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- INDIRIZZO NEUROBIOLOGIA -

"PHARMACOLOGICAL APPROACHES FOR THE TREATMENT OF THE RETT SYNDROME"

Settore scientifico-disciplinare: Scienze Biologiche
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RIASSUNTO

La syndrome di Rett (RTT) è una patologia dello sviluppo neuronale postnatale causata dalle mutazioni del gene MeCP2, situato nel cromosoma X, codificante per la Methyl CpG binding protein 2, un modulatore della trascrizione. La forma classica si manifesta in 1:10,000 bambine ed è caratterizzata da una progressiva regressione generale fisica e mentale, in seguito ad un normale sviluppo nei primi 2 anni di vita. Molti degli aspetti della patologia sono stati riprodotti in diversi modelli murini deleti per il gene MeCP2 ($MeCP2^{-/y}$), inclusi la riduzione della massa cerebrale, l'atrofia neuronale e le disfunzioni cardiorespiratorie, che costituiscono i parametri più robusti e riproducibili tra i diversi modelli murini, accanto ai meno conservati parametri comportamentali, come l'ansia, la socievolezza e l'aspetto motorio. Il fenotipo Rett è caratterizzato inoltre da una riduzione dei livelli di espressione della serotonina (5HT), norepinefrina (NE) e del BDNF (Brain Derived Neurotrophic Factor). Tuttavia, è noto che i farmaci antidepressivi sono in grado di modulare i livelli di BDNF in parte regolando il sistema monoaminergico.

Lo scopo di questo lavoro consiste perciò nel valutare gli effetti del trattamento cronico con antidepressivi in un modello della sindrome di Rett. Abbiamo scelto la Desipramina (DMI) come farmaco di controllo, dal momento che è già stata precedentemente utilizzata per un trial clinico della sindrome di Rett. La Desipramina è un antidepressivo che blocca il recupero di 5HT e NE a livello dello spazio sinaptico, tuttavia presenta delle complicanze cliniche a livello cardiaco. Per evitare tale effetto collaterale della DMI, abbiamo selezionato un antidepressivo altamente tollerabile, la Mirtazapina (MIR), un antagonista degli α2 autorecettori ed eterorecettori centrali e uno specifico inibitore dei recettori 5HT2 e 5HT3.

Il lavoro si divide in 4 fasi:

Fase 1: analisi degli effetti del trattamento con antidepressivi sul peso del corpo e del cervello ed analisi della morfologia dei neuroni piramidali della corteccia somatosensoriale in un modello murino della sindrome di Rett

Fase 2: analisi degli effetti del trattamento con antidepressivi sui parametri vitali, inclusi il battito cardiaco e la frequenza respiratoria nel modello murino della sindrome di Rett

Fase 3: analisi degli effetti del trattamento con antidepressivi sul comportamento nel modello murino della sindrome di Rett

Fase 4: analisi degli effetti del trattamento con antidepressivi sul livello di espressione del BDNF

<u>Fase 1: analisi degli effetti del trattamento con antidepressivi sul peso del corpo e del cervello ed analisi della morfologia dei neuroni piramidali della corteccia somatosensoriale in un modello murino della sindrome di Rett</u>

Prima di tutto abbiamo valutato le caratteristiche generali del modello murino della sindrome di Rett ($MeCP2^{-/y}$), osservando che il peso del corpo e del cervello dell'animale era significativamente ridotto a 42 giorni dalla nascita. Inoltre, come osservato in precedenza (Kishi and Macklis, 2004, Fukuda et al., 2005), abbiamo confermato la significativa riduzione dello spessore totale della corteccia somatosensoriale (la più compromessa in questa patologia), in particolare degli strati II-III e VI a 42 giorni dalla nascita.

Abbiamo quindi trattato gli animali per due settimane a partire dal 28° giorno dalla nascita con DMI alla concentrazione 10 mg/Kg e con MIR a due differenti concentrazioni (10 o 50 mg/Kg) ed analizzato gli effetti del trattamento sul peso del corpo e del cervello. Non abbiamo riscontrato differenze per quanto riguarda il peso del corpo dopo trattamento farmacologico, tuttavia abbiamo notato un significativo aumento del peso del cervello in topi $MeCP2^{-/y}$ dopo 2 settimane di trattamento con MIR 50 mg/Kg, confrontato con il peso del cervello di topi $MeCP2^{-/y}$ della stessa età non trattati.

Per meglio definire le strutture coinvolte nel recupero del peso cerebrale dopo trattamento con MIR 50 mg/Kg, abbiamo effettuato una colorazione Nissl su sezioni coronali di cervello di topo e abbiamo analizzato l'ippocampo e la corteccia somatosensoriale. Abbiamo osservato che non c'erano differenze nelle proporzioni di ogni strato ippocampale rispetto allo spessore totale dell'ippocampo lungo l'asse rostro-caudale. Tuttavia, l'analisi della corteccia somatosensoriale ha rivelato che il trattamento con DMI 10 mg/Kg e MIR 50 mg/Kg fa recuperare lo spessore totale della corteccia in topi $MeCP2^{-/y}$ a 42 giorni dalla nascita ed in particolare lo spessore degli strati II-III e VI che sono principalmente compromessi nel modello murino della sindrome di Rett (Kishi and Macklis, 2004, Fukuda et al., 2005).

Per avere maggiori informazioni sull'effetto della MIR 50 mg/Kg a livello dei neuroni corticali, abbiamo esaminato la morfologia dei neuroni piramidali dello strato II-III della

corteccia somatosensoriale in topi MeCP2$^{-/y}$ a 42 giorni dalla nascita utilizzando la colorazione di Golgi. Abbiamo osservato che il trattamento con MIR 50 mg/Kg induce un recupero dei deficit morfologici presenti nel modello murino (Kishi and Macklis, 2004, Fukuda et al., 2005) inclusi, la ridotta area del soma, il diametro ridotto dei dendriti apicali, l'atrofia dell'albero dendritico apicale ed in particolare quello basale, il numero delle spine "stubby" sia nei dendriti secondari apicali che basali. Infine, dal momento che è stato precedentemento osservato un deficit di rilascio del GABA in topi MeCP2$^{-/y}$ (Chao et al., 2010), abbiamo deciso di valutare se la MIR 50 mg/Kg era in grado di recuperare questo deficit. Abbiamo quindi dimostrato che le correnti GABA sono parzialmente recuperate dopo trattamento con MIR 50 mg/Kg nella corteccia di topi MeCP2$^{-/y}$ a 42 giorni dalla nascita.

Fase 2: analisi degli effetti del trattamento con antidepressivi sui parametri vitali, inclusi il battito cardiaco e la frequenza respiratoria nel modello murino della sindrome di Rett

I pazienti Rett e i topi MeCP2$^{-/y}$ presentano alterazioni cardiache e un respiro anomalo allo stato avanzato della patologia. Attraverso uno strumento non invasivo (MouseOX), abbiamo raccolto i dati relativi alla saturazione dell'ossigeno (percentuale di siti dell'emoglobina occupati dalle molecole di ossigeno), il battito cardiaco e la frequenza respiratoria (numero di battiti e respiri al minuto) e la distensione dell'arteria in base al battito cardiaco in topi Wild Type e MeCP2$^{-/y}$ non trattati e trattati con DMI 10 mg/Kg or MIR 50 mg/Kg. Abbiamo osservato che non ci sono alterazioni nella saturazione dell'ossigeno, tuttavia la frequenza dei battiti cardiaci e del respiro, che è ridotta nei topi MeCP2$^{-/y}$ non trattati, viene recuperata in seguito a trattamento con gli antidepressivi, in particolare con la MIR. Inoltre, l'effetto negativo sulla distensione dell'arteria osservato per la DMI 10 mg/Kg, non viene alterato dal trattamento con MIR 50 mg/Kg.

Fase 3: analisi degli effetti del trattamento con antidepressivi sul comportamento nel modello murino della sindrome di Rett

I topi MeCP2$^{-/y}$ sono caratterizzati dal disturbi motori e una ridotta ansia (Chahrour and Zoghbi, 2007), cosi abbiamo deciso di testare gli effetti degli antidepressivi sul comportamento del modello murino della sindrome di Rett. Attraverso il test dell'"open

field", abbiamo dimostrato che i topi MeCP2$^{-/y}$ trattati con i farmaci trascorrono la maggior parte del tempo del test immobili, e la loro attività in termini di capacità di alzarsi e tenersi sulle zampe posteriori e di cura personale è ridotta. Queste osservazioni sono probabilmente dovute all'effetto sedativo indotto dal trattamento con antidepressivi. Tuttavia, l'ansia che è ridotta nei topi MeCP2$^{-/y}$ non trattati osservata nel test dell'"elevated plus maze", ritorna a valori normali dopo trattamento con gli antidepressivi.

Fase 4: analisi degli effetti del trattamento con antidepressivi sul livello di espressione del BDNF

Precedenti studi hanno dimostrato che il livello di espressione del BDNF totale è significativamente ridotto nel cervello dei topi MeCP2$^{-/y}$ (Chang et al., 2006, Wang et al., 2006). In questo lavoro abbiamo dapprima dimostrato come i livelli delle diverse isoforme del BDNF variano sulla base della mutazione del gene MeCP2 nei pazienti Rett. Successivamente abbiamo valutato le diverse isoforme del BDNF nel prosencefalo di topi MeCP2$^{-/y}$ dimostrando come esse siano significativamente ridotte a 42 giorni dalla nascita. Tuttavia, il trattamento con DMI 10 mg/Kg e MIR 50 mg/Kg non è in grado di recuperare in modo significativo il livello di mRNA. Abbiamo quindi valutato il livello proteico del BDNF, dimostrando un aumento della neurotrofina a livello corticale e una diminuzione a livello ippocampale in topi MeCP2$^{-/y}$ non trattati ma non statisticamente significativo. Tuttavia il trattamento con MIR 50 mg/Kg sembra recuperare il livello del BDNF, sebbene non sia significativo.

ABSTRACT

Rett syndrome (RTT) is an X-linked postnatal neurodevelopmental disorder caused by the mutations on MeCP2 gene which encodes for the Methyl CpG binding protein 2, a transcriptional regulator. The classical form manifests in girls with an incidence of 1:10,000 with a progressive general physical and mental regression after a normal development during the first two years of age. Several clinical features are recapitulated in MeCP2$^{-/y}$ mice, including the reduced brain mass, neuronal atrophy and the cardiorespiratory abnormalities, which are considered the most robust and reproducible parameters among the Rett mouse models and the less conserved alterations on mice behavior. Rett phenotype was characterized by a reduction on serotonin, norepinephrine (5HT; NE) and BDNF (Brain Derived Neurotrophic Factor) expression level. However, it is known that the antidepressants drugs modulate BDNF expression level partly by regulation of monoamine systems.

The aim of the project is to evaluate the effects of repeated antidepressant treatments in a Rett mouse model. We choose Desipramine (DMI) as control drug because it was previously used in a clinical trial of Rett syndrome. DMI blocks the reuptake of 5HT and NE, but it has some cardiac complications. To overcame the cardiac side effect of DMI, we selected the highly tolerable antidepressant Mirtazapine (MIR), which is an antagonist of central α2 autoreceptors and α2 heteroreceptors and a specific blocker of 5HT2 and 5HT3 receptors.

The project comprises four phases:

Phase1: Analysis of the effects of antidepressant treatments on body and brain weight, including the morphology of the somatosensory pyramidal neurons in a model of Rett syndrome (MeCP2$^{-/y}$)

Phase2: Analysis of the effects of antidepressant treatments on the vital signs parameters, including heart and breath rate in MeCP2$^{-/y}$ mice

Phase3: Analysis of the effects of antidepressant treatments on the behavior of the mice (open field and plus maze test) in MeCP2$^{-/y}$ mice

Phase4: Analysis of the effects of antidepressant treatments on brain derived neurotrophic factor (BDNF) expression level

Phase1: Analysis of the effects of the drugs on body and brain weight, including the morphology of the somatosensory pyramidal neurons in a model of Rett syndrome (MeCP2$^{-/y}$)

First of all, we evaluated the general features of the Rett mouse model, observing that the body and the brain weight of MeCP2$^{-/y}$ mice were reduced at postnatal day 42 (p42). We found also that there is a significant reduction on total cortical thickness, in particular of layers II-III and VI at p42 as observed in previous studies (Kishi and Macklis, 2004, Fukuda et al., 2005).

Then, we analyzed the effects of DMI 10 mg/Kg and MIR (at two different concentration: 10 or 50 mg/Kg) treatments on body and brain weight. No difference was observed for body weight, while an increase in brain weight was noticed after treatment with MIR 50 mg/Kg in p42 MeCP2$^{-/y}$ mice compared to MeCP2$^{-/y}$ untreated mice. To better define the brain structures involved in the rescue of the brain weight after MIR 50 mg/Kg treatment, we performed a Nissl staining and we analyzed the hippocampus and the somatosensory cortex. We found that among p42 MeCP2$^{-/y}$ treated mice, there were no differences in the proportion of each hippocampal layer to the total thickness along the rostro-caudal axis. However, the analysis of the somatosensory cortex revealed that DMI 10 mg/Kg and MIR 50 mg/Kg rescued the total cortical thickness in p42 MeCP2$^{-/y}$ mice and in particular the layers II-III and VI which are principally compromised in Rett mouse model (Kishi and Macklis, 2004, Fukuda et al., 2005).

To gain further insight regarding the effect of Mirtazapine treatment on cortical neurons, we investigated the morphology of layer II-III pyramidal neurons of the somatosensory cortex in MeCP2$^{-/y}$ mice using Golgi staining. We observed that MIR 50 mg/Kg treatment was able to recover the neuronal morphology deficits of p42 MeCP2$^{-/y}$ mice (Kishi and Macklis, 2004, Fukuda et al., 2005), including, the small soma area, the reduced diameter of apical dendrites, the atrophy of apical and, in particular, the basal dendritic arborization, the number of secondary basal dendrites, the number of stubby spines both in secondary apical and basal dendrites. Finally, as a deficit on GABA release in MeCP2$^{-/y}$ mice was previously described (Chao et al., 2010), we investigatd if Mirtazapine could rescue this deficit. Indeed, we found that GABA currents were rescued by MIR 50 mg/Kg treatment in the cortex of p42 MeCP2$^{-/y}$ mice, although without reaching full recovery.

Phase2: Analysis of the effects of the drugs on the vital signs parameters, including heart and breath rate in MeCP2$^{-/y}$ mice

Rett patients and MeCP2$^{-/y}$ mice presents cardiac alterations and breathing abnormalities in a later stage of the disorder. Through a non-invasive instrument (MouseOX) we collected the data regarding the Oxygen Saturation (percentage of sites of arterial hemoglobin occupied by oxygen molecules), the Hearth and the Breath Rate (number of beats or breaths per minute) and the Pulse Distention (change in distension of the arterial blood vessels due to a cardiac pulse) on Wild Type and MeCP2$^{-/y}$ mice untreated or treated with DMI 10 mg/Kg or MIR 50 mg/Kg. We found that no alterations was observed for the oxygen saturation, however the frequency of heart and breath are rescued after drug treatments. A negative effect of Desipramine was observed in pulse distention which is not affected with Mirtazapine treatment.

Phase3: Analysis of the effects of the drugs on the behavior of the mice (open field and plus maze test)

MeCP2$^{-/y}$ mice are characterized by motor abnormalities and a decreased anxiety (Chahrour and Zoghbi, 2007), thus, we tested the effects of the antidepressant drugs on the behavior of MeCP2$^{-/y}$ mice. Through an open field test, we found that the MeCP2$^{-/y}$ mice treated with the drugs spent more of the time immobile, and their activity in terms of number of rearing and grooming was reduced. These observations are probably due to the sedative effect of antidepressant treatments. However, the anxiety was recover to normal levels in MeCP2$^{-/y}$ mice treated with the antidepressants in the elevated plus maze.

Phase4: Analysis of the effects of treatments on BDNF expression level

Previous studies showed that total BDNF expression level was significantly reduced in the brain of MeCP2$^{-/y}$ mice (Chang et al., 2006, Wang et al., 2006). First of all, we demonstrated that the levels of BDNF isoforms depend on mutations in MeCP2 gene in Rett patients. Then, we evaluated the BDNF splice variants in the forebrain of MeCP2$^{-/y}$ mice and we demonstrated that they were significantly reduced at p42.

However, treatments with DMI 10 mg/Kg or MIR 50 mg/Kg not rescue significantly the mRNA of BDNF. Therefore, we evaluated the protein level of BDNF and we demonstrated a no statistically significant increase of the neurotrophin in the cortex and a decrease in the hippocampus in MeCP2$^{-/y}$ untreated mice. However, the treatment with MIR 50 mg/Kg seemed to rescue the protein level of BDNF, even if no statistically significant.

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INTRODUCTION

1. Rett Syndrome

1.1 Clinical features

The Rett syndrome (RTT) is a postnatal progressive neurodevelopmental disorder discovered in 1965 by Adrian Rett, a Viennese pediatrician. In its classical form, RTT manifests in girls during their early childhood with an incidence of 1:10,000, with no specific ethnic or geographical preference. The child develop normally up to 6-18 months of life. The earlier indicator of neurological disorder is the reduction of head growth which is accompanied by a general growth retardation, weight loss and mental regression. In a later stage, girls develop some autistic-like features such as repetitive stereotype hand movements, an hallmark of RTT, social withdrawal, loss of language, expressionless face, hypersensitivity to sound, lack of eye to eye contact, indifference to the surrounding environment and unresponsiveness to social cues (Nomura and Segawa, 2005, Kaufmann et al., 2012). A common characteristic of RTT is the presence of breathing abnormalities which include episodes of hyperventilation, severe pauses in breathing and abnormal cardiorespiratory function, due to periods of vasomotor disturbances (usually associated with cold hand and feet), abnormal sweating, decrease heart rate variability and prolongation of corrected QT intervals (an indicator of cardiac electrical activity) (Sekul et al., 1994, McCauley et al., 2011). Seizures are also most common in RTT patients and correlate with the severity of phenotype (Chahrour and Zoghbi, 2007, Katz et al., 2012) (Figure 1).

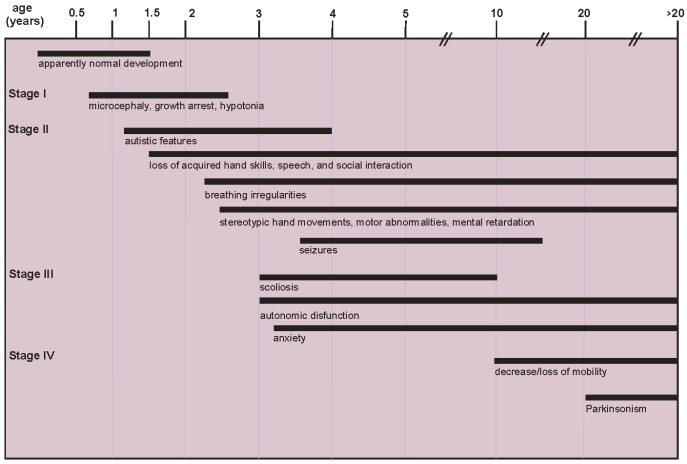


Figure 1. Onset and progression of Rett syndrome (Daniela Zahorava, 2013).

Later in their life, RTT girls enter a stage of motor decline. Gait is almost always disrupted, with evidence ataxia and apraxia. Axial hypotonia is present early in the disease course and later become rigidity. Most of the child present scoliosis (Percy et al., 2010). Also nutrition and gastrointestinal functions are altered in RTT patients (Tarquinio et al., 2012). Females with RTT often survive into adulthood and older age, but their life expectancy is less than that of the healthy population. Approximately 25% of the deaths are sudden and they may occur due to autonomic nervous system disturbances or cardiac abnormalities (Guideri et al., 1999, Kerr and Julu, 1999) (Figure 1).

1.2 Genetic bases of Rett syndrome

The genetic base of Rett syndrome was difficult to identify because more than 99% of the cases are sporadic. The evidence that only females and half-sisters were affected by this disorder, suggested an X-linked inheritance with lethality in hemizygous males (Hagberg et al., 1983). Moreover, an exclusion mapping of the X-chromosome revealed that mutations in MeCP2 (Methyl CpG binding protein 2) gene were the responsible of the disorder (Amir et al., 1999). Most of the mutations arise spontaneously (*de novo*) in the paternal germ line, thus affect females who, owing to X-chromosome inactivation, are somatic mosaics for normal and mutant gene (Katz et al., 2012).

1.3 MeCP2 inheritance and X-chromosome inactivation

Females inherit one X-chromosome from the mother and the other from the father, while males inherit a single X-chromosome, maternally. Therefore, females can inherit MeCP2 mutations from either parents, while males inheritance of MeCP2 mutations is exclusively maternal. Females are subjected to X-Chromosome Inactivation (XCI). X-chromosome inactivation is a process by which one of the two X-chromosome of the females is inactivated to achieve gene expression pattern similar to those found in males, who carry only one copy of the X-chromosome. XCI occurs on a cell-by-cell basis and is predominantly a random process. For this reason, females affected by RTT result as mosaics expressing the normal and the mutant gene. The type of MeCP2 mutation and the degree of inactivation between the mutant and wild-type alleles are both contributing to phenotypic variability of the disorder. However, starting from the inheritance of a single affected X-chromosome, the phenotype in

males result more severe than in females. Generally, males develop neurological disorders like mental retardation, till they die before or soon after birth with a severe encephalopathy (Gonzales and LaSalle, 2010).

2. MeCP2 (Methyl CpG Binding Protein 2)

2.1 From the gene to the protein

MeCP2 (Methyl CpG binding protein 2) gene is located in the Xq28 chromosome and it consists of four exons. It is presented in two protein isoforms that differ only for the N-termini: alternative splicing of exon 2 generates the isoforms MeCP2_e1 and MeCP2_e2; the most abundant MeCP2_e1 has the translation start site in the exon 1 and lacks the exon 2, while MeCP2_e2 has the start site in exon 2. The 3'UTR region of the gene contains multiple poliadenylation sites, that alternatively generate four different transcripts (Chahrour and Zoghbi, 2007) (Figure 2).

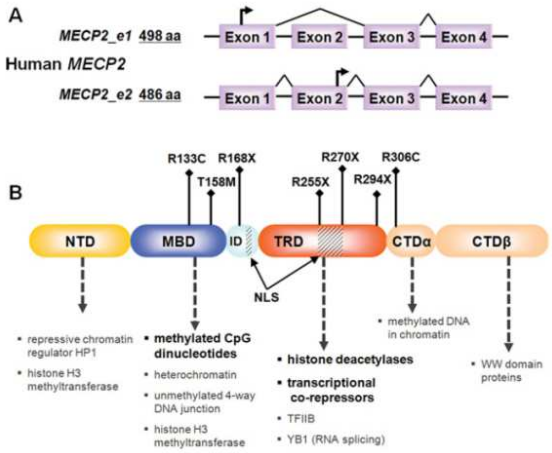


Figure 2. MeCP2 gene and protein. A) The two distinct isoforms derive from the alternative splicing of exon 2. **B)** The principle domains of MeCP2 protein: NTD (N-terminal domain) in which there is a nuclear signal localization, MBD (methylated DNA binding domain), ID (interdomain), TRD (transcription repression domain), CTD (C-terminal domain) (Gadalla et al., 2011).

MeCP2 is a nuclear protein which belongs to the family of Methyl Binding Proteins (MBP). MBP proteins contain the characteristic and functional domain MDB (methylated DNA-binding domain) through it, they bind the methyl groups on the CpG dinucleotides of DNA, modifying the structure of chromatin and repressing the transcription. However, MeCP2 is composed by other distinct domains: NTD (N-terminal domain), in which there is a nuclear signal localization, ID (interdomain), TRD (transcription repression domain), CTD (C-terminal domain). The TRD is the most important domain for the association with the transcriptional co-repressor Sin3a which recruits the histone deacetylases HDAC1 and HDAC2. These histone deacetylases remove acetyl groups from histones and compact the chromatin, inhibiting the binding of the transcriptional complex (Gadalla et al., 2011, Samaco and Neul, 2011) (Figure 2).

The first studies indicate that the main role of MeCP2 is to repress the transcription of specific target genes as described above. However, recent studies suggest that MeCP2 acts as an activator of the transcriptional machinery. In fact, Chahrour and colleagues found that MeCP2 could bind the transcription factor CREB1 and other co-activators to induce the expression of the genes and it was confirmed by the observation that most of the target genes of MeCP2 were activated in MeCP2 overexpressing mice and down-regulated in MeCP2 knock-out mice (Chahrour et al., 2008). In addition, other studies demonstrated that MeCP2 is also involved in chromatin regulation and mRNA processing (Gadalla et al., 2011). In front of these controversial results, nowadays the function of MeCP2 remains unclear (Figure 3).

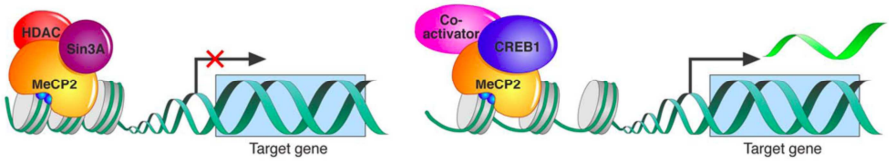


Figure 3. Role of MeCP2 as repressor or activator of transcription. On the left: MeCP2 creates a complex with the co-repressor Sin3A and histone deacetylases (HDACs) to repress transcription of target genes. On the right: MeCP2 associates with the transcriptional activator CREB1 at the promoter of a target gene and activates its transcription.

MeCP2 is expressed in whole body, but it is most abundant in the brain, in particular the isoform MeCP2_e1. The expression level is low during the embryonic development and

increases in adult brain, in particular in mature post migratory neurons. It was found that the expression of MeCP2 protein in some tissues like cortex, hippocampus and cerebellum follows the development and maturation of the same tissues. The abundant presence of MeCP2 in adult brain induces to think that it could play a role in mature neurons regulating neuronal activity and plasticity (Chahrour and Zoghbi, 2007).

2.2 MeCP2 in the embryonic and postnatal cortex development and maturation

Neurogenesis is the process through which neuronal precursor cells differentiate into the different mature neuronal populations including neurons, interneurons, astroglia and oligodendroglia. The fate of neuronal precursors is determined by their interaction with specific signal molecules (like Notch). From the divisions of neuronal precursors originate immature neurons which migrate to reach their final position determining the classical organization in layers of the cortex. The primitive cortex is composed by the marginal zone (MZ; the most external zone which contains the Cajal-Retzius cells that will form the layer I of the cortex), the cortical plate (CP), the subcortical zone (SP), the intermediate zone (IZ) and the ventricular zone (VZ). Neuronal precursors and the immature pyramidal neurons are localized in the ventricular zone of the cortex and after having completed their mitotic cycle, they migrate from the ventricular zone to the cortical plate, using radial glial processes as scaffold (glial cells stretched from the ventricular zone to the pial surface). The first generated neurons stopped in the cortical plate, while the most recently neurons, migrate and stopped in the upper layers. In this way, the process of migration of immature neurons give rise to the different layers of the adult cortex (from II to VI) (Parnavelas et al., 2002).

Alterations of the genes (like Reelin and Cdk5) involved in the mechanisms of neurogenesis and migration of immature neurons generate malformations and disorganization of cortical development, leading to neuronal deficits (Hong et al., 2000, Tissir and Goffinet, 2003). This is not the case of Rett syndrome which is considered a postnatal neurodevelopmental disorder as the children develop neurological deficits after a first period of about two years without symptoms.

This is confirmed by the fact that MeCP2 is involved into the cortical maturation and maintenance of the neurons but not into the fate of neuronal precursor cells (Kishi and Macklis, 2004). In 2004, Kishi and Macklis, demonstrated that MeCP2 was weakly expressed

in the cells of cortical plate (layer V and VI in the adult) in wild type mice at embryonic 16.5 stage with a uniform pattern. However, MeCP2 was more expressed in the superficial layer Cajal-Retzius cells and in the deeper layers of ventricular and intermediated zone. The uniform pattern of the expression of MeCP2 was maintained until postnatal day 0 (p0); from postnatal day 7, it was observed a progression of punctate nuclear staining from the deep cortex to the superficial layers which was extended in brain tissues (hippocampus, striatum, thalamus, cortex) at 9 weeks of age (Figure 4). It was concluded that MeCP2 was expressed and activated after neuronal migration and it increased as neuronal maturation progresses.

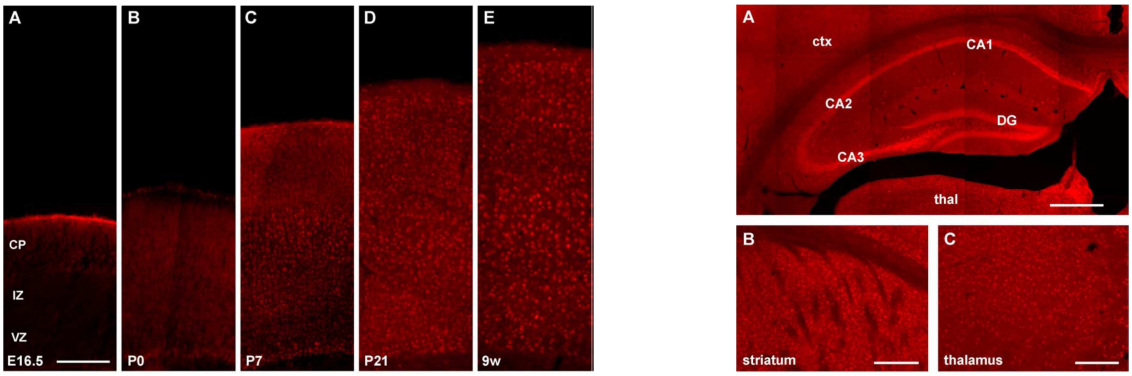


Figure 4. MeCP2 expression pattern. (On the left) MeCP2 is weakly expressed in the cortical plate of E16.5 mice and it expanded at p0. MeCP2 expression staining is punctate in the cortex of wild type mice from p7 to 9 weeks of age. (On the right) MeCP2 is widely expressed in hippocampus, striatum and thalamus in mice at 9 week of age (Kishi and Macklis, 2004).

Moreover, Kishi and Macklis demonstrated that MeCP2 mutation does not affect the proliferation and differentiation of neuronal precursors because they obtained the same number of neurospheres from neuronal precursors derived from E13.5 (E = embryonic) mice (at 9 days in vitro = DIV9), and the same number of generated neurons, astroglia and oligodendroglia comparing wild type (WT), heterozygous ($MECP2^{+/-}$) and hemizygous ($MECP2^{-/y}$) mice.

However, the mutations of MeCP2 gene reduced the complexity but not the number of cortical neurons in terms of cortical thickness, which was reduced in $MECP2^{-/y}$ mice at 6 weeks of age, in particular the layer II-III (Kishi and Macklis, 2004).

2.3 MeCP2 in Rett syndrome

The principle cause in the 90-95% of pathogenic cases of the Rett syndrome is the mutations that occur *de novo* in MeCP2 gene. Over than 200 nucleotide changes are described, however, the most common include missense and nonsense mutations (70%); the others are small deletions in the C-terminal (9%), frame-shift mutation and truncation (Williamson and Christodoulou, 2006). The phenotypes of Rett syndrome are correlated with the domain affected by the mutation. In fact, mutations in the NLS or early truncation cause severe phenotype than missense mutation, while C-terminal mutations are correlated to a milder phenotype (Chahrour and Zoghbi, 2007).

3. Mouse models of Rett syndrome

Classical Rett syndrome patients are characterized by a specific clinical relevance, with a normal development during the first two years of age and then a progressive general mental and physical regression. However, the severity of the disorder is different among the patients from a mild to a severe phenotype (Chahrour and Zoghbi, 2007). This phenotypic variability is principle due to random X-chromosome inactivation and the different mutations that occur in MeCP2 gene, including nonsense, missense, insertion, frameshift mutations or duplication/triplication of the gene (Wang et al., 2013). In front of this variability in Rett patients, different mouse models of the disease were created. Most of them are models based on the deletion of a part of MeCP2 gene that leads to a loss of function of the protein and to a most severe phenotype: $Mecp2^{tm1.1Jae}$ mice with the deletion of exon 3 (Chen et al., 2001b), $Mecp2^{tm1.1Bird}$ with a deletion of exons 3 and 4 (Guy et al., 2001) and $Mecp2^{tm1Tam}$ with a deletion of exon 3 and part of exon 4 (Pelka et al., 2006). These models represent only the 10% of Rett patients. Other models are characterized by truncation and single nucleotide mutation in MeCP2 gene which represent the mutations that occur in RTT individuals: $Mecp2^{308/y}$ with truncation at aminoacid 308, missing the C-terminal domain (Shahbazian et al., 2002); $Mecp2^{tm1.1Coyle}$, $Mecp2^{tm1.1Hup}$, $Mecp2^{tm1.1Joez}$, $Mecp2^{tm1.1HRSF}$ with point mutations that were found in female patients (R168X, T158A, R255X, respectively). Besides, there are also the mouse models carrying point mutation related with neurodevelopmental disorder or with MeCP2 function ($Mecp2^{tm1.1Vnar}$ with the mutation A140V and $Mecp2^{tm1.1Meg}$ with the

mutation S421A). Finally, $Mecp2^{Tg}$, the mouse model with the less severe phenotype, is derived by a two-fold overexpression of human MeCP2 gene (Collins et al., 2004). Although the mouse models recapitulate several RTT clinical features, each MeCP2 mutation confers a discrete disorder profile, supporting the link between heterogeneity in MeCP2 mutations and phenotypic variability in RTT. There are a lot of studies published on the Rett syndrome phenotypes but it is difficult to compare them for the different choice on the mouse model, the background in which the mice are maintained, the environment, the techniques used, the age of the analyzed mice, the brain region considered and particularly the study of different population of neurons.

In 2011, a workshop based on the evaluation of Rett syndrome mouse models and their use for preclinical studies, revealed which were the characteristics in MeCP2 mice more or less similar to those observed in RTT patients. The workshop participants observed that in all strains of MeCP2 deficient mice there is a marked reduced brain size with alteration in neuronal morphology in terms of smaller neurons, higher neuronal density, reduced dendritic arbor and abnormal dendritic spines. In addition, they observed a network hyperexcitability in the brainstem and hippocampus, a network hypoexcitability and synaptic hypoconnectivity in the cerebral cortex, altered intrinsic neuronal electrical properties in the locus coeruleus and substantia nigra, and a dysregulation of transmitter release in cultured hippocampal neurons and chromaffin cells. However, these physiological impairments exhibit significant regional specificity: decreased excitatory synaptic drive is found in cortical circuits, whereas increased neuronal or synaptic excitability is found in the hippocampus and multiple brainstem regions involved in autonomic control. In addition, also GABA synapses are altered (Shepherd and Katz, 2011). Another robust phenotype of RTT patients reproduced in the different mouse models, is the variation in respiratory and cardiac activities with irregular pattern, episodes of apnea, increased mean frequency and prolonged QT interval. The phenotype which is more distant from the RTT individuals concerns the motor, cognitive and social behavior which vary among the different mouse model of the disorder. A reduce lifespan is markedly present in all male null mice. They showed hypoactivity, kyphosis, disheveled fur and weight loss before death (Figure 5). The phenotype observed are explain in details in the next paragraphs.

Core phenotypes in RTT individuals	Similar phenotypes in *Mecp2*-mutant mice
Morphological	
Microcephaly	++
Neuronal hypotrophy (reduced neuronal soma size, smaller dendritic arbors and lower spine density)	++
Respiratory and autonomic control	
Abnormal breathing (irregular pattern, respiratory pauses, increased mean frequency)	++
Prolonged QTc	+
Vasomotor disturbances	nd
Gastrointestinal dysmotility	nd
Cognition and behavior	
Cognitive deficits	++
Social withdrawal	+/–
Increased anxiety	+/–
Loss of speech	?
Repetitive behaviors	? (some indication of repetitive and/or excessive grooming)

++, phenotypes similar to human and reported in more than one mouse model; +, phenotypes similar to human and reported in only one mouse model to date; +/–, conflicting data in different mouse models; ?, suggestive or inconclusive data; nd, not determined. Detailed mouse phenotyping data are available in online supplementary material Tables S3-S6.

Figure 5. Comparison of phenotypes in RTT individuals and in MeCP2 mutant mice (Katz et al., 2012).

4. Rett brain and morphology of the neurons

4.1 Human brain

Rett girls present a reduced head circumference correlating with a decrease in the size of the brain. The analysis of Rett post mortem brain revealed that these small brains are correlated with increased cell-packing density, reduction of both neuronal size and dendritic arborization and global atrophy of gray and white matter (Subramaniam et al., 1997). Armostrong and colleagues performed a Golgi staining to study the morphology of the neurons in Rett patients, revealing that specific brain regions are selectively involved in the developmental arrest. In particular, they observed a reduced and simplified dendritic arborization of the pyramidal neurons of layers II and III belonging to frontal, motor and inferior temporal regions as well as in hippocampal neurons confined in layers II and IV of the subiculum (Armstrong et al., 1995, Belichenko et al., 1997, Kaufmann and Moser, 2000). The sites of "Rett neurons" correlated with the cortical localization of some of its significant motor and behavioral symptoms (Kerr, 1995). There is also a temporal nexus between the time at which

the clinical deficits are observed in the Rett girls and the time of the maturation of cortical layers III and V. The motor delay and emotional instability are observed between 1–3 years (Kerr, 1995), in the same period in which are visible the projection and functional deficits in cortical neurons. Moreover, there are some additional observations supporting the idea that there is an arrest of cortical maturation. Kaufmann and colleagues in 1995 and 1997, demonstrated that in all Rett cortical areas the protein MAP2, a marker that is normally expressed during the period of dendritic branching development and also a second protein, cyclooxygenase 2 (COX 2), which is expressed in distal dendrites during the period of dendrite/synapse pruning, were decreased in frontal and temporal regions, with preservation of the visual cortex (Kaufmann et al., 1995, Kaufmann et al., 1997). Another important aspect observed in Rett brain is the reduction of the number of spines in frontal cortex (Belichenko and Dahlstrom, 1995, Belichenko et al., 1997). In 2009, Chapleau and colleagues presented the first quantitative analysis of dendritic spine density in hippocampus of post mortem brain from Rett patients and revealed that CA1 pyramidal neurons have lower spine density than age-matched non-mentally retarded female control individuals (Chapleau et al., 2009).

4.2 Mouse models brain

In order to clarify the pathogenetic mechanism underlying Rett syndrome, several animal models have been generated. Focusing the attention on size, shape and volumes of different brain areas, Belichenko and colleagues performed a comparative study among male mutant mice belonging to the two most used mouse models: MeCP2 Bird (MeCP2B) and MeCP2 Jaenisch (MeCP2J) strains in comparison to wild-type littermates. This study revealed that both strain show markedly reduced brain weight and volume of the cortex, hippocampus and cerebellum. However, there were much more severe abnormalities in brain volume, area and shape in MeCP2B mice than in the MeCP2J strain and the onset of the symptoms and the time of death were detectable earlier in MeCP2B than in the MeCP2J mutants (Chen et al., 2001b, Guy et al., 2001, Belichenko et al., 2008, Belichenko et al., 2009). Using the MeCP2B mice, Fukuda and colleagues analyzed the thickness of the somatosensory cortex and revealed that it is significantly reduced in MeCP2 KO ($MeCP2^{-/y}$) animals at p42 (postnatal day 42) comparing to wild type (WT), due to probably the reduction of layers II-III and V. Moreover, in the same layers the neuronal density does not decrease with age like in wild type mice andthe neuronal size is significantly smaller. Through a Golgi staining, they also

demonstrated that in p42 mice the diameter of the proximal dendrite on the first branch and the diameter of the distal dendrite at 100 µm from the soma, are reduced. Finally, they observed that the number of spines along 100 µm of apical dendrites from the soma is significantly few at the same age (p42) (Fukuda et al., 2005). Similarly, using both MeCP2B and MeCP2J strains, Kishi and colleagues demonstrated the reduction of the cortical thickness along the barrel cortex in the somatosensory cortex at p56 MeCP2$^{-/y}$ mice and in particular the reduction of the layers II-III, V and VI. They also confirmed the cellular density data of Fukuda in the layers II-III and V, but also for the layers IV and VI. Finally, a Golgi staining revealed a significant reduction of the soma area in MeCP2$^{-/y}$ mice comparing to the wild type, but no significant differences in the number of spines on either primary or secondary dendrites. Sholl analysis revealed also a reduction in the dendritic arborization in MeCP2$^{-/y}$ mice (Kishi and Macklis, 2004). The morphological studies conducted by Belichenko and colleagues confirmed the previous data on dendritic structure and spine density. The studies confirmed also that MeCP2B mutants showed more dramatic deviations from normal than MeCP2J mice (Belichenko et al., 2009).

5. Cardiorespiratory phenotype

Rett patients display abnormal cardiorespiratory pattern in the late stage of the disorder, which contributes to their death. The alteration is characterized by hypoventilation, apnea, shallow breathing, bradycardia or tachycardia during awakeness and sleep. However, there is an interindividual and intraindividual differences among the patients (Rohdin et al., 2007). MeCP2 KO mice show a similar respiratory activity observed in humans, which is characterized by abnormal breathing in terms of irregular pattern, respiratory pauses, periods of tachypnea, decreased expiratory time and an increased mean breathing frequency. Cardiac phenotype is not well characterized, but it was observed that null male mice have a prolonged QT interval and a susceptibility to an increased arrhythmia and cardiac death (McCauley et al., 2011). No vasomotor disturbances was determined. These alterations were observed in null male mice at about 5-6 weeks of age and a later period for heterozygous mice at about 10 weeks of age. Several studies supported an association of abnormal cardiorespiratory with

brainstem dysfunction interconnecting modulatory disturbances and synaptic imbalance (Figure 6).

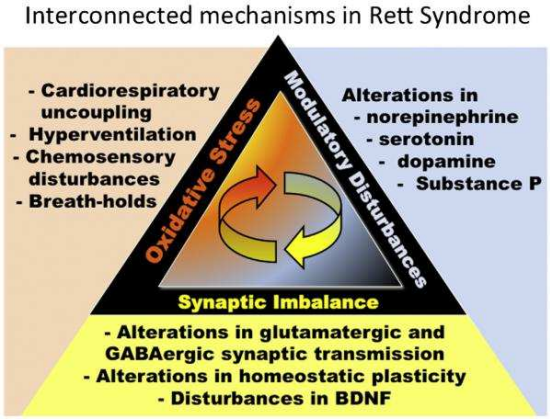


Figure 6. Interconnection mechanism in Rett syndrome. Modulatory disturbances, synaptic imbalance and cardiorespiratory abnormalities are correlated in Rett patients and in the mouse models of the disorder (Ramirez et al., 2013).

In fact, the analysis of sleep-wake rhythm and the analysis of post-mortem Rett brains revealed a reduction in the function of brainstem tissue, in particular about the modulation of serotonin (Itoh and Takashima, 2002, Paterson et al., 2005). Besides, a mouse model of the disorder show a reduction in the number of tyrosine hydroxylase (TH) neurons, norepinephrine (NE) and serotonin (5HT) content at the level of medulla and that, exogenous NE was able to stabilized the irregular respiratory network in brainstem slices derived from the same mice (Viemari et al., 2005). It was also observed that GABA impairment in the neurons of ventrolateral medulla and BDNF in the nucleus of the tractus solitaries have a critical role on the breathing irregularities (Viemari et al., 2005, Wang et al., 2006, Rohdin et al., 2007, Kline et al., 2010).

6. Motor and anxiety phenotype

Studies on Rett patients, revealed that they developed normally during the first two years of life, moreover, some of them show motor deficits during the 6th month of life and abnormalities in the communication during the first 2 years.

MeCP2 Bird strain is the model that represents the more severe aspects of the pathology (Belichenko et al., 2008). MeCP2 Bird mice developed normally until the first postnatal month, when they started to exhibit hypoactivity, stiffness and uncoordinated gait. Subsequently, they developed spontaneous tremors, hindlimb clasping and kyphosis (Ricceri et al., 2008). In the late stage of the disorder, the motor properties are characterized by an increased immobility correlated with a decreased rearing and ambulation observed in the open field test before 5 weeks of age. A reduced latency to fall off the rotarod, indicating a hindlimb coordination deficit, and a reduced grip strength were observed at 6 weeks of age (Santos et al., 2007, Panayotis et al., 2011). The elevated plus maze test revealed that Bird strain mice have less anxiety comparing to wild types at 8-12 weeks, because they spent more time in the open arms, the more anxiety zone (Kerr et al., 2012). Surprisingly, the cognitive and social behaviors are not evaluated in Bird strain mice, different to other models like the Jaenisch strain mice in which it was observed a selective cued fear conditioning deficit at about 6 weeks of age (Stearns et al., 2007), and an increased sociability.

However, cognitive, social and anxiety phenotypes are difficult to replicate by different laboratories and the results of the tests vary depending on the mouse model and the background in which the mice are maintained. It follows a difficult interpretation of the data (Katz et al., 2012).

7. MeCP2 target gene: BDNF (Brain Derivd Neurotrophic Factor)

7.1 MeCP2 target genes

MeCP2 is a modulator of the transcription of specific target genes. Several studies identified the targets of MeCP2 relevant for the pathogenesis of Rett syndrome, through a candidate approach or samples deriving from mice or human tissues. A list of these targets is presented in the table below (Figure 7).

Gene	Function	References
Bdnf	neuronal development and survival	Chen et al., Martinowich et al.
xHairy2a	neuronal repressor	Stancheva et al.
DLX5/ Dlx5	neuronal transcription factor	Horike et al.
Sgk1	hormone signaling	Nuber et al.
Fkbp5	hormone signaling	Nuber et al.
Uqcrc1	mitochondrial respiratory chain	Kriaucionis et al.
ID1-3/ Id1-3	neuronal transcription factors	Peddada et al.
FXYD1/ Fxyd1	ion channel regulator	Deng et al.
IGFBP3/ Igfbp3	hormone signaling	Itoh et al.
Crh	neuropeptide	McGill et al.
UBE3A	ubiquitin ligase	Samaco et al.
GABRB3	GABA-A receptor	Samaco et al.

Figure 7. MeCP2 target Genes. Name of the genes and their relative function are listed (Chahrour and Zoghbi, 2007).

Among the different target genes, there is the neurotrophin Brain derived Neurotrophic Factor (BDNF), identify by a candidate approach.

7.2 BDNF (Brain Derived Neurotrophic Factor)

BDNF is a member of the neurotrophin family of growth factors, which includes also the Nerve Growth Factor (NGF), Neurotrophin 3 (NT-3) and Neurotrophin 4/5 (NT-4/5). Neurotrophins (NTs) are four small secreted proteins that play important roles in the development of the nervous system in vertebrates (Chao et al., 2006). NTs bind with specific affinities to transmembrane-receptors belonging to a small family of tropomyosin-related tyrosine kinases (Trk): TrkA (NGF), TrkB (BDNF and NT-4), and TrkC (NT-3) (Huang and Reichardt, 2003). Trks are receptor tyrosine kinases that dimerize upon ligand binding which results in their activation and subsequent initiation of several signal transduction cascades. In addition, the pro-NTs form can also bind to the p75 neurotrophin receptor (p75NTR) with an equal affinity, mediating apoptosis or cell cycle arrest. The possibility to bind one or the other receptors, allow a good control of the development and function of the neuronal cells.

7.3 BDNF: from the gene to the protein

The gene structure of BDNF is quite complex and is similar in human (Liu et al., 2005) and rodent (Liu et al., 2006, Aid et al., 2007). In rodents, BDNF is composed by nine exons (I-IX) with the coding region in exon IX and eight alternatively spliced 5' untranslated regions

(5'UTR), each preceded by a specific promoter leading to transcription of eleven different BDNF isoforms. All these isoforms contain two polyadenylation sites that can generate a short or a long 3'UTR variant for each transcript. In response to electrical stimuli, BDNF mRNA variants are produced and transported from the soma to the dendrites (Tongiorgi et al., 1997, Jakawich et al., 2010). However, it was shown that BDNF mRNA isoforms are distributed differently in neurons: exon I and IV are restricted at the level of the soma and proximal dendrites, while exon II, and VI are localized into distal dendrites. The different subcellular localization of BDNF mRNA variants represents a specific "spatial code" to direct the synthesis of the a single identical protein in different compartments causing localized effects (Chiaruttini et al., 2008, Tongiorgi, 2008, Chiaruttini et al., 2009, Baj et al., 2011, Autry and Monteggia, 2012, Baj et al., 2013) (Figure 8).

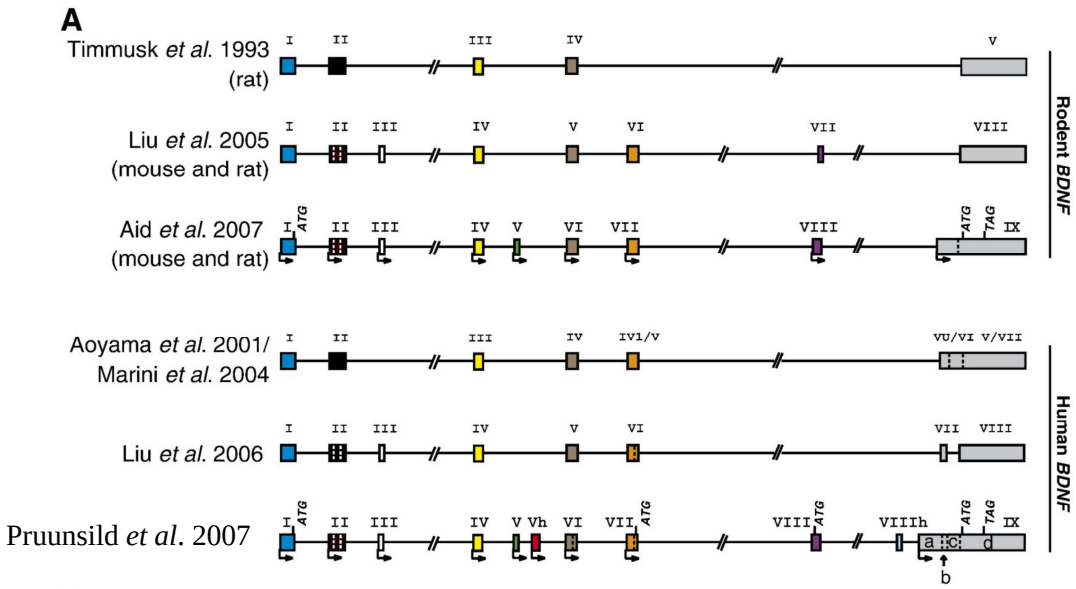


Figure 8. BDNF gene. (A) BDNF gene structure in humans and in rodents individuated during the past, the first version of Timmusk (Timmusk et al., 1993), Liu version (Liu et al., 2005, Liu et al., 2006), Aoyama and Marini version (Aoyama et al., 2001, Marini et al., 2004), the version of Aid (Aid et al., 2007) and finally the version of Pruunsild (Pruunsild et al., 2007). Homologous exons are highlighted with same colors.

BDNF is synthesized as a precursor protein pro-BDNF of 32kDa, which can then be further cleaved into a truncated form of 28kDa which role is unknown, or a mature form of 14kDa (Lessmann et al., 2003). Pro-BDNF functions as a negative regulator when it binds to p75 receptor, mediating apoptosis, inhibiting dendritic complexes, inducing long term depression. On the other hand, mature BDNF has positive effects like neuronal survival, induction of long

term potentiation through the binding with TrkB receptor. When BDNF binds the TrkB, it induces the dimerization and autophosphorilation of the receptor which activates three different signaling pathways involving the mitogen-activated protein kinase (MAPK), the phosphatidyl-inositol 3-kinase (PI3K) and the phospholipase C (PLC) (Li and Pozzo-Miller, 2013). Each of these pathways confers different BDNF actions: MAPK pathway promotes neuronal differentiation, PI3K pathway is involved into the regulation of transcription promoting survival and growth of neurons, while PLC pathway is upstream of ion channel effects in synapses promoting synaptic plasticity (Mattson and Wan, 2008, Yoshii and Constantine-Paton, 2010, Dwivedi, 2012). For this reason, BDNF has different roles in neuronal cells. During the development of nervous system it contributes to dendritic and axonal growth (Yoshii and Constantine-Paton, 2010), then in mature neurons it is involved in neuronal homeostasis, cell survival and death and synaptic plasticity (spine formation and maturation, long terminal potentiation and long terminal depression) (Poo, 2001). BDNF is also crucial for learning and memory processes (Yamada et al., 2002, Lu et al., 2008).

BDNF mRNA and protein are expressed during the development and they reach the highest level by days 10 to 14 postnatally and decrease thereafter. BDNF is widely expressed in the central nervous system, especially in cerebral cortex, hippocampus and amigdala (Hofer et al., 1990, Wetmore et al., 1990). In conclusion a simple alteration in mRNA or protein of BDNF compromised neuronal functions.

7.4 BDNF in Rett syndrome

BDNF was identified as a target of MeCP2 through a candidate approach (Chen et al., 2003, Martinowich et al., 2003). In 2007, Ogier and colleagues demonstrated through a quantitative real time PCR, that BDNF expression in MeCP2 KO mice is reduced comparing to wild type (WT) mice. In particular, total BDNF (exon VIII) and the transcripts containing the exons II, IV and V were markedly decreased (nomenclature of Liu et al., 2006); on the other hand, there is no a significant difference for the transcript containing the exon I. The examination of BDNF expression was performed in cultures of nodose cranial sensory ganglia neurons, which control the cardiorespiratory activity, derived from Jaenisch mice (Ogier et al., 2007).
In the previous year, Wang and colleagues analyzed the level of BDNF protein (through an ELISA assay) in different tissues like nodose ganglia, brainstem, hippocampus and cortex at different ages of WT and MeCP2 null mice (Jaenisch strain). They observed that at p0 there

was no significant differences in BDNF expression level in MeCP2 null mice tissues comparing to the WT; however at p35, when the animals started to present the typical phenotype of Rett syndrome, the neurotrophin protein level was significantly reduced in nodose ganglia and brainstem. No difference was observed for the cortex and hippocampus (Wang et al., 2006).

In the same year, another laboratory used an ELISA assay to evaluate the level of BDNF protein in WT and MeCP2 KO mice (Jaenisch strain) at 2 or 6-8 weeks of age. At p14, no difference was detectable comparing the WT and MECP2 KO mice; however, p42-56 MeCP2 KO brains had less BDNF protein. In particular, a significant reduction was observed in cortex, cerebellum and the rest of the brain (Chang et al., 2006).

7.5 Mechanism of MeCP2 modulation of BDNF expression

A recent study demonstrated that MeCP2 binds BDNF at methylated CpG sites (Klose et al., 2005), modulating its expression. There are some hypotheses on this mechanism, which lead to develop three models: a repressor model, an activator model and a dual operation model (Figure 9).

In neurons that are not stimulated, MeCP2 in its un-phosphorylated form, is linked to BDNF promoter IV, blocking its transcription. Also CREB (cAMP response element binding protein), a well-known activator of BDNF transcription, is in its un-phosphorylated and so inactive form (repressor model). However, after membrane depolarization, MeCP2 is phosphorylated so the promoter of BDNF is free to be translated (Chen et al., 2003). The stimulation of neurons induces MeCP2 phosphorylation on Ser421 (Zhou et al., 2006), and the phosphorylation of CREB (Chen et al., 2003). All these observations were derived from primary neuronal cultures. In hippocampus of MeCP2 KO mice, double point mutations in S421A and S424A of MeCP2 gene, led to high level of expression of BDNF (Zhou et al., 2006). Similarly the mutation T158A reduced the association of MeCP2 with BDNF promoter IV and so its transcription (Goffin et al., 2012) (activator model).

It was also supported that neuronal depolarization is fundamental to reduce the number of methyl groups in the CpG dinucleotide of the BDNF promoter IV (Martinowich et al., 2003). In fact, MeCP2 KO mice studies showed how some regulatory elements which are involved in the methylation or in the demethylation of DNA, are important for the activation or repression of BDNF transcription: for example the lack of DNA methyltransferases (DNMTs)

in MeCP2 KO mice induce an increase of BDNF expression level while the lack of DNA demethylases induce a normal level of methylation of BDNF under basal condition, but it induce a significant decrease when the animals are subjected by electroconvulsive treatment (Martinowich et al., 2003, Ma et al., 2009). The association and dissociation of histone deacetylases (HDACs) and the counterpart histone acetyltransferases (HATs) are also important for the activation or the inhibition of transcription (Martinowich et al., 2003, Zeng et al., 2011), because they contribute to condensed or relaxed form of chromatin.

In addition, it was not observed a direct interaction between MeCP2 and BDNF, suggesting a possible action by miRNA132, which is responsible of the block of MeCP2 translation and consequently the repression of BDNF transcription (Klein et al., 2007). Other miRNAs which react with the 3'UTR of BDNF, are found to have a negative effect on its transcription in the cerebellum of MeCP2 KO mice (Wu et al., 2010). In conclusion, two factors contribute to influence BDNF transcription: neuronal activity that induces the transcription machinery, and the age of the MeCP2 KO mice. In fact presymptomatic mice show normal BDNF levels (Chang et al., 2006), while some evidences demonstrated that symptomatic MeCP2 KO mice have a reduction or not significant decrease of BDNF expression level (McGraw et al., 2011).

Furthermore, the levels of the different isoforms of BDNF often do not reflect the BDNF protein level. For example MeCP2 KO mice shown BDNF exon IV upregulated but BDNF exon II downregulated and probably it is due to the action of the gene repressor REST (RE1 silencing transcription factor) and its corepressor CoREST which bind the BDNF promoter IV (Abuhatzira et al., 2007). Similarly Ogier and colleagues in 2007, showed how BDNF promoter I was upregulated in MeCP2 KO mice but all the others isoforms were reduced, resulting in low level of BDNF protein. The contrasting roles of MeCP2 suggest a dual control of BDNF, switching from the activation to repression and viceversa. This was confirmed in the modulation of expression of the gene EGR2 (early growth response factor-2): in fact the phosphorylation and dephosphorilation of Ser80 of MeCP2 control the repression of EGR2 transcription in SHSY5Y neuroblastoma cells and its activation after cell differentiation. A similar mechanism was observed for the gene RET (receptor tyrosine kinase), but involving phosphorylation in Ser229 (Gonzales et al., 2012). However, the mechanism of phosphorylation that occurs in MeCP2 does not change its association with the target genes, rather it was hypothesized a mechanism through which it recruits different regulatory complexes to regulated gene expression. In fact, the phosphorylation of Ser80 in MeCP2 is known to associate with Sin3a and the RNA binding protein YB-1, while phospho-

Ser229 MeCP2 with Sin3a, HP1 (heterochromatin protein 1) and SMC3, a component of the cohesion complex; however, the function of these associations is not clear yet.

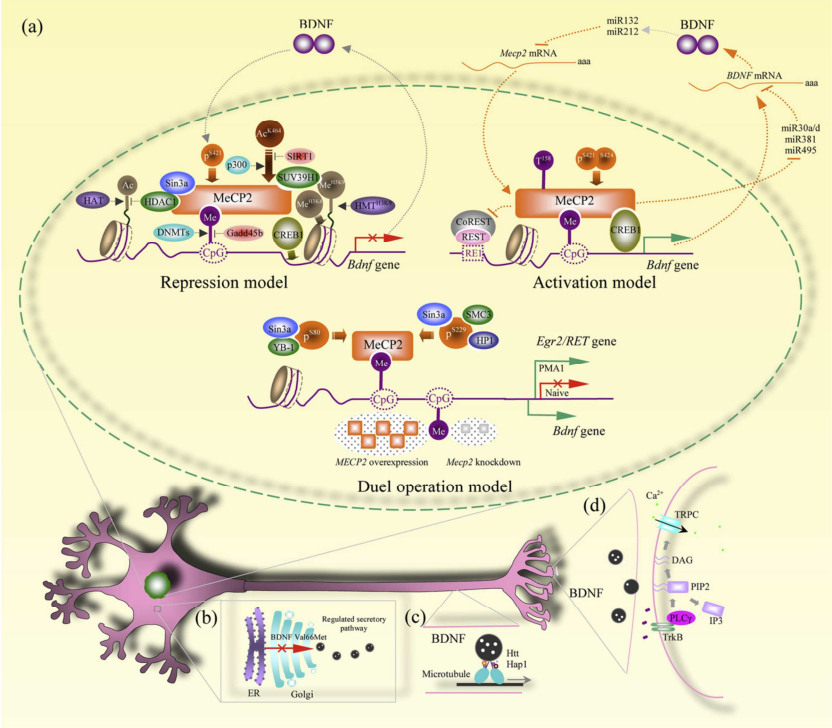


Figure 9. **Three models depict how MeCP2 regulate BDNF transcription.** BDNF deregulation in multiple regions of a neuron.

8. Molecular rescue of RTT phenotype

8.1 MeCP2 reactivation and overexpression

MeCP2 is an important factor involved in neuronal development and maintenance of the neurons (Palmer et al., 2008, Degano et al., 2009), and the absence of this protein leads to Rett syndrome. Therefore, it is evident that the reintroduction of MeCP2 could reverse the Rett phenotype (Gadalla et al., 2011). In this regard, Luikenhuis and colleagues placed MeCP2 cDNA under the control of endogenous promoter of the microtubule-binding protein, Tau. Tau-MeCP2 fusion protein was expressed at a level 2- to 4-fold higher than endogenous MeCP2, thus they demonstrated that a modest overexpression of the transgene in the MeCP2 null mice (Jaenisch strain) rescue the Rett phenotype in terms of lifespan, physical development and brain weight, however, a severe overexpression of the transgene induced

motor dysfunction in wild type and MeCP2 null mice (Luikenhuis et al., 2004). This study highlight the importance of the presence of MeCP2 protein in a just expression level. In another study, Giacometti and colleagues generated a number of Cre mice lines which expressed MeCP2 in specific brain region and at a different developmental time points (from 2 to 4 weeks of age). When the transgene was activated early they observed a prolongation of the lifespan, a delay of motor deficits and an improvement of motor activities. It also restored the normal body and brain weight and neuronal size (Giacometti et al., 2007). Another evidence by Guy and colleagues, showed how MeCP2 was important for the nervous system to ameliorate the Rett phenotype. In fact, they created a mouse model in which the endogenous MeCP2 was silenced, but it can be conditionally activated following tamoxifen injection. In this way, they can lead the expression of MeCP2 at different ages. They showed that MeCP2 null male mice treated with tamoxifen in the presymptomatic and postsymptomatic stages reversed RTT phenotype and enhanced the survival (Guy et al., 2007). In 2009, Larimore and colleagues confirmed that pyramidal neurons transfected with a plasmid encoding a small hairpin RNA (shRNA) to knockdown endogenous MeCP2 had shorter dendrites than control untransfected neurons, without detectable changes in axonal morphology. On the other hand, overexpression of wild type human MeCP2 increased dendritic branching, in addition to axonal branching and length. Consistent with reduced neuronal growth and complexity in Rett brains, overexpression of human MeCP2 carrying missense mutations common in Rett individuals (R106W or T158M) reduced dendritic and axonal length (Larimore et al., 2009).

Different strategies were developed at the level of the gene to ameliorate Rett phenotype: the reactivation of normal allele and the gene therapy (Gadalla et al., 2011). Concerning the first one, some studies tried to induce the reactivation of the inactivated X-chromosome to allow the expression of normal allele of MeCP2, using 5-azacytidine that reduced genomic methylation in cell culture models (Mohandas et al., 1981). However, the principle problem was the possibility of the reactivation of the entire X-chromosome and not only the normal allele of MeCP2. Actually, there are no ways to induce a target activation and also specifically for a type of cells. The other approach at the level of the gene is gene therapy through the correct gene is introduced in the cells to treat the pathology. However, this technique have some challenges like the delivery and the dosage of the level of the gene.

8.2 BDNF overexpression

Different studies suggest that BDNF expression levels change in Rett brain, therefore probably there is a link between the loss of function of MeCP2 and the alteration of BDNF levels. On the basis of this theory in 2006, Chang and colleagues increased the neurotrophin levels in MeCP2 KO mice (Jaenisch strain) using a conditional BDNF transgene. Several aspects of the MeCP2 mutant phenotype were ameliorated like the locomotor function improving the possibility to access to food and water, which may, at least in part, explain the significant extension of lifespan. In addition, the overexpression of BDNF partially rescue the brain weight and the activity of the neurons, in fact it increased spontaneous firing of layer V pyramidal neurons (Chang et al., 2006). In 2009, Larimore and colleagues demonstrated that the BDNF scavenger TrkB-Fc prevented the increase in dendritic branching caused by wild type human MeCP2 overexpression, while overexpression of the BDNF gene reverted the dendritic atrophy caused by MeCP2 knockdown. These results demonstrate that BDNF is able to revert the dendritic atrophy caused by Rett-associated MeCP2 mutations (Larimore et al., 2009).

9. Pharmacological rescue of Rett phenotype

An alternative approach to rescue the Rett phenotype is to act on the factors that are downstream of MeCP2 function with a pharmacological treatment. These factors included neurotrophins (like BDNF) and neurotransmitters (like noradrenaline, serotonin and dopamine).

In 2007, Ogier and colleagues tested the CX546, a modulator of AMPA receptor and a known enhancer of BDNF levels in MeCP2 KO mice. They focused on respiratory dysfunctions and observed that the treatment with CX546 ameliorated breathing abnormalities. They concluded that respiratory deficits could be rescued by probably the increase of BDNF, due to the modulation of AMPA receptors (Ogier et al., 2007). In 2009, Tropea and colleagues used another factor to induce an increase of BDNF levels, an active peptide of IGF1 (Insulin-like Growth Factor 1). This factor extended the lifespan of the MeCP2 KO mice, improved locomotor function, ameliorated breathing patterns, and reduced irregularity in heart rate. In

addition, treatment with IGF-1 peptide increased the brain weight of the mutant mice (Tropea et al., 2009).

In addition to alteration in BDNF expression, a deficit on monoamine levels, including noradrenaline (NE), serotonin (5HT) and dopamine (DA) was reported in the brain and cerebrospinal fluid of both Rett patients and MeCP2 KO mice (Santos et al., 2010). The alteration in monoamine systems, in particular for NE and 5HT, was related to prefrontal and the motor cortices deficits in mice at p21. However, the alterations in monoamines was extended in mice at p56 to hippocampus and cerebellum, who have probably a role in the progression of the disorder. The reduction of monoamines could be the responsible of the motor deficits and breathing abnormalities observed in Rett syndrome (Santos et al., 2010).

The alteration of monoamines levels was characteristic of major depressed patients, in which also the BDNF was reduced in their serum; an antidepressant (ADs) treatment in these patients increased serum BDNF levels up to the level found in healthy controls (Karege et al., 2002, Shimizu et al., 2003b, Gervasoni et al., 2005, Gonul et al., 2005). Furthermore, in postmortem human brain tissues an increase in hippocampal BDNF immunoreactivity has been described in subjects treated with antidepressants compared to untreated subjects (Chen et al., 2001a). A parallel situation has been shown in rodents. Local infusion of BDNF into the midbrain and hippocampus caused antidepressant-like activity in two behavioral models of depression: the forced swimming and the learned helplessness (Shirayama et al., 2002, Hoshaw et al., 2005). The above findings support the idea that increasing the expression of endogenous BDNF may have an antidepressant effect. Since noradrenergic and serotonergic blockade inhibited BDNF mRNA upregulation following exercise and antidepressant (Ivy et al., 2003), it was concluded that BDNF may exert an antidepressant effect partly by regulating the noradrenergic and/or serotonergic systems. Moreover, the loss of forebrain BDNF (in BDNF knockout mice) attenuated the action of the antidepressant in behavioral tests (Monteggia et al., 2004). All these findings focused the attention on a role of BDNF in depression and/or in the mechanism of action of ADs as one of their main targets.

In 2003, Coppell and colleagues evaluated the effect of some antidepressants on BDNF mRNA in rats. They observed that the ADs, which enhanced serotonergic neurotransmission (Fluoxetine, Paroxetine sertraline or Tranylcypromine), excluding Desipramine, Maprotyline (two noradrenaline re-uptake inhibitors) or Mianserin (atypical antidepressant), induced a bi-phasic change in BDNF mRNA levels after repeated treatment (twice daily for 14 days): they observed a decrease at 4 hours and an increase at 24 hours after the last injection (Coppell et

al., 2003). Unlike to what was previously believed, the effect of ADs on BDNF gene expression may be more complex and less widespread across treatments. In contrast to what was found by Coppell, some authors reported a significant increase caused by the specific reuptake inhibitor NE (Desipramine) on BDNF expression in the hippocampus (Nibuya et al., 1995, Dias et al., 2003, Jacobsen and Mork, 2004). In marked contrast to the findings that Desipramine increases BDNF mRNA expression, was the observation that denervation of NE axons increased BDNF mRNA expression in the hippocampus (Hutter et al., 1996), which suggested that NE tonically inhibited BDNF mRNA expression. Thus, the noradrenergic regulation of BDNF mRNA and protein expression seems to be a complex phenomenon and may explain the discrepancies between studies. In 2007, Roux and colleagues tested in MeCP2 KO mice Desipramine, the antidepressant which block the reuptake of NE. This study revealed that Desipramine ameliorated respiratory dysfunction, in particular reduced the apneas and expanded the lifespan of the animals (Roux et al., 2007). However, when it was introduced in a clinical trial for Rett syndrome, it induced some cardiac dysfunctions with a premature heart failure. In the same way, Voituron and Hilaire tested the benzodiazepine Midazolam, which modulate $GABA_A$ receptor subtype, since in Rett mice the dysfunction of GABA signaling is associated with autistic like behaviors, motor and respiratory dysfunction. They found that a pretreatment with Midazolam reduced the number of apneas, albeit transiently (Voituron and Hilaire, 2011). Another study, demonstrated that increasing GABA levels, using a reuptake blocker, improved the respiratory phenotype, reducing apneas, and prolonged also the survival of the animals (Abdala et al., 2010).

In 2005, Rogóz and collaborators investigated the influence of repeated treatment (twice daily for 14 days) with the antidepressant Mirtazapine on BDNF mRNA level in rat hippocampus and cerebral cortex. Mirtazapine (Org 3770, Remeron) (1,2,3,4,10,14b-hexa-hydro-2-methylpyrazinol [2,1-alpyridol[2,3-c][2]benzazapine), enhances noradrenergic and serotonergic 5-HT1A neurotransmission via an antagonistic action at central α2-adrenergic autoreceptors and heteroreceptors and the blockade of 5-HT2 and 5-HT3 receptors (de Boer, 1995, de Boer et al., 1996, Holm and Markham, 1999). In contrast to imipramine or other tricyclic ADs, this drug does not inhibit NE or 5-HT reuptake (de Boer et al., 1988). Rogóz collected the data 24 hours after the last oral administration and observed that repeated treatment with Mirtazapine 10 mg/Kg significantly elevated BDNF mRNA levels compared to the vehicle treated control in both rat hippocampus (26.5%) and cerebral cortex (29.9%). Remarkably, the up-regulation of CREB is a common effect of repeated AD treatment

(Nibuya et al., 1995) that may lead to regulation of specific target genes such as BDNF and TrkB, and to a long-term impact of these treatments on brain functions. These finding provide a strong evidence that the increased expression of BDNF is a downstream effect of increased 5-HT/NA neurotransmission, and that it may be partially responsible for the therapeutic effect of ADs that stimulate these systems (Rogoz et al., 2005).

AIM OF THE PROJECT: A CURE FOR THE RETT SYNDROME

Currently, there is no cure for Rett syndrome but since 2004 at least three clinical trials have been carried: Dextromethorphan, which acts by blocking NMDA/glutamate receptors to reduce the toxic excess of glutamate, that was observed in Rett patients, Desipramine, and IGF-1 treatment. However, these clinical trials are still ongoing and as it was said above, some of them have important side effects.

Rett syndrome is characterized by alterations in neuronal morphology, cardiorespiratory activities, motor and anxiety behavior which were reproduced in different mouse models. These clinical relevancies were associated to a reduction of monoamines and of some modulators of synaptic transmission like BDNF and GABA.

Since antidepressants were described to increase monoamines and BDNF or GABA levels, we hypothesized that they could be used to rescue the Rett phenotype.

Therefore, the aim of this project is to investigate the effects of a chronic antidepressant treatment on a mouse model of the Rett syndrome.

We selected two antidepressant drugs: Desipramine as control drug previously used for a clinical trial of Rett syndrome, and Mirtazapine. Mirtazapine is an antagonist of central α2 autoreceptors and α2 heteroreceptors and a specific blocker of 5HT2 and 5HT3 receptors, which are the main responsible of antidepressants side effects. Mirtazapine is one of the 12 antidepressants with an higher tolerability and it is approved by Food and Drug administration for severe depression. Mirtazapine has very little side effect (little orthostatic hypotension) and an overdose of this drug induces an evident sedative effect.

We selected the $MeCP2^{-/y}$ Bird strain, which represents the more severe phenotype of Rett syndrome and we treated male null mice with the antidepressants. These mice present a marked reduced brain and body weight.

We evaluated the effects of the antidepressant treatments on:

1. Brain weight and morphology of the layer II-III pyramidal neurons
2. Neuronal function: GABA currents
3. Cardiorespiratory phenotype, motor and anxiety behavior
4. mRNA and protein BDNF expression level

MATERIALS AND METHODS

Mice. All animal experimental protocols were approved by the Italian Ministero della Sanità (D.L.vo 116/92). Wild-type (WT) C57/BL6 mice were purchased from Charles River Laboratories (Calco, LC, Italy). Female MeCP2 heterozygous mice (Guy et al., 2001) were purchased from Jackson Laboratories, Bar Harbor, Maine (strain name: B6.129P2(C)-Mecp2tm1.1Bird/J, stock number: 003890), and they were crossed with Wild Type C57/BL6 to obtain MeCP2 KO mice. In order to overcome the poor breeding performance of these mice and improve pups survival rate, we used a cross-fostering approach in which mice offspring were raised by FVB foster mothers (from Harlan Laboratories, Udine, Italy).

B6.129P2(C)-Mecp2tm1.1Bird/J mice genotyping. The genotypes of mice were identified by PCR on tail genomic DNA. The samples were incubated with 300 µl of Extraction Buffer (Tris-HCl 10 mM pH 8.0, SDS 0,5%, EDTA 0,1 M pH 8.0, RNAse 0,2%) at 37°C for 1 hour, followed by an incubation with Proteinase K (30 µg) at 50°C overnight. Then, 300 µl of Phenol-Chloroform:Isoamyl alcohol (25:24:1) at pH 8.0 were added to the samples and they were mix for 20 minutes; samples were centrifuged at 12,000 rpm for 10 minutes and the upper aqueous phase, containing the genomic DNA, was recovered and diluted with two volumes of ethanol 100%. Samples were stored at -80°C for 30 minutes to enable DNA precipitation. Samples were centrifuged at 4°C for 10 minutes at 12,000 rpm and the DNA pellet was suspended in 30 µl of DNAse-free water. The genotypes were assessed by PCR preparing two different mixes with a specific reverse primer to either amplify the mutant or the wild type (WT) form of MeCP2 gene (forward common primer oIMR1436 5'- GGT AAA GAC CCA TGT GAC CC -3', reverse mutant primer oIMR1437 5'- TCC ACC TAG CCT GCC TGT AC -3', reverse wild type primer oIMR1438 5'- GGC TTG CCA CAT GAC AA -3'). The PCR reactions were performed with 1U GoTaq Polymerase (Promega Corporation), 1X Green GoTaq Buffer, 0,2 mM dNTPs each, 2,5 mM $MgCl_2$, 0,5 µM of each primer and 100 ng of genomic DNA. The PCR conditions were set as follows: 5 minutes at 95°C, 30 cycles of 45 seconds at 95°C, 50 seconds at 57,5°C, 50 seconds at 72°C, followed by 10 minutes at 72°C. The PCR generates a 400-bp product for WT genomic DNA, and 400 and 416-bp products for heterozygous mice ($MeCP2^{-/+}$), and a 416-bp product for hemizygous mice ($MeCP2^{-/y}$).

Mice treatment. Beginning from p28, wild type (WT) males and MeCP2$^{-/y}$ (KO) littermates were treated for 14 days, between 10-11 a.m., with Vehicle (Vehic = 1% aqueous solution of Tween80, Sigma-Aldrich), Desipramine 10 mg/Kg (DMI10; Vinci-Biochem), Mirtazapine 10 or 50 mg/Kg (MIR10/50, Abcam) throughout an intraperitoneal injection. Each group was randomized on the basis of weight.

Nissl staining. Wild Type (WT) and MeCP2$^{-/y}$ mice untreated (UNT) and treated with the vehicle or the drugs (Desipramine and Mirtazapine) were euthanized at p42. Brains were removed and were freshly frozen in isopentan at -80°C (n = 3-8 mice for each genotype). Coronal sections at the level of the primary somatosensory barrel cortex S1 (approximately from bregma 1,32 mm to -1,64 mm) were cut with a cryostat (Leica) at a thickness of 20 µm and mounted on 26x76 mm gelatin treated microscope slides. Sections were post-fixed in PBS/PFA 4% for 30 minutes at 4°C, washed 3 times in PBS and pre-incubated for 30 minutes in a solution containing 0,2% gelatin, 0,2% Tween20 in PBS. Nissl staining was performed with cresyl violet (0,2% cresyl violet, 0,5% acetic acid, 0,01 M sodium acetate in sterile water) for 20 minutes at 37°C. Sections were dehydrated with rapid washes in ethanol 70%, 95%, 100%, methanol, methanol-xylene (50:50), xylene and mounted with Eukitt (Sigma Aldrich).

Human brain samples from the cortex of 3 donors and 3 Rett patients, including Brodmann Areas from 1 to 5, which represented the primary somatosensory cortex, the primary motor cortex and the somatosensory association cortex, were processed as above.

Brain sections pictures were acquired by a Nikon AMX1200 digital camera on a Nikon E800 Microscope (10X for mice samples, 10X – 40X magnification for human samples).

Total cortical thickness and layers thickness. ImageJ program was used for quantitative imaging of the thickness of the cortex in brain sections. The cortical thickness of p42 WT and MeCP2$^{-/y}$ untreated mice were measured on three adjacent sections, every 400µm, from the most rostral position of the barrel cortex that was visually determined on the basis of the peculiar barrel appearance after Nissl staining (n = 4-8). p42 WT and KO mice untreated and treated with Vehic, DMI10, MIR10 and MIR50 were measured in the first 400 µm of the barrel cortex (n = 6 mice for each group). Mouse brain sections pictures were acquired by a Nikon AMX1200 digital camera on a Nikon E800 Microscope (10X magnification). The

thickness of layers from I to VI of the cortex was measured at the middle of the barrel cortex, on three adjacent sections. The ImageJ program was used to determine the gray level profile along a line (width = 300) spanning the barrel cortex perpendicularly from the pial surface to the white matter underlying layer VI.

Hippocampal measurements. The same brain sections labeled with Nissl were used for the hippocampal measurements with ImageJ program. The thickness of hippocampus and its cellular layers (*stratum pyramidalis, stratum radiatum, stratum lacunosum moleculare, stratum molecularis, stratum granularis*) were measured as above and the percentage of each layer compared to the total thickness (100%) was calculated (n = 3-4 for each group).

Golgi staining. We used a modified protocol inspired from Ranjan and Mallick (Ranjan and Mallick, 2010). Brains (n = 3 mice for each group) were divided into the two emispheres along the midline and after a wash in PBS, they were immersed in the Golgi solution containing 5% of Potassium Dichromate, 5% of Mercuric Chloride and 5% of Potassium Chromate, in the dark for 27 hours at 37°C. The brain was washed in distillated water and cut with a vibratome (Campden Instruments, MA752 motorised advance vibroslice) in 200 µm coronal sections. The sections were treated as described by Ranjan and Mallick. Finally, the sections were dehydrated in 70%, 80%, 95% ethanol, methanol, methanol-xylene (50:50), xylene and mounted in Eukitt on gelatinized slides. The slides were allowed to dry at room temperature and were observed under the microscope Nikon E800. Ten neurons for each animal were analyzed and a series of stack images every 1 µm were collected at 60X magnification. The stacks were compressed with ImageJ program to permit the measure of soma area, diameter of apical dendrite, number and type of spines. The soma area was analyzed through a polygonal selection of the soma including the hillock. At ten or a hundred microns from the soma were measured the diameter of the apical dendrite. For each neuron, two segment of 50 µm of secondary basal dendrites and two segment of 50 µm of primary and secondary apical dendrites were collected to evaluate the number of spines. In the same segments of secondary basal and apical dendrites, all the spines were divided on the basis of the category: stubby, mushroom and thin according to Harris and colleagues (Harris et al., 1992).

Sholl analysis. Images of neurons (n = 10 for each mouse; n = 3 mice for each group) were collected at 20X magnification with Nikon E800 microscope. A series of stacks every 2 µm were used to obtain a bidimensional image to analyze with Neurostudio program. The Sholl analysis was performed separately for the basal and apical dendrites with concentric circles of 10 µm.

Measurement of GABA currents. Membranes from WT (n = 4), MeCP2$^{-/y}$ untreated (n = 3) and KO MIR50-treated (n = 3) mice cortices were prepared and injected into *Xenopus* oocytes by using the procedures described by Miledi and colleagues (Miledi et al., 2002). Each membrane preparation was obtained from one animal and injected separately. Xenopus *laevis* follicles were dissected from segments of ovary, defolliculated with collagenase (0,5 mg/ml, 35 minutes, Type I, Sigma, St Louis, MO, USA) and maintained at 16°C in Barth's solution (containing 0,5 mg/ml gentamicin, Sigma). The next day, the membrane preparations were injected into the animal pole of the oocyte in close proximity to the equatorial band, at a fixed protein concentration of 1 mg/ml (50nl) (Miledi et al., 2006). One to four days after injection, GABA-currents were recorded from voltage-clamped oocytes, using two microelectrodes filled with 3M KCl (Miledi et al., 1982). The oocytes were superfused continuously at room temperature with Ringer solution (115 mM NaCl, 2 mM KCl, 1,8 mM $CaCl_2$, 5 mM Hepes, adjusted to pH 7 with NaOH) in a purpose-designed recording chamber (RC-3Z, Warner Instruments, Hamden, CT, USA). Data acquisition and analyses were performed using WinWCP version 3.5 Strathclyde Electrophysiology software (kindly provided by John Dempster, Glasgow, UK). GABA (1 mM) was applied using a constant perfusion system (5–10 ml/min, VC-8 perfusion system, Warner Instruments). To reduce the variability, the GABA current amplitude of each membrane preparation was normalized among oocytes injected with membrane of KO UNT or KO MIR50 cortices and those recorded in WT injected-oocytes of the same batches at the same day post injection. For dose/current–response curves, the GABA was repeatedly applied at 5 minutes intervals, and the half-dissociation constants (EC_{50}) and Hill coefficients (n_H) were estimated by fitting the data to Hill equations. The oocyte membrane potential was held at - 80 mV.

Quantification of protein concentration. Protein concentrations were quantified by Quant-iT Protein Assay kit (Invitrogen) according to manufacturer's instructions. One microliter of each sample was added to 199 µl of Working Solution and measured with Qbit Fluorometer.

Statistical analysis for morphological analysis. The Student's t-test was used for two-sample comparisons. For multiple sample comparisons, One Way ANOVA was performed, followed by Tukey test and Dunn's method post-tests using the SigmaStat software.

Vital parameters with MouseOX Plus instrument. MouseOX is a non-invasive instrument which measures vital signs of awake mice. We analyzed WT and KO untreated animals (n = 14-10 respectively) and KO mice treated with Desipramine 10 mg/Kg (n = 9) or Mirtazapine 50 mg/Kg (n = 10). Before the experiment, a small collar clip is placed in the neck of the mouse for 30 minutes to reduce the stress due to the collar; after that, the collar is replaced by a new small collar which has an infrared light sensor. This collar is connected to the MouseOX instrument and a computer through a cable that is enough long to allow the movements of the animal. The vital signs are measured for at least 20 minutes by the MouseOX and the computer develops a profiles for each of these parameters: the Oxygen Saturation (O2 sat) which corresponds to the percentage of sites of arterial hemoglobin occupied by oxygen molecules, (an indirect measure of the quantity of oxygen that arrives on tissues), the Hearth Rate (HR) that is the number of beats per minute (it changes on the body necessary to absorbed O_2 and expel CO_2, for example during a physical exercise, sleep or illness), the Breath Rate (BR) that is the number of breaths per minute (the brain controls the respiratory activity and commands to the body when breath on the basis of the quantity of O_2 and CO_2 presents in the blood) and the Pulse Distention (PD) that corresponds to the change in distension of the arterial blood vessels due to a cardiac pulse (a direct measurement of changes in local blood volume; it measures the blood flux from the head to the hearth and vice versa). The profiles obtained were converted in numerical raw data and were analyzed. We decided to analyzed only ten minutes of the data collected, this because we eliminated the first minutes of the test, in which the animal has to get used to the collar with the cable and the last minutes when the animal seems to be tired. A plot profile was performed by the binning of the data every 1 minute to evaluate the general course of the parameter; the histogram was obtained by taking the mean of the averages of each individual mouse within the same group and the plot was performed putting together the averages of single mouse of the same group. One way ANOVA was performed for the oxygen saturation parameter (normal data) and ANOVA on ranks with Dunn's test was performed for the other parameters.

Open Field Test. The open field test was performed to evaluate the exploratory drive and the anxiety of the following groups of p40 mice: WT and KO untreated (n = 10), KO treated with Desipramine 10 mg/Kg (n = 10) or Mirtazapine 50 mg/Kg (n = 10). Before the test, the animals were acclimated for 30 minutes. The animal was placed at the center of the open arena and it is free to move for 20 minutes. The test was performed from 10 to 12 a.m. After the test, the arena was clean with ethanol 70% to eliminate any smell of mouse and avoid influencing the performance of the following mouse. The data were analyzed with Any-maze program (Ugo Basile Instruments): the arena was divided into three zones: a central zone, the most anxiety zone (16% of total area), a border zone, the less anxiety zone (8 cm wide) and a middle zone (the rest). Distance travelled, time of immobility, entries and time in the zones, number of freezing episodes, time of freezing, number of rearing (the animal was standing on his hind legs), grooming (the animal took care of itself) and hopping behavior (the animal seemed crazy and jumped on the wall with a very high frequency without control) were analyzed.

Elevated Plus Maze Test. The elevated plus maze was performed to test the anxiety of the animals when they were placed on a cross to a height of 40 centimeters from the floor. The cross has two open arms (the more anxious) and two closed arms (the less anxious). We tested the following p41 mice: WT and KO untreated (n = 10/9), KO treated with Desipramine 10 mg/Kg (n = 9) or Mirtazapine 50 mg/Kg (n = 10). The test was performed for 5 minutes from 10 to 12 a.m. The animals were putted into the center of the cross at the beginning of the test. After the test, the cross was clean with ethanol 70% to eliminate any smell of mouse and avoid influencing the performance of the following mouse. The data were analyzed with Any-maze program (Ugo Basile Instruments) dividing the cross into three zones: central zone, open arms (the more anxious), closed arms. Distance travelled, time of immobility, entries and time in the zones were analyzed.

Human brains . Human brain tissues from 3 healthy donors (HD) and 3 Rett (RTT) patients were acquired from NICHD Brain and Tissue Bank for Developmental Disorders, at University of Maryland Baltimora, USA. RTT patients were all of the classical RTT syndrome type and all females. The clinical information on brain specimens used are described in Table 1.

Table 1. Clinical information on brain specimens.

UMB#	AGE (YEARS)	CAUSE OF DEATH	MECP2 MUTATION	FINAL NEUROPATHOLOGIC DIAGNOSIS
HD1584	18	Multiple Injuries	-	-
HD1347	19	Multiple Injuries	-	-
HD1846	20	Multiple Injuries	-	-
RTT1815	18	Complication of the disorder	Het.IVS3-2A>G	Generalized atrophy and multifocal maldevelopment. Contusion, right temporal tip (clinical history of seizures).
RTT4852	19	Seizures	Het G451T missense mutation in exon4	Pallor of the substantia nigra compacta. Subcortical neurons, temporal lobe. Long (>14 years) history of epilepsy. Mineralization, frontal cortex and periventricular area. Capillary telangiectasia, hillum of right dentate nucleus, incidental.
RTT4516	20	Natural	No genetic analysis	Slight cerebral atrophy. Slight cerebellar and nigral degeneration.

RNA extraction and reverse transcription. Total RNA was extracted from human brain samples of healthy donors and Rett (RTT) patients (n = 1 for each sample), using RNeasy Lipid Tissue Mini Kit (QUIAGEN) according to the manufacturer's instructions. A quality control of the RNA was performed in an agarose gel and with a PCR amplifying the GAPDH gene. Mice were sacrificed by transcranic dislocation, the brain extracted to isolate the hippocampus and the somatosensory cortex. RNA was extracted from tissues using TriZol Reagent (Invitrogen) according to the manufacturer's instruction. 1,5 µg of total RNA was then heat, denaturated and reverse-transcribed into cDNA using two mix. The first one contained 100 ng oligodT primers (Roche Diagnostic), 40 U/µl RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen) and H_2O DEPC. 2 µl of RNA were added to reach a final volume of 12,5 µl; samples were hoven 10 minutes at 70°C, 45 seconds in ice, spinned for 5 seconds. At this point mix 2 was added (5X first strand buffer (250 mM Tris-HCl pH 8.3 at room temperature, 375 mM KCl, 15 mM $MgCl_2$) (Invitrogen), 0,1 M DTT (Invitrogen), 10 mM each dNTP (Promega)), samples were spinned for 5 seconds and put for 10 minutes at room temperature in order to allow the Annealing process. Finally, 200 U/µl Superscript III (Invitrogen) was provided in each sample and the reaction was carried out at 50°C for 50 minutes followed by Superscript III inactivation for 15 minutes at 70°C.

Quantitative real time PCR. A quantitative real time PCR (qRT-PCR) was performed using a Biorad CFX96^{TM} Real-Time System according to the manufacturer's instructions. The PCR reactions were performed in a final volume of 20 µl with 2X Master SYBR Green I Mix (Fluocycle, Euroclone), 0,5 µM of each primer and 100 ng of cDNA was added as PCR template. The qRT-PCR was used to evaluate the amount of each BDNF transcript presents in RTT patient's brains compared to the corresponding control condition. Primer sequences for human BDNF splice variants amplification are listed in Table 2 on the left. The primers pairs used, the relative expected amplicons size and the PCR condition for each amplification are reported in Table 3.

The amount of each BDNF transcript present in MeCP2 null mice (n = 4) compared to the corresponding control condition (n = 4 wild-type C57/BL6 mice) was measured at three different ages (p35, p42, p49). Primer sequences for mouse BDNF splice variants amplification are listed in Table 2 on the right. The primers pairs used, the relative expected amplicons size and the PCR condition for each amplification are the same reported in Table 4. Four groups (n = 3 each one) have been analyzed at p42: MeCP2 null mice treated with vehicle, with Desipramine 10mg/Kg, with Mirtazapine 10 mg/Kg or 50 mg/Kg.

Table 2. Primer sequence for human and mouse BDNF splice variants.

HUMAN	SEQUENCE	MOUSE	SEQUENCE
exI fwd	CTTCCAGCATCTGTTGGGGAGACG	**exI fwd**	CCTTCCTGCATCTGTTGGGGAG
exII fwd	CCAGCGGATTTGTCCGAGGTGG	**exII fwd**	CCAGCGGATTTGTCCGAGGTGG
exIII fwd	AGCCCAGTTCCACCAGGTGAG	**exIII fwd**	AGCCCAGTTCCACCAGGTGAG
exIV fwd	ACCGAAGTCTTCCCCAGAGCAG	**exIV fwd**	ACCGGTCTTCCCCAGAGCAG
exV fwd	ACCAATAGCCCCCATGCTCTG	**exV fwd**	ACCATAACCCCGCACACTCTG
exVI fwd	TGGAGCCAGAATCGGAACCAC	**exVI fwd**	AGGGACCAGAAGCGTGACAAC
exVII fwd	CCACATCTCTACCCATCCTGC	**exVII fwd**	CTCTGTCCATCAGCGCACC
exVIII fwd	TGGCATGACTGTGCATCCCAG	**exVIIIfwd**	GGTATGACTGTGCATCCCAGG
exIXa fwd	ACAATCAGATGGGCCACATG	**exIXa fwd**	ACAATCAGATGGGCCACATG
com rev	ACGCTCTCCAGAGTCCCATG	**com rev**	CACGCTCTCCAGAGTCCCATG
cds fwd	AAACATCCGAGGACAAGGTGGC	**cds fwd**	AAACGTCCACGGACAAGGCA
cds rev	GGCACTTGACTACTGAGCATCACC	**cds rev**	TTCTGGTCCTCATCCAGCAGC
gapdh fwd	GGTGAAGGTCGGAGTCAACGGA	**gapdh fwd**	ACCACAGTCCATGCCATCAC
gapdh rev	GAGGGATCTCGCTCCTGGAAGA	**gapdh rev**	TCCACCACCCTGTTGCTGTA

Table 3. The primers pairs used for human samples, the relative expected amplicons size and the PCR condition for each amplification.

HUMAN TRANSCRIPT	ACCESSION NUM.	PRIMERS	PRODUCT	PCR CONDITIONS
exI	EF689021.1	exI fwd/com rev	205	45X(95°C 20",56°C 20",72°C45")
exII	EF674517-8-9.1	eXII fwd/com rev	192-404-487	45X(95°C 20",56°C 20",72°C45")

exIII	EF674520.1	eXIII fwd/com rev	165	45X(95°C 20",56°C 20",72°C45")
exIV	EF674521.1	eXIV fwd/com rev	219	45X(95°C 20",56°C 20",72°C45")
exV	EF689011.1	eXV fwd/com rev	227	45X(95°C 20",55°C 20",72°C45")
exVI	EF689014-5.1	eXVI fwd/com rev	209-227	45X(95°C 20",56°C 20",72°C45")
exVII	EF689018.1	eXVII fwd/com rev	305	45X(95°C 20",56°C 20",72°C45")
CDS	"	CDS fwd/CDS rev	230	45X(95°C 20",56°C 20",72°C45")

Table 4. The primers pairs used for mouse samples, the relative expected amplicons size and the PCR condition for each amplification.

MOUSE TRANSCRIPT	ACCESSION NUM.	PRIMERS	PRODUCT	PCR CONDITIONS
exI	EF125669.1	exI fwd/com rev	226	45X(95°C 20",56°C 20",72°C45")
exII	EF125670-1-2.1	eXII fwd/com rev	195-407-490	45X(95°C 20",56°C 20",72°C45")
exIII	EF125681.1	eXIII fwd/com rev	165	45X(95°C 20",56°C 20",72°C45")
exIV	EF125673.1	eXIV fwd/com rev	218	45X(95°C 20",56°C 20",72°C45")
exV	EF125682.1	eXV fwd/com rev	237	45X(95°C 20",55°C 20",72°C45")
exVI	EF125674.1	eXVI fwd/com rev	227	45X(95°C 20",56°C 20",72°C45")
exVII	EF125683.1	eXVII fwd/com rev	229	45X(95°C 20",56°C 20",72°C45")
exVIII	EF125684.1	eXVIII fwd/com rev	253	45X(95°C 20",56°C 20",72°C45")
exIXa	EF125685.1	eXIXa fwd/com rev	238	45X(95°C 20",56°C 20",72°C45")
CDS	"	CDS fwd/CDS rev	161	45X(95°C 20",56°C 20",72°C45")

Data analysis of qRT-PCR. A normalization of the C_t of the target gene (Exon) to that of the reference gene (GAPDH) (= ΔC_t) to adjust for small differences in input DNA for both human and mice samples; the ΔC_t of the RTT samples were normalized to the mean of ΔC_t of all HDs patients in the same experiment ($\Delta\Delta C_t$). For the untreated animal samples, the ΔC_t of a MeCP2 null mouse sample (KO UNT) was normalized to the ΔC_t of a wild type mouse (WT UNT) in the same experiment ($\Delta\Delta C_t$). For the BDNF transcripts in all treated conditions, we normalized the ΔC_t of MeCP2 null mouse treated sample (Desipramine 10 mg/Kg; Mirtazapine 10 mg/Kg; Mirtazapine 50 mg/Kg;) to the mean of ΔC_t of all the MeCP2 null mice treated with the vehicle ($\Delta\Delta C_t$). The resultant $2^{-\Delta\Delta C_t}$ values were transformed in logarithmic scale to better appreciate the increase/decrease of BDNF isoforms expression.

Statistical analysis of qRT-PCR. A Mann-Whitney test was performed using GraphPad Prism5 software. The population of HDs is homogeneous but each of the RTT patient carries a different mutation, so each RTT patient is tested versus all the HDs. The same test was used to test all WT UNT mice versus all KO UNT mice and to test all MeCP2 KO treated mice (KO DMI10 - KO MIR10 - KO MIR50) versus all KO mice treated with vehicle (KO VEHIC).

Mice hippocampal and cortical tissues preparation for ELISA assay and Western Blot. Hippocampus and cortex were dissected from mice brain and lysed using a buffer containing 500 mM NaCl; 0,2% Triton X-100; 0,1% NaN_3; EDTA 2 mM. For the ELISA assay, it was added also 2% BSA to the buffer (Szapacs et al., 2004). It was added freshly the protease inhibitors PMSF 200 µM, Leupeptin 10 mg/ml and Aprotinin 0,3 µM. The samples were sonicated for 15 seconds, pulse 1 second, power 4 (Sonoplus Ultrasonic Homogenizers HD 2070). Samples were centrifuged at 16,000 g for 30 minutes at 8°C and then, the supernatants were conserved at -80°C.

ELISA for mice hippocampal and cortical tissues (Promega and Chemikine kit). BDNF from mice hippocampus and cortex was quantified by ELISA using the BDNF Emax® ImmunoAssay System (Promega) or the Chemikine kit (Millipore), according to manufacturer's instructions. 100 µl of each lysate in duplicate was used. BDNF concentration was measured at absorbance at 450 nm using the Glomax multidetection system (Promega).

Western blot. BDNF from mice hippocampus and cortex was quantified by western blot. The same lysis buffer used for ELISA was employed, the only difference was the absence of BSA. Samples were boiled and analyzed on 12% polyacrylamide gel electrophoresis at 12 mA (15 µl of sample + 15 µl DTT 1M in laemly buffer were loaded). Proteins were then electroblotted at 100 mA for 45 minutes onto nitrocellulose membrane with a semidry system. Aspecific sites were blocked in 10% milk in TBS-Tween 0,1% for one hour (Garcia et al., 2012). In order to allow different primary antibody binding, the part of the membrane containing BDNF was separated from the one containing tubulin (55kDa). The two membranes were then washed with TBS-Tween 0,1% (BDNF) and PBS-Tween 0,1% (Tubulin) to remove blocking solution. Membranes were incubated overnight at 4°C with shaking respectively with rabbit anti-BDNF N20 (Santa Cruz) 1:500 in TBS-Tween 0,1% and

mouse anti-Tubulin (Sigma-Aldrich) 1:10000 in 5% milk in PBS-Tween 0,1% pH 8.0. Membranes were washed 3x10-15 minutes in TBS-Tween 0,1% pH 8.0 and PBS-Tween 0,1% pH 8.0, respectively and then, they were incubated for 1 hour at room temperature with the secondary antibodies: anti-rabbit HRP (Dako) 1:2000 in TBS-Tween 0,1% pH 8.0 on BDNF membrane and anti-mouse HRP (Sigma-Aldrich) 1:10000 in 5% milk in PBS-Tween 0,1% pH 8.0 on Tubulin membrane. Three washes were executed as explained before and membranes were maintained in their respective washing solution until development. The experiment was completed by a development with Amersham ECL Prime Western Blotting Detection Reagents (GE Healthcare) dilute 1:5 for Tubulin and 1:2 for BDNF. Bands were quantified using Quantity-one software.

RESULTS

Reduction in body and brain weight and cortical thickness in MeCP2$^{-/y}$ mice and rescue of the brain weight by Mirtazapine treatment. Rett mouse models display loss of body and brain weight (Chen et al., 2001b, Guy et al., 2001). To obtain a reference before pharmacological treatment, we evaluated the body and the brain weight of MeCP2$^{-/y}$ mice (KO) and their Wild Type (WT) littermates at postnatal day 42 (p42). Compared to WT mice, p42 KO mice showed a significant reduction of body weight (-34,7%; *t-test*) and brain weight (-17,6%; *t-test*) (Figure 1A, B respectively).

Previous data demonstrated a reduction in the cortical thickness of the somatosensory cortex in p42 and p56 MeCP2$^{-/y}$ mice as compared to WT (Kishi and Macklis, 2004, Fukuda et al., 2005). To better define the onset of these cortical structure alterations, we perfomed a Nissl staining of WT and KO mice brains at p42. As shown in Figure 1C, a densitometric profile was used to measure the thickness of the total somatosensory cortex and its individual layers from I to VI. Thickness of the somatosensory cortex (S1-M1, barrel cortex) was measured on 3 sections collected at regular intervals every 400 µm from the beginning of the barrel cortex (referred as to 0 µm), until 1,200 µm along the antero-posterior axis. At p42, KO mice cortices were reduced compared to WT, at all intervals (Figure 1C). To further evaluate the thickness of the individual layers we considered the sections at 400 µm and we observed that at p42, there was a significant reduction in layers II-III (the most affected layers in Rett syndrome patients) and VI (Figure 1C). These results showed a reduction in body and brain weight of p42 MeCP2$^{-/y}$ mice compared to WT littermates, and a reduction in the thickness of the somatosensory cortex, where the layer II-III is the most affected one, in agreement with previous observations (Kishi and Macklis, 2004, Fukuda et al., 2005).

To evaluate if Mirtazapine has an effect on body and brain weight, WT and MeCP2$^{-/y}$ mice were assigned randomly to groups of n = 8-11 animals which were treated for 2 weeks with vehicle (Vehic), or the control drug (Desipramine, 10 mg/Kg) or the testing drug (Mirtazapine) at 2 different concentrations (10 or 50 mg/Kg). Mice were weighted every day during the 14 days of treatment and at p28, the KO groups showed, in average, 30% lower body weight with respect to the WT groups and this difference was maintained until p41 (Figure 1D). Considering the body weight at p28 and p41, we observed that the 30%

difference between WT and KO mice was maintained at all time points irrespective of the drug (Figure 1E). These results confirmed that Desipramine does not increase the body weight as was previously described by Zanella and colleagues (Zanella et al., 2008). The reduction in body weight observed after Mirtazapine treatment could be explain by the reduced capacity of the mice to reach food and water deriving from the sedative effect due to the high concentration of Mirtazapine (Fawcett and Barkin, 1998b, a).

After 24 hours from the last injection, mice were euthanized and brains were weighted. The brain weight was not affected by the different treatments in WT treated mice compared to the WT untreated mice (WT UNT) (Figure 1F). The brain weight in KO untreated (KO UNT) mice was significantly lower than the average brain weight in WT untreated mice (KO UNT = 80%; WT UNT = 100%). Treatment with vehicle had no effect (KO UNT = 80%; KO vehic = 82,4%) and Desipramine and Mirtazapine 10mg/Kg induced a slight increase of the brain weight (DMI10 = 84,2%, MIR10 = 86,2%). However, treatment with Mirtazapine at 50mg/Kg increased the brain weight to 91,6% of KO untreated mice, reaching values close to the WT untreated (Figure 1G). Thus, Mirtazapine has a positive effect on the weight of Rett brains.

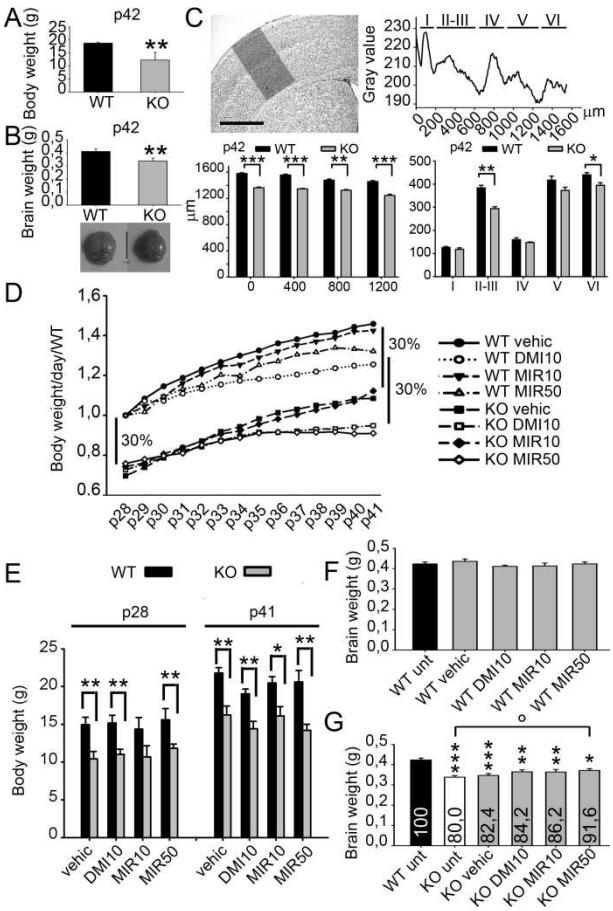


Figure 1. Reduction in body and brain weight and cortical thickness in MeCP2$^{-/y}$ mice and rescue of the brain weight by Mirtazapine treatment. (A; B) Body and brain weight in grams of p42 WT and KO mice (n = 8-9). Below, photographs of representative brains. Values are represented as mean ± SEM. **p<0.01 (*t-test*). **(C)** A gray scale section stained with Nissl and the perpendicular line crossing the somatosensory cortex (scale bar = 1000 µm). On the right, the densitometric plot in gray scale (Y-axis) to measure the total cortical thickness in µm and its individual layers, from I to VI. Below, total cortical thickness in µm (Y-axis) in p42 wild type (WT) and MeCP2$^{-/y}$ (KO) mice from the beginning of the barrel cortex (0 µm) to 1200 µm (at intervals of 400 µm) (n = 4-8). Values are represented as mean ± SEM. *p<0.1; **p<0.01; ***p<0.001 (*t-test*). On the left, the thickness of cortical layers (from I to VI) in µm (Y-axis) of p42 WT and KO mice (n=4-8). Values are represented as mean ± SEM. *p<0.1; **p<0.01 (*t-test*). **(D)** Body weight of WT and KO mice treated with vehicle (vehic), Desipramine 10 mg/Kg (DMI10) and Mirtazapine 10-50 mg/Kg (MIR10-50), measured every day from p28 to p41 (n = 8-11). Body weight of KO treated mice is normalized to the corresponding WT treated mice (= 1 at p28). **(E)** Body weight evaluated at p28 and p41 in WT and KO treated mice. Values are represented as mean ± SEM. *p<0.1; **p<0.01 (*t-test*). **(F)** WT brain weight of untreated (UNT) and treated mice with vehic, DMI10, MIR10 and MIR50 (*One way ANOVA*). **(G)** KO brain weight of untreated (KO UNT) and treated mice with vehic, DMI10, MIR10 and MIR50. Values are represented with bars as mean ± SEM. *referred to WT UNT mice; °referred to KO UNT mice; */°p<0.1; **p<0.01; ***p<0.001 (*One way ANOVA*). The values inside the bars are represented as percentage based on WT UNT (= 100%).

Hippocampal structure is not affected by the loss of MeCP2 nor Mirtazapine treatment.

To investigate which brain structures are involved in the rescue of brain weight by

Mirtazapine, we evaluated first the effects on the hippocampus (Figure 2). In a previous study, we observed that the specific contribution of each layer to the total thickness (=100%) is constant along the rostro-caudal axis of the hippocampus in both mouse and rat (Baj et al., 2012). Using the same strategy, (Figure 2B) we performed a Nissl staining and analyzed three sections for each animal (WT UNT, KO UNT, KO DMI10, KO MIR10-50). Through a line drawn perpendicularly to the CA1 pyramidal layer, we measured the thickness of the layers and determined the proportion of each hippocampal lamina in WT UNT as follows: CA1 pyr. l.=8,7%, CA1 st. rad.=32,5%, st. lac. mol.=19,6%, mol. l.=27,3%, gran.l.=10,6% (Figure 2B). There were no differences in the hippocampal layers thickness between WT and KO untreated or treated mice (Figure 2C). Hence, the gross anatomical structure of the hippocampus was not affected by the loss of MeCP2 nor treatment with antidepressants.

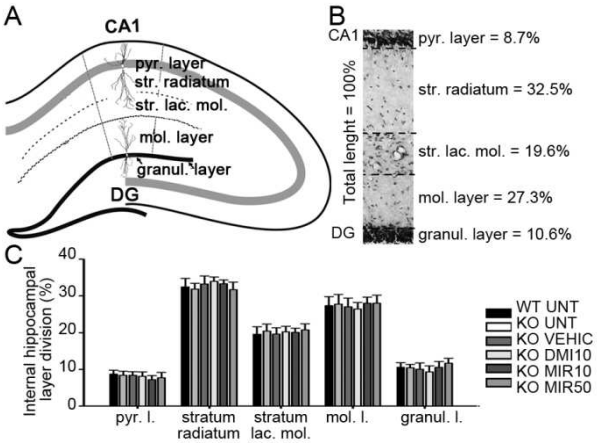


Figure 2. Hippocampal structure is not affected by the loss of MeCP2 or Mirtazapine treatment. **(A-B)** A representation of hippocampal structure and layers (*pyramidal layer, stratum radiatum, stratum lacunosum moleculare, molecular layer, granular layer*). **(C)** The proportion of each hippocampal layer based on the total thickness (= 100%) in WT and KO untreated (UNT) mice and in KO mice treated with vehicle (vehic), Desipramine 10 mg/Kg (DMI10) and Mirtazapine 10-50 mg/Kg (MIR10-50) (n = 3-4). Values are represented as percentage ± SEM (*One way ANOVA*).

Mirtazapine rescues the somatosensory cortical thickness in MeCP2$^{-/y}$ mice. Since previous studies (Kishi and Macklis, 2004, Fukuda et al., 2005) and our own data (Figure 1C) showed a reduction in the structure of the somatosensory cortex and in particular of the layer II-III in MeCP2 KO mice, we investigated if the pharmacological treatment could rescue the cortical organization (Figure 3). First of all, we analyzed the effects of antidepressants on WT animals and we did not found differences compared with WT untreated or treated with vehicle

mice, both in the total cortical thickness and in all of its layers (Figure 3B, C respectively) (Total cortical thickness = mean ± SEM: WT UNT = 1601.34 µm ± 8.66; WT VEHIC = 1561.89 µm ± 12.03; WT DMI10 = 1566.60 µm ± 10.62; WT MIR10 = 1567.08 µm ± 10.96; WT MIR50 = 1600.03 µm ± 19.10). However, a highly significant increase in total cortical thickness was observed in MeCP2$^{-/y}$ mice treated with Desipramine 10 mg/Kg (90,6%; 1451.28 µm ± 12.84) or Mirtazapine 50 mg/Kg (92,9%; 1488.74 µm ± 23.33) compared to the KO UNT mice (85,7%; 1372.87 µm ± 7.85) or KO vehic (85,2%; 1364.10 µm ± 8.73) and WT (100%; 1601.34 µm ± 8.66) (Figure 3B). When single cortical layers were analyzed, Mirtazapine 50 mg/Kg was found to induce partial rescue of the layer II-III and a complete rescue of layer VI (Figure 3D). In conclusion, the recovery of the brain weight of MeCP2$^{-/y}$ mice observed with Mirtazapine can be largely accounted by a rescue of the somatosensory cortex cytoarchitectonic.

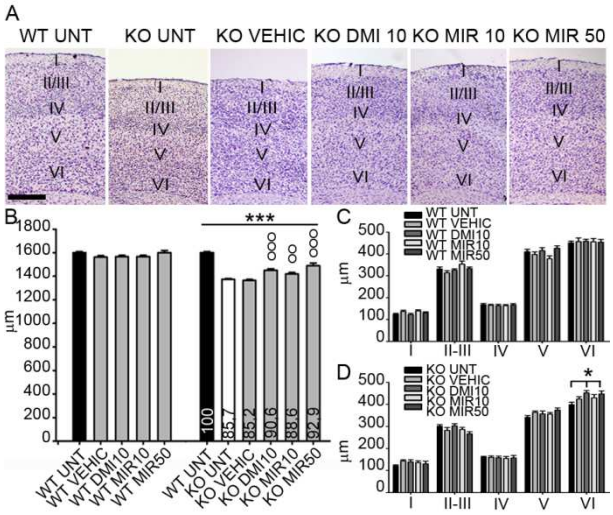


Figure 3. Total cortical thickness is rescued after the treatment with Mirtazapine 50 mg/Kg. WT UNT (wild type untreated mice; black bars); KO UNT (MeCP2$^{-/y}$ untreated mice; white bars); KO treated (vehic, DMI10, MIR10-50; gray bars) (n = 6) **(A)** Nissl staining of the somatosensory cortex in WT UNT and KO UNT mice and in KO treated mice (scale bar = 400 µm). **(B)** Total cortical thickness in µm in WT UNT and KO UNT mice and KO treated mice (n = 6). Values are represented as mean ± SEM. *referred to WT UNT mice; °referred to KO UNT mice; °°p<0.01; ***/°°°p<0.001 (*One way ANOVA*). The values inside the bars are reported in percentage respected to WT UNT mice (= 100%). **(C)** Thickness of the layers in WT UNT mice and in WT mice treated with VEHIC, DMI10 and MIR50. Values are represented as mean ± SEM. (*One way ANOVA*). **(D)** Thickness of the layers in KO UNT mice and in KO mice treated with VEHIC, DMI10 and MIR50. Values are represented as mean ± SEM. *referred to WT mice; *p<0.1 (*One way ANOVA*).

Mirtazapine treatment rescues MeCP2$^{-/y}$ cortical neurons morphology. To gain further insight regarding the effect of Mirtazapine treatment on cortical neurons, we investigated the morphology of layer II-III pyramidal neurons of the somatosensory cortex in MeCP2$^{-/y}$ and WT untreated mice using Golgi staining. We did not perform the Golgi staining for the WT mice treated with Mirtazapine since they presented no differences in the thickness of the cortex and of its layers compared with WT untreated mice (Figure 3B, C). In MeCP2$^{-/y}$ mice, pyramidal neurons showed smaller area of their somata with respect to WT mice but it was restored by the treatment with Mirtazapine 50 mg/Kg to the levels of WT mice (Figure 4A). A Sholl analysis was performed separately on basal and apical dendrites and results were plotted on the same graph (Figure 4B). With respect to WT, MeCP2$^{-/y}$ mice showed a reduced number of crossings in the region from 120 to 150 µm from the soma in apical dendrites, and also a marked reduction along the basal dendrites between -40 and -140 µm from the soma. However, treatment with Mirtazapine 50 mg/Kg rescued these deficits back to WT levels (Figure 4B).

A previous study demostrated a diameter reduction of the apical dendrite of MeCP2$^{-/y}$ mice neurons with respect to WT (Fukuda et al., 2005). We measured the diameter of the apical dendrites of layer II-III pyramidal neurons at 10 and 100 µm from the soma and found a significant diameter reduction of apical dendrites in KO mice at both distances (Figure 4C, D; *One Way ANOVA*). Treatment with Mirtazapine led to a full recovery of this morphological deficit (Figure 4C, D respectively). These data suggest that Mirtazapine can restore the fine morphology of cortical neurons.

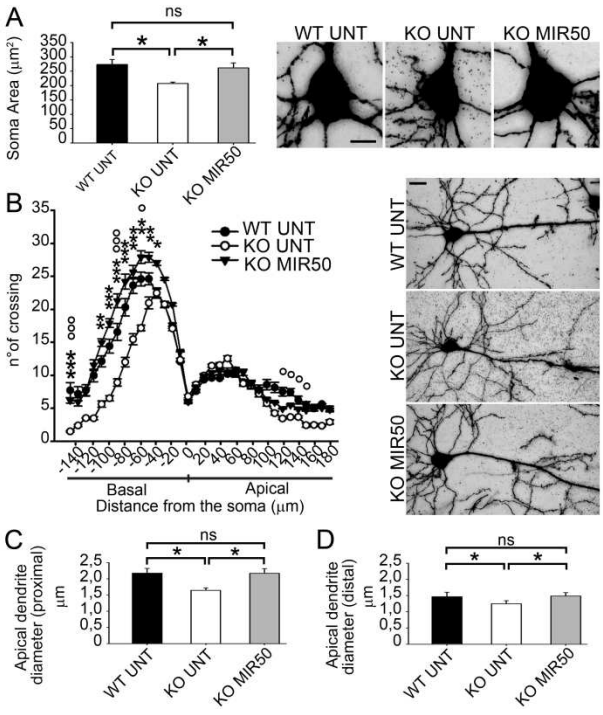


Figure 4. Golgi staining of layer II-III pyramidal neurons (somatosensory cortex) in WT and MeCP2$^{-/y}$ untreated and treated mice. WT UNT (wild type untreated mice; black bars); KO UNT (MeCP2$^{-/y}$ untreated mice; white bars); KO treated with Mirtazapine 50 mg/Kg (MIR50; gray bars); (n = 3 mice; n = 10 neurons for each mouse) **(A)** Soma area of the neurons in WT/KO UNT, and KO MIR50. Values are represented as mean ± SEM. *p<0.1; ns = no significant (*One way ANOVA*). On the right a representation of soma area of each group of mice (scale bar = 10 µm). **(B)** Sholl analysis: number of basal and apical dendritic crossing through a series of concentric circles centered at the soma and spaced at 10 µm intervals of WT/KO UNT and KO MIR50 mice. Values are represented as mean ± SEM. *KO MIR50 respect to KO UNT mice; °WT UNT respect to KO UNT mice; */°p<0.1; **p<0.01; ***/°°°p<0.001 (*t-test*). On the right, a representation of neurons of each group of mice (scale bar = 20 µm). **(C)** The apical dendrite diameter (proximal) at 10 µm from the soma of WT/KO UNT mice and KO MIR50. *p<0.1; ns = no significant (*One way ANOVA*). **(D)** The apical dendrite diameter (distal) at 100 µm from the soma of WT UNT, KO UNT and KO MIR50. *p<0.1; ns = no significant (*One way ANOVA*).

Mirtazapine rescues the number and type of spines in MeCP2$^{-/y}$ neurons dendrites. It was previously reported that the number of spines in primary and secondary apical dendrites were similar between WT and KO mice (Jaenisch and Bird strains) (Kishi and Macklis, 2004). Using sections labeled with Golgi staining, we investigated not only the number of spines in primary and secondary apical dendrites, confirming the previous study, but also in secondary basal dendrites wherein we observed a significant reduction in KO mice compared to WT. Treatment with Mirtazapine 50 mg/Kg caused a complete rescue of secondary basal dendrite spines (Figure 5B). However, when the spine types were considered, we observed no differences in the number of mushroom and thin spines. In contrast, stubby spines were

significantly reduced in both in apical and basal dendrites from KO mice and were completely rescued by Mirtazapine treatment (Figure 5C). Probably the cognitive deficits observed in Rett girls, reduce the inputs needed to promote postnatal maturation of dendritic spines and that these remain in the immature state of stubby spines. In conclusion, Mirtazapine treatment acts on the number and spine types to rescue the Rett phenotype on both apical and basal dendrites.

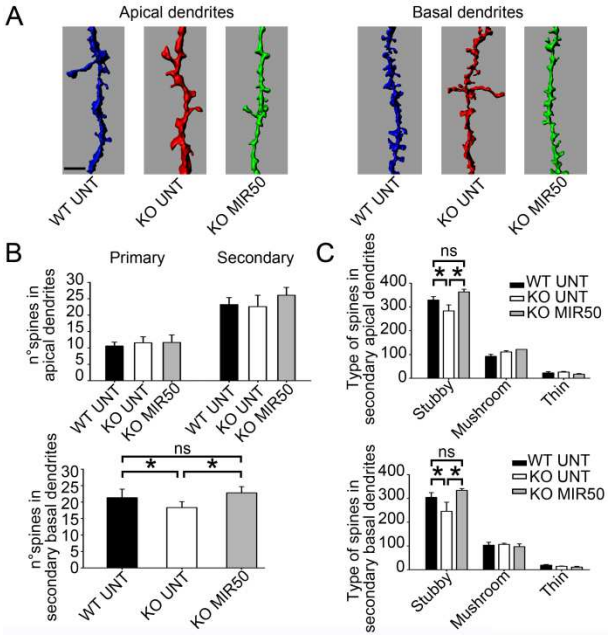


Figure 5. Mirtazapine rescues the number and type of spines in MeCP2$^{-/y}$ neurons dendrites. WT UNT (wild type untreated; black bars); KO UNT (MeCP2$^{-/y}$ untreated; white bars); KO treated with Mirtazapine 50 mg/Kg (MIR50; gray bars); (n = 3 mice; n = 10 neurons for each mouse) **(A)** A 3D representation of secondary apical and basal dendrites of WT/KO UNT mice and KO MIR50 (scale bar = 5 µm). **(B)** The number of spines in primary and secondary apical dendrites and in basal dendrites in WT/KO UNT and in KO MIR50. Values are represented as mean ± SEM. *p<0.1; ns = no significant (*One way ANOVA*). **(C)** The type of spines (stubby, mushroom and thin) in WT/KO UNT and in KO MIR50. Values are represented as mean ± SEM. *p<0.1; ns = no significant (*One way ANOVA*).

Mirtazapine rescues GABA currents in MeCP2$^{-/y}$ cortical neurons. Several lines of evidence suggest that in the pathophysiology of Rett syndrome are involved deficits in GABAergic transmission. The loss of MeCP2 in transgenic mice induces a reduction of GABA release in brain neurons (Chao et al., 2010, Zhang et al., 2010) but also reduces the expression of $GABA_A$ receptor subunits (Samaco et al., 2005). Antidepressant treatments are known to rescue GABA levels and GABA deficits in patients with mood disorders (Krystal et

al., 2002). To investigate whether the antidepressant Mirtazapine could affect the $GABA_A$ receptor functionality in $MeCP2^{-/y}$ mouse cortex, cell membranes isolated from cortices of WT UNT, KO UNT and KO mice treated for two weeks with 50 mg/Kg Mirtazapine (KO MIR50) and were microtransplanted into *Xenopus* ooctyes. This method overcomes the critical limitation of brain slides which are extreme difficult to prepare from adult animal (Chao et al., 2007, Huang and Uusisaari, 2013), as those used in this study (p42). Besides, the microtransplantation technique offers many advantages: *1)* the oocyte plasma membrane incorporates the foreign membranes and efficiently acquires functional neurotransmitter receptors and voltage-operated channels (Bernareggi et al., 2007, Bernareggi et al., 2012), *2)* the foreign receptors are still embedded in their natural lipid membrane and the properties of the receptors are the same as those of the receptors while still in the ‘‘donor’’ cells (Palma et al., 2002), *3)* a limited number of animals is required since the same membrane preparation can be injected in to oocytes many times (Miledi et al., 2006, Eusebi et al., 2009), and *4)* this approach can be extended to post-mortem human tissues (Bernareggi et al., 2007, Palma et al., 2007, Limon et al., 2008, 2012). Figure 6A shows typical $GABA_A$-current traces recorded 2 days after injection. Currents could be detected in all injected oocytes. No response was obtained in non-injected oocytes.

To characterize the effect of Mirtazapine on $GABA_A$ receptors, membrane preparations from different treatment and genotype groups were injected at the same protein concentration (1mg/ml), and the $GABA_A$-currents were recorded from 1 to 4 days after injection. Since GABA-elicited amplitudes were variable among oocytes isolated from different frogs, $GABA_A$-currents from KO UNT and KO MIR50-injected oocytes were normalized to those recorded in WT-injected oocytes isolated from the same frog and recorded at the same time after injection. Figure 6B shows that $GABA_A$-current amplitude was significantly lower in KO-injected oocytes (n = 80 oocytes, 4 WT UNT cortices and n = 46, 3 KO UNT cortices, respectively). Mirtazapine treatment significantly increased the current amplitude (n = 48, 3 KO MIR50 cortices). Dose-current response curves showed a left shift for KO-injected oocytes revealing that the receptor affinity for GABA was altered in $MeCP2^{-/y}$ animals. Half maximal effective GABA concentration (EC_{50}) of KO-injected oocytes was significantly different from the WT UNT (WT UNT, EC_{50} = 98 ± 4.38 μM, n_H = 1.11 ± 0.04, n = 39; KO UNT, EC_{50} = 78.73 ± 4.92 μM, n_H = 1.15 ± 0.08, n = 36). In KO MIR50-injected oocytes the EC_{50} was not significantly different from the WT (EC_{50} = 104.14 ± 11.2 μM, n_H = 1.02 ± 0.11, n = 14).

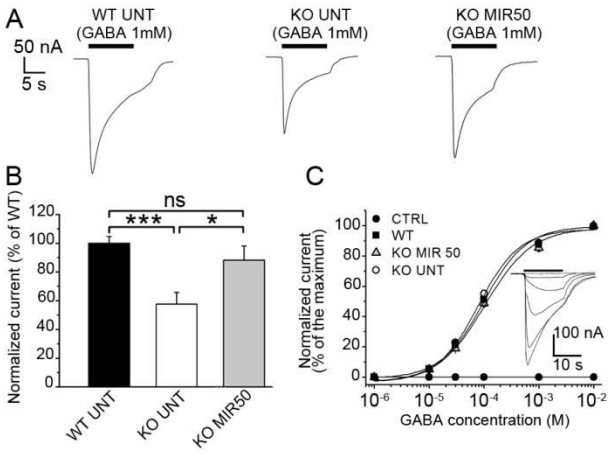


Figure 6. Mirtazapine treatment rescues GABA currents in oocytes injected with membranes of WT and MeCP2$^{-/y}$ mouse cortex. (n = 3 mice each group) **(A)** GABA currents (1 mM) recorded in oocytes injected with membranes isolated from WT UNT, KO UNT and KO MIR50 mouse cortices. **(B)** GABA currents (% of WT UNT) recorded in oocytes injected with KO UNT and KO MIR50 cortex membranes. *p<0.05; ***p<0.001; ns = no significant (*t-test*). **(C)** GABA-induced dose-response currents of WT UNT, KO UNT and KO MIR50-injected oocytes and no- injected oocytes. The values were normalized to the maximum value of inward currents. In the inset, representative traces of GABA-currents in MeCP2 KO-injected oocyte. Oocyte membrane potential was held at -80 mV.

Mirtazapine restores the heart and breath rate to healthy level, without altering the oxygen saturation and pulse distention. In the late stage of the disorder, Rett patients develop cardiorespiratory dysfunction with apnea, episodes of hyperventilation, abnormal sweating, decrease heart rate variability and prolongation of corrected QT intervals (McCauley et al., 2011, Kaufmann et al., 2012). To investigate the functional effects of the antidepressant treatment on MeCP2$^{-/y}$ mice, we used the MouseOX instrument based on an infrared collar which collects in real time the data regarding the oxygen saturation, the heart and breath rate and the pulse distention. The data recorded by the MouseOX, during 10 minutes of the test are shown in Figure 7A. Averaging of 10 minutes recording of each individual mouse within the same group, showed that the oxygen saturation (O_2 saturation), which was not significantly compromised in KO untreated mice, was not altered after 14 days of Desipramine 10 mg/Kg or Mirtazapine 50 mg/Kg treatment (Figure 7B). Moreover, we observed a reduction of heart and breath rate in KO untreated mice (p<0.05; *One Way ANOVA* and *Kruskal wallis test*, respectively), which was restored to normal levels partially after Desipramine treatment and completely after Mirtazapine treatment, significant only for the breath rate (p<0.05; *Kruskal wallis test*) (Figure 7B). For the first time, we evaluated in an

animal model of the Rett syndrome the pulse distention, an indirect measure of the blood flux. We observed that pulse distention was not reduced in KO untreated mice comparing to the WT animals, and Mirtazapine treatment did not affect the blood flux, unlike the negative effect observed after Desipramine treatment ($p<0.05$; *One Way ANOVA*) (Figure 7B). The averages of single mouse of the same group were plotted in Figure 7C to evaluated the trend of each group of animal, which correspond to the observations described above.

In conclusion, Mirtazapine has a positive effect on heart rate, unlike Desipramine which is known to induce cardiac complications, and a positive effect on breath rate which is most important for the irregular pattern of respiration observe in Rett patients. Moreover, in contrast to Desipramine, Mirtazapine did not affect oxygen saturation and did not alter pulse distention.

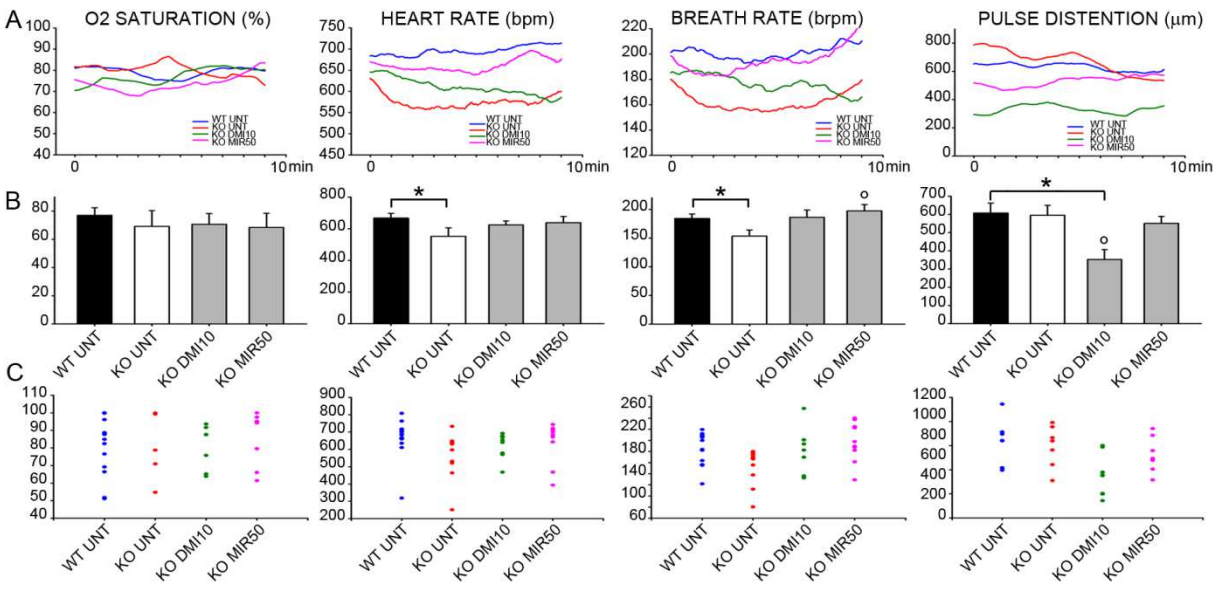


Figure 7. Mirtazapine restores the heart and breath rate to healthy level, without altering the oxygen saturation and pulse distention. n = 14 WT UNT; 11 KO UNT; 11 KO DMI10; 12 KO MIR50. **A)** The data of O2 saturation, heart rate, breath rate and pulse distention recorded by the MouseOX during 10 minutes of the test in WT and KO untreated mice (WT UNT; KO UNT) and in KO mice treated with Desipramine 10 mg/Kg (KO DMI10) and Mirtazapine 50 mg/Kg (KO MIR 50). **B)** Averaging of 10 minutes recording of each individual mouse within the same group (WT UNT; KO UNT; KO DMI10; KO MIR 50). *referred to WT UNT; °referred to KO UNT; */°$p<0.05$; *One Way ANOVA* and *Kruskal wallis test*. **C)** The plotted averages of single mouse of the same group (WT UNT; KO UNT; KO DMI10; KO MIR 50).

Antidepressant treatments reduced motor function and restored the anxiety behavior. The Bird strain of MeCP2$^{-/y}$ mice are known to show hypoactivity, impaired balance and

coordination, spontaneous tremors and hindlimb clasping from the first month of age (Ricceri et al., 2008). From 5 weeks of age, Bird MeCP2$^{-/y}$ mice exhibit clearly motor disabilities, characterized by a reduction in the activity during the open field test while, sensory and social deficits, anxiety-like behavior and cognitive abilities were not investigated in this animal model of Rett syndrome (Katz et al., 2012). To evaluate activity and anxiety in the Bird MeCP2$^{-/y}$ mice, animals were tested in an open field and in an elevated plus maze, respectively, at basal conditions and after two weeks treatment with Desipramine 10 mg/Kg and Mirtazapine 50 mg/Kg. The open field test revealed that the distance travelled by MeCP2 KO mice was reduced with respect to the WT animals, but not significantly. Antidepressant treatments reduced significantly the activity of the mice, increasing the time in which they stay immobile (Figure 8A, B; *One Way ANOVA*). MeCP2$^{-/y}$ mice exhibited reduced movements with the reduction of the number of entries around the different zones of the arena (border, middle, center), even if the proportion of the entries were maintained: all the groups of animals moved from the border to the middle zone and sometimes passed in the center of the arena (Figure 8C). Moreover, there was not a reduction of anxiety since the time spent in the different zones (border, middle, center) was comparable for all the groups of animals (Figure 8D).

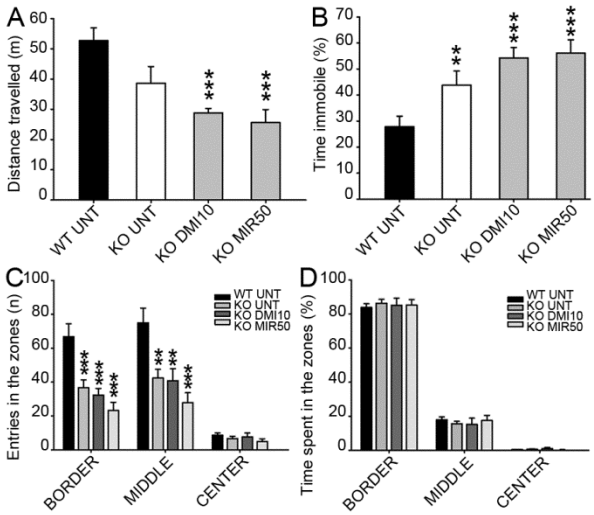


Figure 8. The motility in the open filed test is reduced by antidepressants treatment. n = 11 WT UNT; 10 KO UNT; 11 KO DMI10; 10 KO MIR50. **A)** Distance travelled in meters of Wild type and MeCP2$^{-/y}$ mice untreated (WT UNT; KO UNT) and treated with Desipramine 10 mg/Kg (KO DMI10) and Mirtazapine 50 mg/Kg (KO MIR50). Values are represented as mean ± SEM. (***p<0.001; *One way ANOVA*). **B)** The time spent immobile in percentage (%) respected to the total time of the test (= 20 minutes) by WT UNT, KO UNT, KO DMI10; KO MIR50 mice. (**p<0.01; ***p<0.001; *One way ANOVA*). **C)** The number of entries in the different zones (border, middle, center) by WT UNT, KO

UNT, KO DMI10; KO MIR50 mice. Values are represented as mean ± SEM. (**p<0.01; ***p<0.001; *One way ANOVA*). **D)** The time spent in the different zones by WT UNT, KO UNT, KO DMI10; KO MIR50 mice expressed in percentage (%) to the total time of the test.

We also evaluated the activity of MeCP2$^{-/y}$ mice: we observed that the number of episodes in which the animals remained completely immobile (freezing) were significantly reduced in MeCP2$^{-/y}$ mice treated with the antidepressants (Figure 9A). However, the time of freezing increased in MeCP2$^{-/y}$ mice untreated and treated with the antidepressants compared to wild type animals even if not significantly (Figure 9B). The capacity of the mice to stand on its hind legs (rearing) was significantly reduced in MeCP2$^{-/y}$ mice treated with Desipramine and Mirtazapine compared to wild type, but not significantly reduced in MeCP2$^{-/y}$ untreated mice (Figure 9C). On the contrary, the number of grooming was reduced in MeCP2$^{-/y}$ untreated mice and rescued to wild type level in KO mice treated with the antidepressants, in particular with Mirtazapine 50 mg/Kg (Figure 9D). Then, we observed the typical behavior of MeCP2$^{-/y}$ mice to jump on the wall without control (hopping behavior), which is less evident in WT animals and in MeCP2$^{-/y}$ mice after antidepressant treatments (Figure 9E).

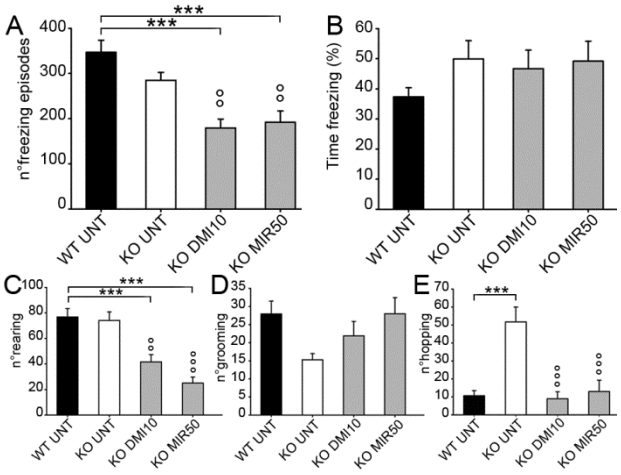


Figure 9. The activity in the open field test. n = 11 WT UNT; 10 KO UNT; 11 KO DMI10; 10 KO MIR50. **A)** The number of freezing episodes in wild type and MeCP2-/y untreated mice (WT UNT; KO UNT) and in KO mice treated with Desipramine 10 mg/Kg (KO DMI10) and Mirtazapine 50 mg/Kg (KO MIR50). Values are represented as mean ± SEM. (*referred to WT UNT; °referred to KO UNT; ***p<0.001; °°p<0.01; *One way ANOVA*). **B)** Time freezing in percentage (%) to the total time of the test (= 20 minutes) of WT UNT, KO UNT, KO DMI10 and KO MIR50 mice (*One way ANOVA*). **C, D, E)** Number of rearing, grooming and hopping behaviour of WT UNT, KO UNT, KO DMI10 and KO MIR50 mice. Values are represented as mean ± SEM. (*referred to WT UNT; °referred to KO UNT; ***p<0.001; °°p<0.01; °°°p<0.001 *One way ANOVA*).

To investigate the anxiety-like behavior we tested MeCP2$^{-/y}$ mice with the elevated plus maze and then, we evaluated the effects after Desipramine and Mirtazapine treatment. Like open field test, we observed a reduction in the distance travelled, and accordingly an increase immobility in KO untreated mice, more evident after drugs treatment (Figure 10A, B). We observed the same proportion of the entries in the zones (open arms, center, closed arms) for all the animal groups: they entered more times in the center because it was an obligated passage to move in the closed and opened arms (Figure 10C). However, when we analyzed the time spent in the different zones, we observed that MeCP2$^{-/y}$ untreated mice spent more time in the open arms respected to WT mice, and that this situation was significantly recovered after drug treatments (Figure 10D).

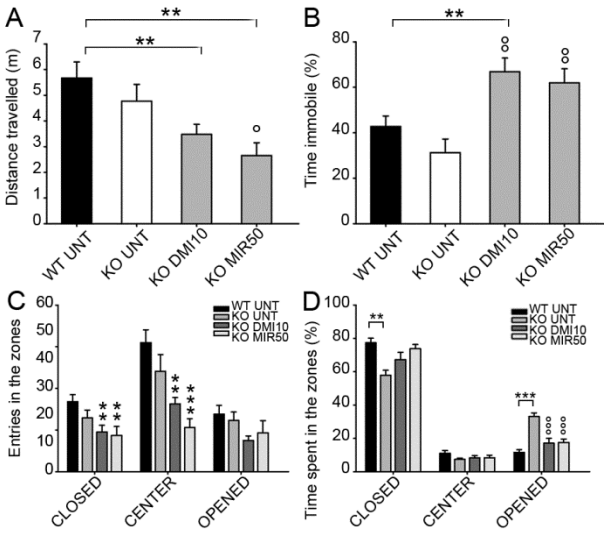


Figure 10. The anxiety in the elevated plus maze test. n = 11 WT UNT; 10 KO UNT; 11 KO DMI10; 10 KO MIR50. **A)** Distance travelled in meters of Wild type and MeCP2$^{-/y}$ mice untreated (WT UNT; KO UNT) and treated with Desipramine 10 mg/Kg (KO DMI10) and Mirtazapine 50 mg/Kg (KO MIR50). Values are represented as mean ± SEM. (*referred to WT UNT; °referred to KO UNT; **p<0.01; °p<0.1; *One way ANOVA*). **B)** The time spent immobile by WT UNT, KO UNT, KO DMI10; KO MIR50 mice expressed in percentage (%) respect to the total time of the test (= 5 minutes). (°°/**p<0.01; *One way ANOVA*). **C)** The number of entries in the different zones (closed, center, open) by WT UNT, KO UNT, KO DMI10; KO MIR50 mice. Values are represented as mean ± SEM. (**p<0.01; ***p<0.001; *One way ANOVA*). **D)** The time spent in the different zones by WT UNT, KO UNT, KO DMI10; KO MIR50 mice expressed in percentage (%) to the total time of the test (**p<0.01; ***/°°°p<0.001; *One way ANOVA*)..

In conclusion, we observed a reduction in the motility and in the activity in MeCP2$^{-/y}$ mice, which were more evident after antidepressant treatments. However, a positive effect on

anxiety was observed when MeCP2$^{-/y}$ mice treated with Mirtazapine spent their time more in the closed arms like WT animals.

BDNF expression levels in Rett patients and in MeCP2$^{-/y}$ mice. Previous studies have highlighted a possible role of BDNF to rescue the neuronal and respiratory phenotype in Rett syndrome (Ogier et al., 2007, Kline et al., 2010). Accordingly, we investigated the expression of BDNF in Rett mice before and after antidepressants treatment.

BDNF was first analyzed in post mortem samples from 3 Rett patients and 3 healthy donors. To verify the integrity of the samples of human somatosensory cortex obtained from three healthy donors and three Rett patients (Table 1), we performed Nissl staining on a series of fresh frozen sections and we run two tests of RNA quality. Brain sections labeled with Nissl staining showed normal cortical lamination and did not reveal any tissue damage (Figure 11A). The RNA quality was verified by agarose gel electrophoresis. The two bands of ribosomal RNA (28S and 18S) were clearly visible and the ratio 28/18S was within normal values for all samples (Figure 11B). The quality of the retro-transcribed cDNAs was verified via PCR amplification of the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which showed similar band intensity at the densitometric analysis (Figure 11B). As the histological appearance of the different human brain samples and the RNA quality were comparable, we concluded that there was no obvious effect of post-mortem delay, which was comprised between 5 and 18 hours.

To investigate BDNF isoforms expression in Rett patients and healthy controls, we performed semi-quantitative real time PCR (qRT-PCR) using specific pair of primers (Table 2 on the left). The expression levels of BDNF isoforms were different among the three patients which had different MeCP2 mutations (Figure 11C), while they were comparable between healthy donors (Supplementary Figure 1). In patient RTT1815 (mutation Het.IVS3-2A<G) all isoforms resulted increased but only exon V and VII were significantly higher than in control subjects (ex V $p = 0.015$; ex VII $p = 0.004$; Mann-Whitney U test). In patient RTT4516 (mutation not determined = N.D.) there was no significant regulation of the different BDNF isoforms with respect to healthy donors, while patient RTT4852 (missense mutation Het.G451T in exon 4) presented a global reduction of BDNF (CDS), which was particularly marked for ex I, ex III, ex V transcripts (CDS $p = 0.012$; ex I $p = 0.004$; ex III $p = 0.031$; ex V $p = 0.005$; Mann-Whitney U test). Additionally, this patient presented a significant increase in

the ex VII isoform (p = 0.002; Mann-Whitney U test) (Figure 11C). This last case was the only one in agreement with the data described by Wang and collaborators (Wang et al., 2006) in the Jaenisch MeCP2 KO mouse model of the Rett Syndrome, in which they observed a significant reduction of total BDNF in the brain.

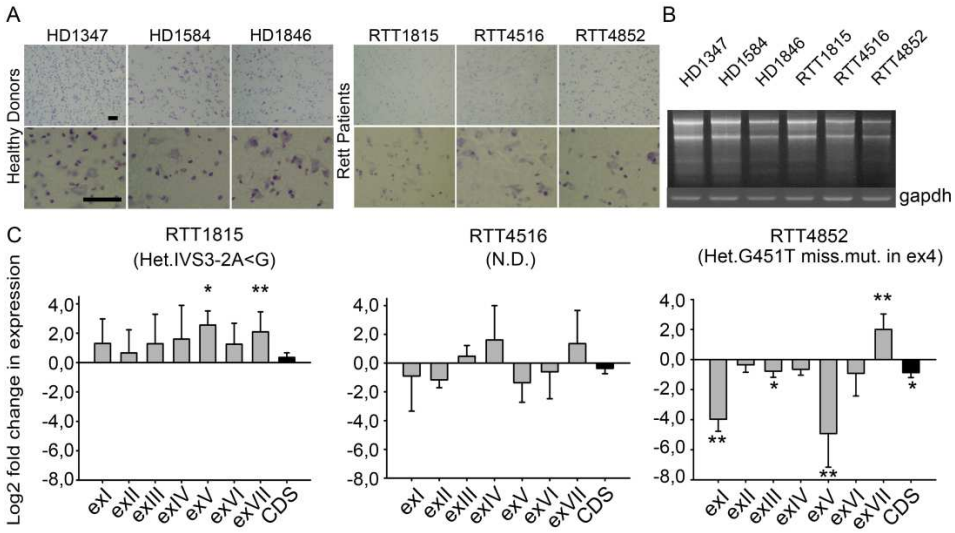


Figure 11. Quality control of human brain sample tissues (Healthy Donors: HD1347;HD1584;HD1846; Rett Patients: RTT1815;RTT4516; RTT4852) and qRT-PCR analysis of BDNF isoforms and total BDNF expression in RTT patients. Human brain samples were received from NICHD Brain and Tissue Bank for Developmental Disorders. Histological samples correspond to human somatosensory and motor cortex (Broadmann areas 1-5) and were conserved as frozen samples to allow biochemical analysis. **A)** Nissl staining of 20 µm sections. Pictures were taken at different magnification (10X and 40X), scale bar = 50 µm. **B)** Upper part: agarose gel electrophoresis to test the integrity of extracted RNAs. The two bands at 28S and 18S are clearly distinguishable. Lower part: agarose gel electrophoresis of PCR products corresponding to GAPDH gene was performed as quality control for cDNA retrotranscribed from the extracted RNAs. **C)** qRT-PCR: results are represented in logarithmic scale: positive and negative bars indicate respectively an increase/decrease of BDNF isoforms expression, comparing to healthy donors (= 0 ± SD). Variations in expression among the RTT patients are due to their different MeCP2 mutation. Data are analyzed with a Mann-Whitney U test. *p<0.1; **p<0.01. RTT1815 has a global increase of the isoforms, particularly significant for the ex V and ex VII; RTT4516 has an up/down regulation of the exons but not significant; RTT4852 has a global reduction, particularly evident for the total BDNF, ex I-III-V. The patient has also a significant increase of the ex VII isoform.

To investigate the expression levels of BDNF in the Bird MeCP2 KO mouse model (males MeCP2$^{-/y}$), we quantified by qRT-PCR the different BDNF transcripts at postnatal days p35, p42, p49 (n = 4 for each genotype and age). In MeCP2$^{-/y}$ mice of all ages analyzed, we found a generalized decrease in the abundance of BDNF isoforms with respect to wild type littermates. However, at p35 the difference was significant only for ex VIII, while at p42,

when all mice in the colony were showing clear Rett syndrome symptoms, there was a massive decrease in most isoforms, in particular of ex I ($p = 0.037$), ex II ($p = 0.049$), ex III ($p = 0.020$), ex IV ($p = 0.014$) and ex VI ($p = 0.023$) ($n = 4$ animals per group, PCR in quadruplicates, Mann-Whitney U test). Surprisingly, at p49, BDNF isoform expression levels were not significantly different between MeCP2$^{-/y}$ and wild type animals (Figure 12).

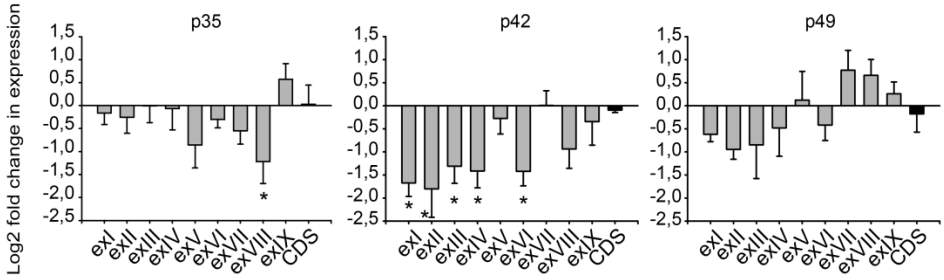


Figure 12. qRT-PCR on BDNF transcripts levels in cortex and hippocampus from wild-type and MeCP2$^{-/Y}$ mice at different ages (p35-42-49). MeCP2 null mutation is associated with a dynamic and deregulated expression of total BDNF and specific BDNF transcripts in cortex and hippocampus; more evident is the decreasing of BDNF isoforms I-II-III-IV and VI in the symptomatic mice p42. Results are represented in logarithmic scale: positive and negative bars indicate respectively an increase/decrease of BDNF isoforms expression, comparing to WT mice (= 0 ± SD). A Mann-Whitney U test is performed comparing all WT mice vs all KO mice (*$p<0.1$).

Effects of antidepressant treatments on BDNF transcripts levels in MeCP2$^{-/y}$ mice. BDNF up-regulation was described as a possible treatment for the Rett syndrome (Chen et al., 2003, Martinowich et al., 2003). Since several antidepressants are known to increase BDNF expression (Castren and Rantamaki, 2010), we hypothesized that a pharmacological treatment could be used as a possible therapeutical strategy to rescue BDNF expression to a normal level in Rett mice. We selected Desipramine as control drug and Mirtazapine because in addition to enhance BDNF expression (Coppell et al., 2003, Dias et al., 2003, Rogoz et al., 2005, Balu et al., 2008), they can also stimulate serotonergic and noradrenergic systems which are severely affected in Rett syndrome patients (Nibuya et al., 1995, Berendsen and Broekkamp, 1997, Dazzi et al., 2002, Marek et al., 2003, Nakayama et al., 2004, Yamamura et al., 2011). In addition, Desipramine was previously tested in Rett syndrome (Roux et al., 2007, Roux and Villard, 2007). Considering that most BDNF transcripts were significantly reduced at p42, the mice started to show the first signs of Rett around p30 and the effect of the treatment with antidepressant is visible after two weeks, we decided to treat MeCP2$^{-/y}$ mice from p28 to p42. Semi-quantitative real time PCR analysis was performed on total BDNF

mRNA (CDS) and on the transcripts that were decreased in MeCP2$^{-/y}$ mice at p42 coding for ex I, ex II, ex III, ex IV, ex VI. Desipramine and Mirtazapine 10 mg/Kg induced slight increase in the mRNAs encoding these BDNF isoforms, except exon III which resulted decreased, although none of these variations was statistically significant with respect to WT animals (n = 3 animals per group, PCR in quadruplicates, Mann-Whitney U test; Figure 13). Even the higher concentration of Mirtazapine (50 mg/Kg) was unable to produce a significant rescue of BDNF mRNA levels for these isoforms (n = 3 animals per group, PCR in quadruplicates, Mann-Whitney U test; Figure 13).

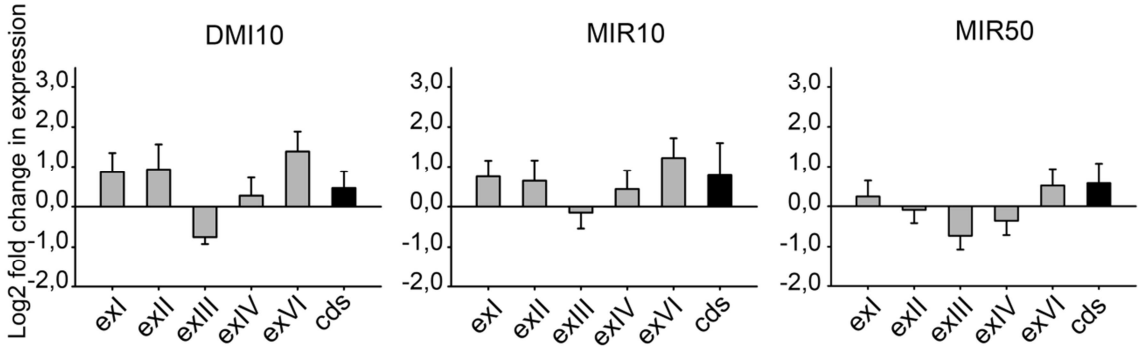


Figure 13. qRT-PCR on BDNF transcripts levels in cortex and hippocampus from wild-type and MeCP2$^{-/Y}$ mice after drug treatments. The isoforms I-II-III-IV- VI and CDS are not significant altered in MeCP2 KO mice after the treatment with DMI10, MIR10 and MIR50. Results are represented in logarithmic scale: positive and negative bars indicate respectively an increase/decrease of BDNF isoforms expression, comparing to WT mice (= 0 ± SD). A Mann-Whitney U test is performed comparing all WT mice vs all KO mice.

Effects of antidepressant treatments on BDNF protein level in the forebrain of MeCP2$^{-/y}$ mice. Previous studies showed that the mRNA levels encoding BDNF do not correspond to the actual levels of the protein (Tropea et al., 2001). Therefore, we evaluated if BDNF protein level in MeCP2$^{-/y}$ mice could be rescued after Mirtazapine treatment, even if the mRNA isoforms were not significantly up-regulated by the treatment. Therefore, mature BDNF protein level in the forebrain were quantified using Western blot analysis of homogenates from hippocampus and cortex pooled together. Moreover, the pharmacological treatment with Desipramine 10 mg/Kg, Mirtazapine 10 mg/Kg, or Mirtazapine 50 mg/Kg had no significant effect on the levels of mature BDNF in WT or MeCP2$^{-/y}$ mice forebrains (n = 1 animal per

group, Western-blot in triplicates, *One Way ANOVA*; Figure 14A). To further verify this result, we performed an ELISA assay with two different kits that recognize all BDNF isoforms (Promega and Chemikine; Figure 14 B, C, respectively). We observed that with both ELISA kits, MeCP2$^{-/y}$ mice treated with vehicle showed an increase of BDNF compared to WT mice, and the protein level was reported to control level in MeCP2$^{-/y}$ mice treated with Desipramine and Mirtazapine (n = 3 animals per group, in triplicates, *One Way ANOVA.* Figure 14B, C).

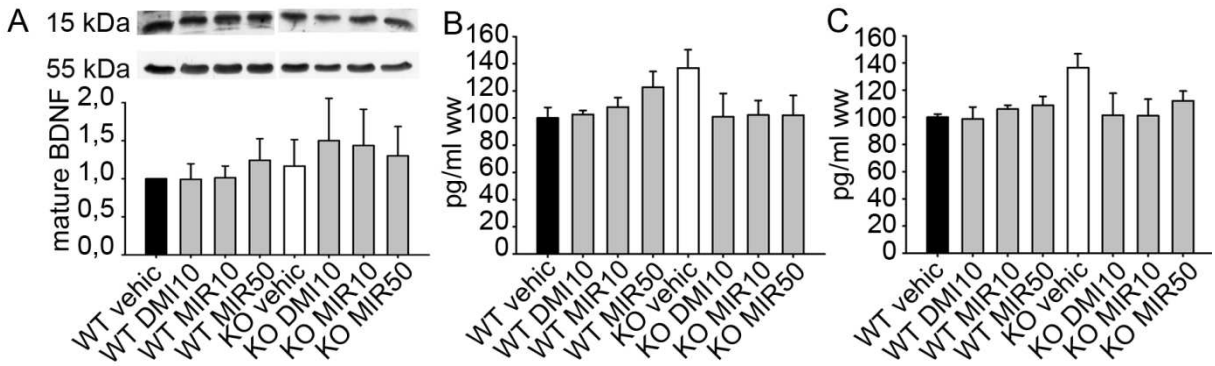


Figure 14. BDNF protein level in the forebrain of WT and MeCP2$^{-/y}$ mice. A) Western blot for BDNF on hippocampus and cortex lysates from wild type and MeCP2 KO mice treated with vehicle (vehic), Desipramine 10 mg/Kg (DMI10), Mirtazapine 10 mg/Kg (MIR10) and Mirtazapine 50 mg/Kg (MIR50). *One Way ANOVA*. **B)** ELISA assay Promega kit on hippocampus and cortex of wild type and MeCP2 KO treated mice treated with vehicle (vehic), Desipramine 10 mg/Kg (DMI10), Mirtazapine 10 mg/Kg (MIR10) and Mirtazapine 50 mg/Kg (MIR50). *One Way ANOVA*. **C)** ELISA assay Chemikine kit on hippocampus and cortex of wild type and MeCP2 KO treated mice treated with vehicle (vehic), Desipramine 10 mg/Kg (DMI10), Mirtazapine 10 mg/Kg (MIR10) and Mirtazapine 50 mg/Kg (MIR50). *One Way ANOVA*.

Effects of antidepressant treatments on BDNF protein level in the cortex and hippocampus of MeCP2$^{-/y}$ mice. In front of the results obtained on pooled lysates from cortex and hippocampus, we hypothesized that specific variations could be undetectable in case of contrasting regulation of BDNF between these two brain areas. Therefore, we investigated BDNF protein levels in the two brain regions separately using the ELISA kit from Promega. We observed an increase of BDNF level in the cortex of MeCP2$^{-/y}$ mice comparing to WT and an opposite effect in the hippocampus, but not significant. However, BDNF levels both in hippocampus and cortex seemed to return at the level of the control after

Desipramine 10 mg/Kg or Mirtazapine 50 mg/Kg treatment (n = 4 animals per group, in duplicates *One Way ANOVA*. Figure 15A).

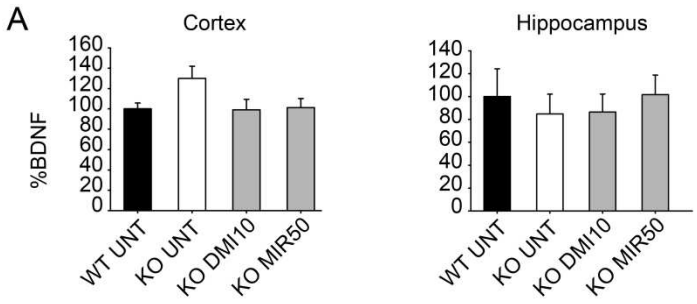


Figure 15. BDNF protein level in the cortex and hippocampus of WT and MeCP2$^{-/y}$ mice. A) ELISA assay Promega kit for BDNF on hippocampus and cortex separately of wild type and MeCP2 KO untreated mice (WT UNT; KO UNT) and treated mice with Desipramine 10 mg/Kg (KO DMI10) and Mirtazapine 50 mg/Kg (KO MIR50). Values are represented as percentage (%) on WT level. *One Way ANOVA.*

DISCUSSION

The clinical course of the Rett syndrome is characteristic for the girls affected by this disorder. However, the severity of Rett syndrome is different among patients, ranging from a mild to a severe phenotype (Chahrour and Zoghbi, 2007). This phenotypic variability was reproduced in different mouse models. In 2011, a workshop was organized to evaluate the variability in the genetic of MeCP2 mutant mice, the methodologies used by the laboratories to produce and analyze data and in particular, to define the physiological and behavioral phenotypes of the different mouse models which were considered more or less near to the clinical observations in Rett human individuals. The aim of the workshop was to define the aspects in the different mouse models which were important for preclinical studies of the Rett syndrome. The participants observed that in all strains of MeCP2 deficient mice there was a marked reduction in brain size with alterations in neuronal morphology and a quite reproducible variation in respiratory and cardiac activities. The phenotype which was more distant from the Rett individuals concerned the motor, cognitive and social behavior which varied among the different mouse models of the disorder.

Thus, in this project, we analyzed neuronal morphology, cardiorespiratory function, motor function and anxiety behavior in MeCP2$^{-/y}$ mice in a pharmacological preclinical study. We evaluated the effects of two weeks treatment with the antidepressant Mirtazapine at 50 mg/Kg and we found that the drug treatment rescued brain weight, the micro and macro architecture of the somatosensory cortex, cortical GABAergic synaptic transmission and cardiorespiratory activities which were altered in MeCP2$^{-/y}$ mice. However, the sedation caused by the high concentration of Mirtazapine induced a reduction of the motility and activity of MeCP2$^{-/y}$ mice, although the anxiety behavior was comparable to the controls. In addition, Mirtazapine had no effect on the synaptic transmission in terms of mRNA expression of the different splice variants of BDNF in MeCP2$^{-/y}$ mice, even if Mirtazapine seemed to recover the neurotrophin protein level.

We found that Mirtazapine restored normal brain weight and thickness of the somatosensory cortex, especially of layer II-III, in which pyramidal neurons recovered size of somata, diameter of apical dendrites, arborization of basal dendrites and density of spines in particular, of stubby spines. Previous experiments showed that p42 MeCP2$^{-/y}$ mice exhibited a reduction

in the thickness of the somatosensory cortex especially in layers II-III and V (Fukuda et al., 2005). A reduction in the total thickness of the somatosensory cortex in layers II-III, V and VI was observed also in p56 MeCP2$^{-/y}$ mice by Kishi and colleagues in 2004 (Kishi and Macklis, 2004).

We examined p42 MeCP2$^{-/y}$ mice, i.e. at the time when they already present the signs of Rett syndrome but are not close to death and we used the Bird strain because it presents more significant alterations in brain weight, volumes of cortex and cerebellum, than those observed in the Jaenisch strain (Belichenko et al., 2008, Belichenko et al., 2009). The alterations in cortical morphology in MeCP2$^{-/y}$ mice are highly reproducible in different laboratories, including ours, and closely resemble those observed in patients thus, they are considered strong evidence of face validity of these Rett mouse models (Katz et al., 2012). Accordingly, measurement of cortical morphology represents a robust parameter to evaluate possible pharmacological treatments.

Post-mortem brains from Rett patients showed reduced thickness and simplified structure of layers II-III pyramidal neurons in frontal, motor and inferior temporal regions (Armstrong, 2005). Previous studies using Golgi staining in brain tissue of p42 MeCP2$^{-/y}$ mice demonstrated that pyramidal neurons exhibit a reduction of the soma area, the diameter of the proximal dendrite on the first branch and the diameter of the distal dendrite at 100 µm from the soma. However, no significant differences were observed in the number of spines on either primary or secondary dendrites. A Sholl analysis revealed also a reduction in the apical dendritic arborization in MeCP2$^{-/y}$ mice (Kishi and Macklis, 2004, Fukuda et al., 2005). In our study, analysis of the morphology of layer II-III pyramidal neurons using Golgi staining confirmed the results obtained by Fukuda and Kishi. However, we extended the findings of previous studies, by analyzing the arborization of the basal dendrites which resulted to be strongly affected in p42 MeCP2$^{-/y}$ mice and showed reduced number of spines on of stubby spines.

At present, there is no cure for the Rett syndrome. Some approaches tried to reverse the symptoms of this disorder starting from the reintroduction of MeCP2 gene or through reactivation of the normal allele (Luikenhuis et al., 2004). An alternative approach to rescue the Rett phenotype is to act on the factors that are downstream of MeCP2 function through a pharmacological treatment. These factors include neurotrophins (like BDNF or IGF-1) and neurotransmitters (like noradrenaline, serotonin and dopamine). A deficit in monoamine levels, including noradrenaline, serotonin and dopamine was reported in the brain and

cerebrospinal fluid of both Rett patients and MeCP2$^{-/y}$ mice (Santos et al., 2010). We considered antidepressants as possible candidates because they are able to reestablish monoamine systems functioning. Effects of antidepressants become evident from 2-3 weeks onward and therefore we chose 2 weeks as the possible minimum time period for a treatment (Quitkin et al., 1984). We selected Desipramine, a tricyclic antidepressant previously used in a clinical trial for Rett syndrome. Desipramine reduced apneas and prolonged the survival in MeCP2$^{-/y}$ mice (Roux and Villard, 2007). However, it was recently reported that Desipramine may induce cardiac failure and death. Thus, we selected Mirtazapine, which is a tetracyclic antidepressant approved by food and drug administration (FDA) for severe depression and it shows high tolerability and few side effects (very little orthostatic hypotension). An overdose of this drug induces only an evident sedative effect. Mirtazapine ameliorated the neurocognition observed in schizophrenic patients (Stenberg et al., 2010, 2011) and this could be explained by the capacity of this drug to increase the release of noradrenaline and dopamine in prefrontal cortex (Devoto et al., 2004, Nakayama et al., 2004), a region involved in the control of emotion and cognitive functions. In this study, Mirtazapine (50 mg/Kg) treatment restored the deficits observed in MeCP2$^{-/y}$ brains and neurons. In conclusion, due to its pleiotropic effects and reduced side-effects, Mirtazapine appears as a strong candidate drug for Rett syndrome.

In addition to induce morphological neuronal changes, Mirtazapine treatment was found to restore the functionality of $GABA_A$ sygnalling lost in MeCP2$^{-/y}$ neurons. The GABA-induced current reduction observed in oocytes injected with cortical membranes from MeCP2$^{-/y}$ mice could be a consequence of many factors such as an impaired receptor–channel function, a decreased number of receptors, or an altered GABA receptors subunit composition. Abnormalities in the density and composition of $GABA_A$ receptors have been reported in young human brains affected by Rett syndrome as well as in the cerebrum of MeCP2 deficient mouse (Yamashita et al., 1998, Samaco et al., 2005). Interestingly, the loss of MeCP2 in mouse and in human Rett cortex caused a reduction in the expression of GABRB3 gene encoding the β3 $GABA_A$ subunit (Samaco et al., 2005). Such change could be also related to the different GABA affinity of the GABA receptors similarly to what was observed in cortical tissue deriving from Angelman syndrome affected patients (Roden et al., 2010).

In this study, we showed that Mirtazapine induced an increase in GABA currents in MeCP2$^{-/y}$ mice, likely due to an increase in the density of GABA receptors. In 2004, it was shown that the antidepressant Fluoxetine, increases monoamine levels in prefrontal cortex, and this effect

was secondary to a desensitization of 5-HT_2 receptors leading to a positive modulation of GABAergic transmission (Zhong and Yan, 2004). Similarly, Mirtazapine which is an inhibitor of 5-HT_2 receptors, in addition to regulating release of norepinephrine (NE), serotonin (5HT) and dopamin (DA) in the cortex, can also regulate GABAergic transmission, causing a return of neuronal activity to its homeostatic set point with restoration of normal neuronal morphology.

Monoamines networks have an important role in the neuronal control of breathing (Richter et al., 2003, Hilaire et al., 2004). Changes in NE and 5HT systems alter the maturation of respiratory networks during embryonic development with severe consequences in the postnatal stages (Hilaire et al., 2004). Around postnatal day 30 and successively, $MeCP2^{-/y}$ mice exhibited an evident reduction of NE and 5HT levels in medulla with irregular breathing characterized by respiratory pauses (Viemari et al., 2005, Ogier et al., 2007, Abdala et al., 2010, Voituron et al., 2010). In addition, neuronal regulation of the respiratory network is closely linked to cardiovascular system, and it explains the peripheral vasomotor and the cardiac disturbances observed in Rett patients (Julu et al., 1997, Guideri et al., 1999). In MeCP2 mutant mice was observed a prolonged QT interval, with a reduction of the heart rate but the vasomotor disturbances were not determined (McCauley et al., 2011).

In this work, we used the non-invasive MouseOX instrument to evaluate the heart and breath rate in $MeCP2^{-/y}$ mice and the effects of Mirtazapine treatment. We observed that heart and breath rates which were significantly reduced in $MeCP2^{-/y}$ untreated mice, were recovered after Mirtazapine treatment without alterations in the level of oxygen saturation. In addition, the negative effect on pulse distention due to the treatment with Desipramine, was not observed after Mirtazapine treatment. The cardiorespiratory phenotype observed in the different MeCP2 mutant mice is similar to that observed in Rett patients, and with our study we demonstrated that Mirtazapine treatment could ameliorate some symptoms of this disorder, with no cardiorespiratory side-effects.

The phenotypes which are more variable among the MeCP2 mutant mice and are more distant from Rett individuals, are the motor, cognitive, social and anxiety behavior. Only a reduced latency to fall off the rotarod was observed in $MeCP2^{-/y}$ Bird mice at 5 weeks of age. No data are available on cognitive performance for $MeCP2^{-/y}$ Bird mice, although recent studies described variable cognitive impairments in heterozygous female (Santos et al., 2012). On the other hand, sociability differs among the different strains of MeCP2 mutant mice, depending on the mutation and the background. $MeCP2^{-/y}$ Bird mice showed an increased sociability in

terms of time spent to explore unfamiliar mice (Kerr et al., 2008). Open field and elevated plus maze test revealed that anxiety behavior was not altered in 4 week-old MeCP2$^{-/y}$ Bird mice (Santos et al., 2007). In contrast, MeCP2$^{-/y}$ Jaenish mice showed reduced anxiety behavior at 8 weeks of age, spending more time in the open arms both in the zero maze and elevated plus maze but spending more time freezing on zero maze (Stearns et al., 2007).

In our study, we observed that, both in open field and elevated plus maze tests, p42 MeCP2$^{-/y}$ mice reduced their motility and spent more time freezing. Also the activity in terms of rearing and grooming was reduced, unlike the hopping behavior which may the consequence of seizures which are often observed in Rett patients. The anxiety behavior was evident in both tests when p42 MeCP2$^{-/y}$ mice spent more time than wild type mice in the less anxiety zones (border zone for open field and closed arms for elevated plus maze). However, in the elevated plus maze MeCP2$^{-/y}$ mice were less anxious because they mainly explored the open arms where they spent more time compared to wild type animals. The effects of Mirtazapine treatment were observed in the recovery of a normal level of anxiety in the elevated plus maze, and in the rescue of the grooming and the hopping behavior. The evident reduction in the distance travelled, in the animals treated with the antidepressant drugs could be explained by a sedative effect (Glass et al., 1982, Fawcett and Barkin, 1998b). The sedation was also present in a few wild type animals treated with the antidepressants drugs.

The other phenotype observed both in Rett patients and in MeCP2 mouse models is the cardiorespiratory dysfunction. Two important mechanisms have an effect on the progression of cardiac and breathing irregularities: the alteration in monoamine levels and the disturbances of BDNF expression (Ramirez et al., 2013). In this study, we found that BDNF splice variants expression was variable among Rett patients depending on the mutations in MeCP2 gene. In MeCP2$^{-/y}$ mice, BDNF isoforms levels were changing depending on the age of animals and were particularly reduced at p42, especially exons I, II, III, IV and VI. However, total levels of BDNF protein were not significantly altered in MeCP2$^{-/y}$ mice. In addition, antidepressant treatments with Desipramine 10 mg/Kg or Mirtazapine 10 or 50 mg/Kg were unable to produce any appreciable change in BDNF expression at both mRNA and protein levels.

Previous studies showed lower BDNF mRNA levels in post mortem brain samples from Rett individuals (Abuhatzira et al., 2007, Deng et al., 2007). In 2007, Abuhatzira and colleagues observed a reduction in BDNF exon II and total BDNF, and an increase in BDNF exon I and III in Rett males and females. The difference in the expression of BDNF isoforms was

attributed to the alteration in the repression complexes which bind to the different BDNF promoters (Abuhatzira et al., 2007). In our study, we analyzed female Rett patients, each with a different mutation in MeCP2 gene. We observed that BDNF splice variants were expressed at different level among the patients. The IVS3-2A>G of the patient RTT1815 is a frameshift mutation and this patient showed a general increase in BDNF isoforms levels (although only two were statistically significant). The G451T in the patient RTT4852 is a missense mutation in the exon 4 of MeCP2 and she showed a general decrease in BDNF splice variants expression except for exon VII. The mutation in the patient RTT4516 was not determined and she presented a variable expression of BDNF variants that were not significantly different from normal girls. All the patients were characterized by the development of seizures in their life. Our results demonstrated that Rett patients have a different expression of BDNF splice variants depending on the mutation occurring in MeCP2 gene, even if the clinical course had some common features.

For the first time, we evaluate BDNF expression level in hippocampus and cortex of the Bird's model of Rett syndrome, and we observed a significant reduction of the exon I, II, III, IV and VI at postnatal day 42.

The different knock-out mouse models of Rett syndrome reproduced the typical characteristics of the pathology although showed some subtle phenotype differences, possibly due to strain or construct differences (Katz et al., 2012). There are a few studies on BDNF expressions levels in the different mouse models. In 2007, Ogier and colleagues used cultures of nodose cranial sensory ganglia neurons (NGs) of Jaenisch strain mice (deletion of exon 3 of MeCP2)(Chen et al., 2001b) to demonstrate that total BDNF and the transcripts containing the exons II, IV and V were markedly decreased in $MeCP2^{-/y}$ mice (Ogier et al., 2007). Guy and colleagues developed a mouse model of Rett syndrome based on the deletion of exon 3 and 4 of MeCP2, resulting in a total loss of function of the gene but BDNF levels were not investigated in this mouse (Guy et al., 2001).

In our study, we investigated the effect of the antidepressant treatments on BDNF level in $MeCP2^{-/y}$ mice and we observed no significant rescue of mRNA variants in the hippocampus and cortex (forebrain) compared to wild type littermates.

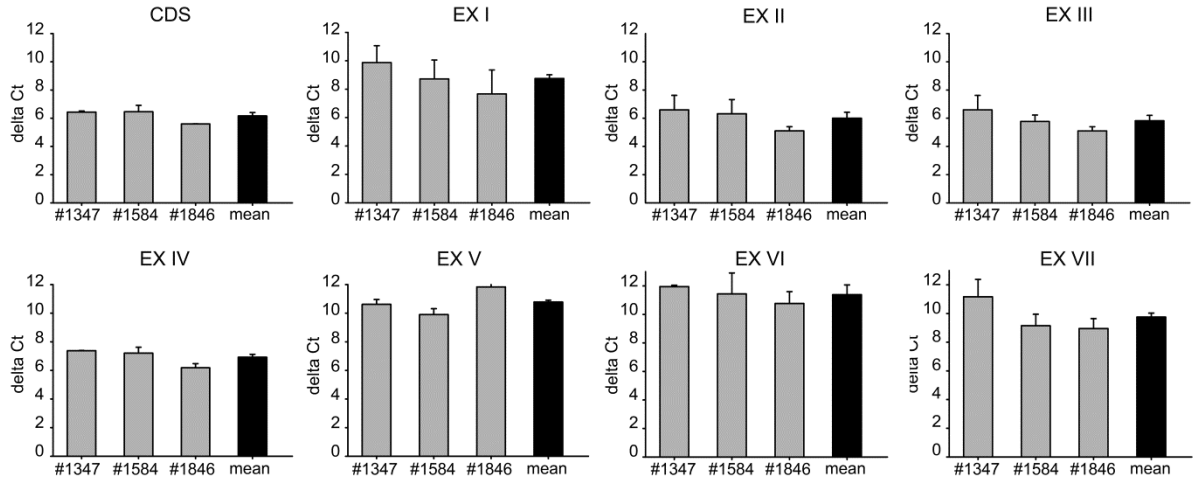
Previous studies reported that BDNF level increased in hippocampus and in serum of depressed patients after an antidepressant treatment (Chen et al., 2001b, Shimizu et al., 2003a, Gonul et al., 2005). In the same way, repeated (but not acute) administration of antidepressant increases mRNA of BDNF and TrkB in both rat hippocampus and cortex (Coppell et al.,

2003, Dias et al., 2003, Jacobsen and Mork, 2004). It was demonstrated that Mirtazapine was able to increase BDNF expression level after a chronic treatment of depressed patients in hippocampus and cerebral cortex (Engel et al., 2013). Rogóz and colleagues, showed that Mirtazapine elevated BDNF mRNA (24%) and protein (30%) levels in both rat hippocampus and cerebral cortex after a chronic treatment (Rogoz et al., 2005).

Using the Jaenisch model, Wang and colleagues showed that the BDNF protein level was significantly reduced in the brainstem and nodose ganglia, but not in cortex and hippocampus, of p35 MeCP2$^{-/y}$ mice compared to wild type littermates (Wang et al., 2006). In the same year, Chang and colleagues using the same mouse model of Wang, demonstrated that BDNF protein level was significantly reduced in the cortex, cerebellum and the rest of the brain in MeCP2$^{-/y}$ mice at 6-8 weeks of age. Differently from these previous results, we observed that BDNF protein levels increased in the forebrain of p42 MeCP2$^{-/y}$ Bird's mice treated with the vehicle compared to wild type, both in western blot (mature BDNF) and in ELISA assays (total BDNF). In addition, Mirtazapine treatment did not altered the levels of BDNF. We reasoned that perhaps, these results were difficult to interpret because BDNF protein levels were measured in homogenates obtained by pooling together hippocampus and cortex. In 2012, Deogracias showed that BDNF protein expression level in the cortex was comparable to wild type animals and was significantly reduced in the hippocampus of p30 MeCP2$^{-/y}$ Bird's mice (Deogracias et al., 2012). BDNF protein was not affected also in the cortical neurons derived from p55 MeCP2$^{-/y}$ mice with respect to wild type (Roux et al., 2012). Considering the cortex and the hippocampus separately, we observed an increase in BDNF protein level in the cortex of p42 MeCP2$^{-/y}$ mice and a reduction in the hippocampus of p42 MeCP2$^{-/y}$ mice with respect to wild type littermates even if they were not statistically significant. Particularly important was the observation that in cerebrospinal fluid and blood serum of Rett patients, BDNF protein level was similar to unaffected individuals (Vanhala et al., 1998, Riikonen, 2003).

In conclusion, our work underline the importance of Mirtazapine treatment in MeCP2$^{-/y}$ mice to restore the main symptoms of the Rett syndrome. However, other studies are necessary to understand if Mirtazapine could be introduced in a clinical trial. In particular, some questions have remained unanswered: 1) can Mirtazapine extends the lifespan of Rett animals? 2) is sedative effect see only in Rett animals or is it present also in Wild type? 3) does the improvement in heart and respiratory rates correlate with restored GABAergic transmission in the brainstem?

Supplementary figure



Supplementary figure 1. Delta Ct of total BDNF and BDNF splice variants in Healthy donors. Delta Ct of the total BDNF (CDS) and of BDNF isoforms (EX I, II, III, IV, V, VI and VII) are not different among the healthy donors (#1347; #1584; #1846). In black bars the mean of delta Ct of the healthy donors.

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