptomatic severity was assessed through the RCSS.

Patient	MTZ dose (mg / day)	MTZ mg / kg bw / day	MTZ treatment duration (years)	MTZ Tolerance
#1	7.5	0.170	2	T
#2	15	0.375	2	T
#3	30	1.071	2	T
#4	30	0.428	5	T
#5	30	0.806	2	T
#6	15	0.395	1	T
#7	15	0.288	2	T
#8	22.5	0.625	3	T
#9	7.5	0.306	0.5	NT
#10	15	0.288	2	T
#11	15	0.441	0.08	NT

Table 4. Dosages and duration of MTZ treatment on patients. Dosages were personalized for each patient according to clinical observations, also in terms of mg of MTZ per kg of body weight (bw) per day. Duration of MTZ treatment was always longer than one year, with only two exceptions (patients #9 and #11), in which adverse effects made evident an intolerance (NT) to the drug. In all the other cases, MTZ treatment was well tolerated (T).

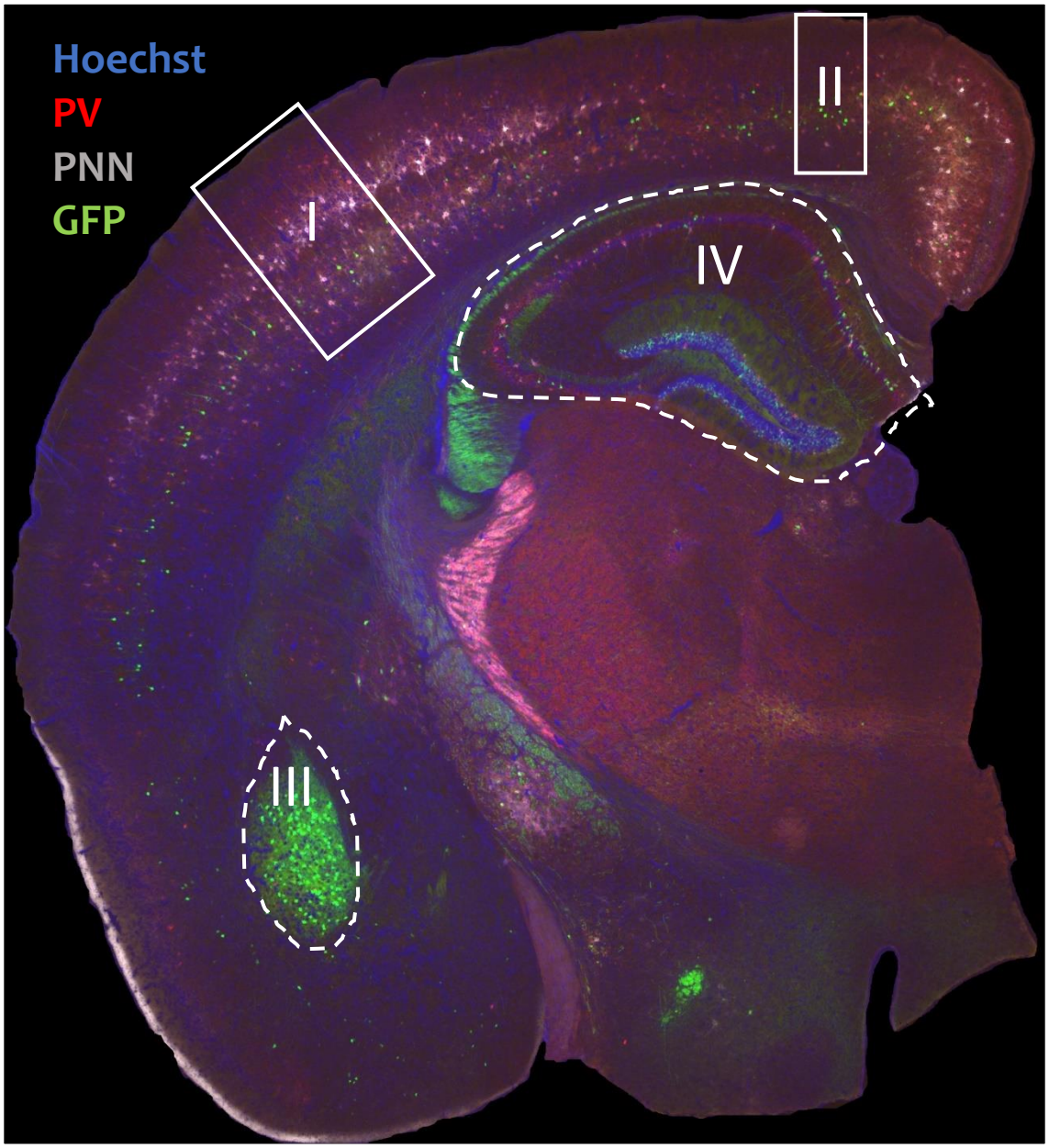

Figure 11. Regions of interest for histological analyses. A 20-µm brain slice from a wild-type 11-month-old Tg(Thy1-EGFP)-*Mecp2*$^{tm1.1Bird}$ mouse is shown. This mouse line expresses constitutively the green fluorescence protein (GFP) under the control of the Thy1 promoter. In addition, we used α-PV antibodies (in red) to stain a subpopulation of GABAergic interneurons and lectin from *Wisteria floribundia* to stain perineuronal nets (PNNs, in grey). Most of these structures surround PV cells, this creating a pinkish staining which indicates colocalization. We finally used Hoechst (in blue) to unspecifically stain cell nuclei. The four different ROIs studied in this project are shown: (I) primary somatosensory-barrel cortex, (II) primary motor cortex, (III) basolateral amygdala and (IV) dorsal hippocampus. **S**cale bar: 1000 µm