VNIVERSITAT DE VALÈNCIA

SOCIAL AND SENSORY DEFICITS IN RETT SYNDROME:
NEUROANATOMICAL AND BEHAVIOURAL ANALYSES IN A MOUSE MODEL
DEFICIENT FOR *MECP2*

Elena Martínez Rodríguez

2023

Doctoral Programme in Neurosciences

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Supervisor:

Dra. Carmen Agustín Pavón

February 2023

Carmen Agustín Pavón, Profesora Contratada Doctora, del Departamento de Biología Celular, Biología Funcional y Antropología Física de la Universidad de Valencia

CERTIFICA

que Dña. **Elena Martínez Rodríguez**, Máster Euromediterráneo en Neurociencias y Biotecnología por la Universidad de Valencia, ha realizado bajo su dirección el trabajo titulado *"**Social and sensory deficits in Rett syndrome: neuroanatomical and behavioural analyses in a mouse model deficient for Mecp2**"* para la obtención del grado en Doctora en Neurociencias.

Para que conste, en cumplimiento de la legislación, firmo el presente certificado en Burjassot, a 09 de febrero de 2023.

Dra. Carmen Agustín Pavón

Carmen Agustín Pavón, Professora Contractada Doctora, del Departament de Biologia Cel·lular, Biologia Funcional i Antropologia Física de la Universitat de València

CERTIFICA

que Na. **Elena Martínez Rodríguez**, Màster Euromediterrani en Neurociències i Biotecnologia per la Universitat de València, ha realitzat sota la seua direcció el treball amb títol *"**Social and sensory deficits in Rett syndrome: neuroanatomical and behavioural analyses in a mouse model deficient for Mecp2**"* per l'obtenció del Grau de Doctora en Neurociències.

Per què conste, en compliment de la legislació, signe el present certificat en Burjassot, a 09 de febrer de 2023.

Dra. Carmen Agustín Pavón

VNIVERSITAT DE VALÈNCIA Facultat de Ciències Biològiques

Carmen Agustín Pavón, Associate Professor, of the Department of Cell Biology and Physical Anthropology of the University of Valencia

HEREBY CERTIFIES

That Ms. **Elena Martínez Rodríguez**, MSc in Neurosciences and Biotechnology at the University of Valencia, has conducted under her direction the work entitled *"**Social and sensory deficits in Rett syndrome: neuroanatomical and behavioural analyses in a mouse model deficient for Mecp2**"* to obtain the degree of Doctor in Neurosciences.

For the record, in compliance with the legislation, we sign this certificate in Burjassot, on February the 09th, 2023.

Dra. Carmen Agustín Pavón

The present thesis has been funded by 'Ayudas FinRett 2019', the Spanish Ministry of Education and Competitiveness (BFU2016-77691-C21-P) and Conselleria d'Educació, Investigació, Cultura i Esport (PROMETEO/2016/076), Universitat Jaume I (UJI-B2016-45) and the E-Rare-2 JTC 2014 by the German Federal Ministry of Education and Research; Bial Foundation, Grants for Scientific Research, 85/18

AGRADECIMIENTOS

Todavía me cuesta creer que, después de 5 años, finalmente haya llegado hasta aquí. Lo que pensaba que sería sencillo ha resultado ser un viaje bastante largo, con sus altibajos y sus momentos de alegría y frustración. Por suerte, he contado con la ayuda de bastante gente para poder continuar adelante. Tengo claro que nada de esto habría sido posible de no haber sido por el apoyo y la ayuda de muchos de vosotros, y creo que nunca seré capaz de agradecéroslo a todos por completo, aunque lo voy a intentar. Porque todos vosotros habéis creído en mí, incluso cuando yo dudaba, y me habéis allanado el camino.

En primer lugar, quiero agradecérselo a mi tutora y mentora, Carmen, poque nada de esto habría sido posible sin ti. Gracias por remover cielo y tierra para conseguir que pudiese quedarme con vosotros y darme la oportunidad de hacer esta tesis. Gracias por creer en mí, por demostrarme que no hace falta ser una "investigadora excelente" para hacer ciencia necesaria y de calidad. Por todo lo que me has enseñado, tanto dentro como fuera del laboratorio. Porque desde que empecé en el máster contigo hasta el final de este recorrido, me has dado facilidades en todo momento, me has entendido, me has apoyado y me has ayudado mucho más de lo que podría siquiera escribir aquí. Porque mantengo lo que dije hace cinco años, eres la única persona con la que me plantearía hacer una tesis, y me alegro de haberlo hecho.

Tengo claro que todo esto ha sido posible también gracias al apoyo de mis padres, tanto económico como moral. Siempre habéis apostado por mi futuro académico, y habéis hecho todo lo posible para que pudiese seguir adelante. Y, por eso, muchas gracias. A Vivi, por tu compañía durante estos dos últimos años que tan cuesta arriba se me han hecho. Por ayudarme a salir de mis bucles, y enseñarme a priorizar y cuidar de mi salud mental.

A mis “niños” queridos de Valencia: Carlos, Danae, Rafa, Javi, Darren, y en especial a Franchi y Miguel, por todas esas tardes de bravas y cervezas en las que me habéis hecho de guía espiritual. Por acompañarme en mis peores momentos y alentarme a seguir adelante cuando sólo podía pensar en dejarlo todo. Por vuestra gran aportación a esta tesis y a mi vida en general. También quiero agradecérselo a toda la gente del lab que me ha acompañado durante todos estos años, especialmente a Quique, María y a Jose, por todo lo que he podido aprender de vosotros y todas las cosas que habéis hecho por mí. A Mónica, por permitirnos llevar a cabo gran parte de esta investigación gracias a sus muestras y consejos, y por acogerme en su laboratorio en Coimbra. A las asociaciones FinRett, por creer en nosotras y ayudarnos a financiar nuestro proyecto.

A todos vosotros, y mucha más gente que ha estado ahí, muchísimas gracias, y sabed que siempre tendréis un huequito en mi corazón.

CONTENTS

LIST OF ABBREVIATIONS

3V	Third ventricle
AC/ADP	Nucleus of the anterior commissure/anterodorsal preoptic nucleus region
Aca	Anterior commissure
Acb	Accumbens nucleus
AcbC	Nucleus accumbens, core
AcbSh	Nucleus accumbens, Shell
Amy	Amygdaloid nucleus
AOB	Accessory olfactory bulb
AOL	Anterior olfactory nucleus, lateral
AOM	Anterior olfactory nucleus, medial
AON	Anterior olfactory nucleus
AOV	Anterior olfactory nucleus, ventral
BLA	Basolateral amygdaloid nucleus, anterior part
BST/BNST	Bed nucleus of the stria terminalis
BSTMPI	Bed nucleus of the stria terminalis, medial division, posterointermediate part
CC	Corpus callosum
Ce	Central amygdaloid nucleus
CPu	Caudatus putamen
dEn	Dorsal endopiriform cortex
DG	Dentate gyrus of the hippocampus
dlPAG	Dorsolateral periaqueductal grey
DMH	Dorsomedial hypothalamic nucleus
DR	Dorsal raphe nucleus
dSt	Dorsal striatum

DTg	Dorsal tegmental nucleus
En	Endopiriform cortex
GlOB	Glomerular cell layer of the olfactory bulb
GrDG	Granular layer of the dentate gyrus
GrOB	Granular cell layer of the olfactory bulb
Hyp	Hypothalamic nucleus
ICj	Islands of Calleja
LHb	Lateral habenular nucleus
LS	Lateral septum
LV	Lateral ventricle
MCx	Motor cortex
Me	Medial amygdaloid nucleus
MeA	Medial amygdaloid nucleus, anterior part
MePD	Medial amygdaloid nucleus, posterodorsal part
MHb	Medial habenular nucleus
Mi	Mitral cell layer of the olfactory bulb
MOB	Main olfactory bulb
Mol	Molecular layer of the dentate gyrus
OB	Olfactory bulbs
Opt	Optic tract
Pa	Paraventricular hypothalamic nucleus
PAG	Periaqueductal gray
Pe	Periventricular hypothalamic nucleus
PGl	Periglomerular layer of the olfactory bulb
Pir	Piriform cortex
PoDG	Polymorphic-oriens layer of the dentate gyrus
PV	Paraventricular thalamic nucleus

PVN	Paraventricular nucleus
RMS	Rostral migratory stream
SCh	Suprachiasmatic nucleus
SON	Supraoptic nucleus
SOR	Retroquiasmatic part of the supraoptic nucleus
St	Striatum
SVZ	Subventricular zone
Tu	Olfactory tubercle
vHip	Ventral hippocampus
vlPAG	Ventrolateral periaqueductal grey
VMH	Ventromedial hypothalamic nucleus
vmStP	Ventromedial striatopallidum
VP	Ventral pallidum
vSt	Ventral striatum
VTA	Ventral tegmental area

GENERAL INTRODUCTION

Rett syndrome: a rare neurodevelopmental disorder mainly affecting females

Rett syndrome (RTT, OMIM #312750) is a rare neurodevelopmental disorder first described by Andreas Rett in 1966 [(Rett, 2016) translation of the original article (Rett, 1966)]. This syndrome affects 1 in 10-15.000 girls, so it is considered a rare disease. Nonetheless, it represents the second cause of intellectual disability of genetic origin in females, right after Down syndrome (Christodoulou, 2001).

Symptomatology of Rett syndrome

Girls affected with RTT are born apparently healthy and show normal development until the age of 6 to 18 months. At this moment, they show an arrest in their development and start to manifest the typical symptomatology of the disorder. Symptomatology arises progressively, becoming more severe with age, and can be divided in four different stages (see Table 1 and Fig. 1; reviewed in Christodoulou, 2001):

- **Stage I:** a phase characterized by the stagnation of normal development.
- **Stage II:** during this period, changes may be acute and similar to those described in neurodegenerative disorders. It is most defined by devastating cognitive and motor regression. Seizures are also reported in 30-50% of cases of RTT, with electroencephalographic abnormalities by the age of 3-5 years.
- **Stage III:** in this stage, seizures become predominant and intractable in many cases. In addition, 75% of patients start manifesting respiratory abnormalities (hyperventilation, breath-holding, aerophagia, and abdominal bloating). These symptoms, together with the appearance of stereotypic hand movements, become the primary diagnostic features in stage III. By

contrast, there is a partial recovery of hand use, with girls acquiring minimal skills. These girls also show an improvement in social interaction during this period.

- **Stage IV:** clinical evolution reaches a plateau in this stage. There is a cognitive stability, and seizures and respiratory abnormalities start to reduce or even disappear. Half of the patients also develop scoliosis, and the diffuse hypotonia changes to rigidity and dystonia in a majority of the affected girls.

Table 1: Description of symptomatology of Rett syndrome classified according to the four clinical stages (see Baikie et al., 2014; Downs et al., 2016; Hagberg, 1995, 2002, 2005; Hagberg et al., 1983; Leonard et al., 2016; Lombardi et al., 2015; Neul et al., 2010; Rett, 2016; Zoghbi, 2016).

	MAIN SYMPTOMATIC MANIFESTATION
STAGE I	• Difficulties in word development, social interaction, or motor ability
STAGE II	• Functional decline affecting body and cognition • Loss of previously acquired abilities • Stereotypic hand movements (clasping or wringing) • Respiratory abnormalities
STAGE III	• Seizures • Scoliosis • Defects in social interaction • Altered olfactory response • Loss of ability to ambulate • Changes in brain morphology
STAGE IV	• Wheelchair dependency

Other symptoms of RTT in girls include lower weight and general size and height (Schultz et al., 1993), sleep disturbances with night laughing and screaming spells (Ellaway et al., 2001), and altered sensitivity to pain (Downs et al., 2010). There are also disbalances in the normal microbiota (Borghi et al., 2017; Strati et al., 2016) and low bone health (osteopenia and fractures are common; Haas et al., 1997; Pecorelli et al., 2021). Girls also develop problems in heart rate with lower rate variability on average, autonomic dysfunction, and peripheral circulation. Autonomic dysfunction includes peripheral vasomotor disturbances causing cold hands and feet (Acampa &

Guideri, 2006; Hagberg, 2002). A decreased heart rate variability has been described in those patients, and seems to correlate with age and clinical severity (Acampa & Guideri, 2006; Guideri et al., 2001). Girls also manifest difficulties in swallowing and abnormal vagal tone (supporting the autonomic impairment hypothesis; Merbler et al., 2020). Many of them retain their long-term memory, revealing a predilection for objects, people, and music from their childhood. In some cases, memory function is not even affected (Christodoulou, 2001; Downs et al., 2010; Hagberg, 2002; Merbler et al., 2020).

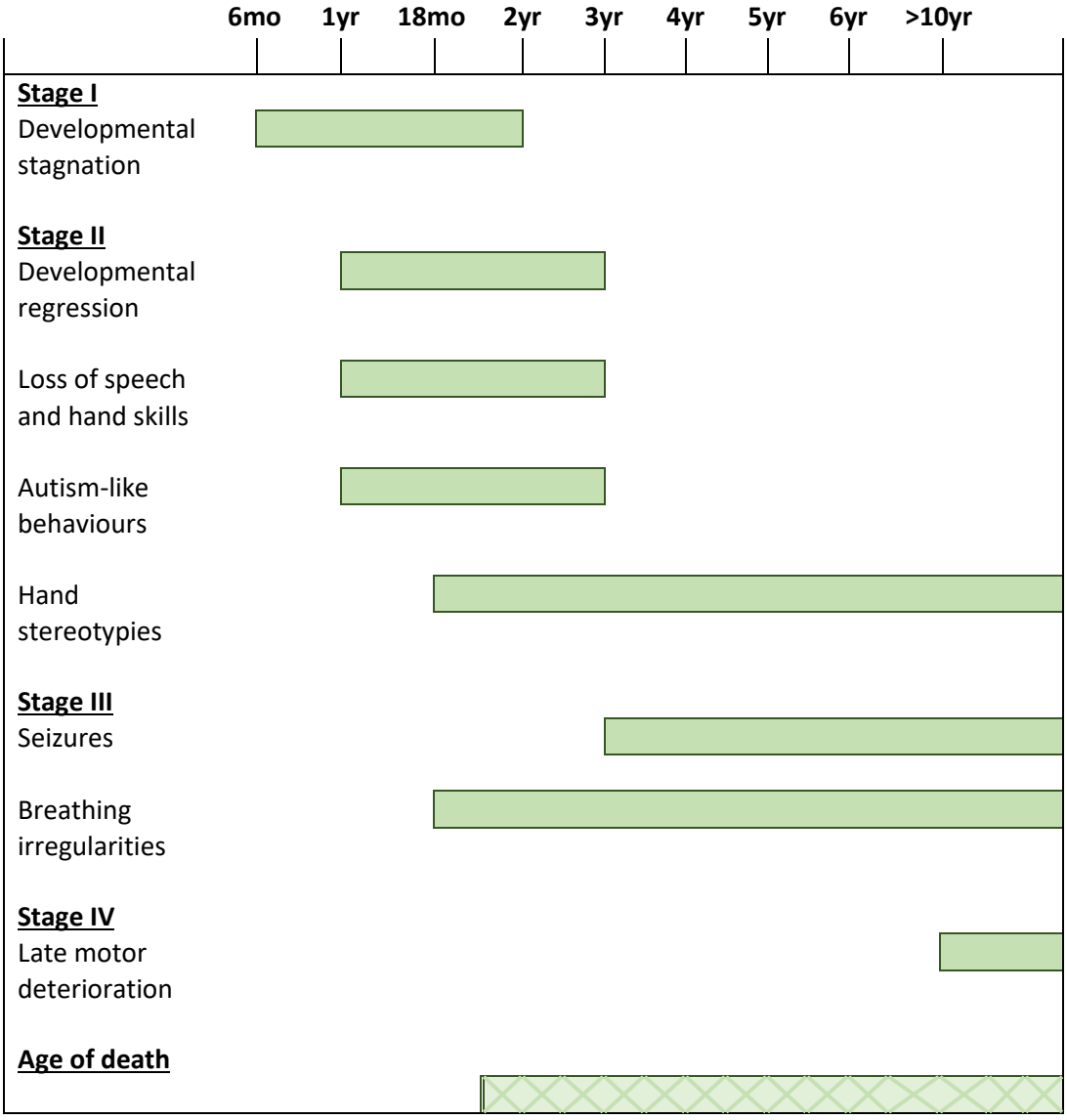


Figure 1: **age of onset of main symptoms in four stages and range of death in girls with Rett syndrome** (adapted from Christodoulou, 2001; Zoghbi, 2016).

Deceleration in head growth takes place even before onset of stage I (by the age of 2-4 months of life). Despite its weight and size, no obvious morphological alterations have been described in the brain of RTT patients. Indeed, there is no evidence of cell loss, inflammation, or gliosis within this organ (D. D. Armstrong, 2001; Jellinger et al., 1988; Pecorelli et al., 2020). However, cortical development seems to be defective, with neurons with small soma and reduced dendritic branching, specifically in the layers III and IV in the motor and frontal cortex, and layer IV of the subiculum (Duncan Armstrong, 2005; Reiss et al., 1993).

As with the onset of symptomatology, age of death differs from one patient to another in ranges from 2 years to adulthood. One third of deaths usually happen before the age of 20 years, primarily during sleep. Only some isolated and relatively healthy cases are able to survive to quite an advanced age (about 40-50 years of age; reviewed in Christodoulou, 2001).

Classical and atypical phenotypes of Rett syndrome

According to the symptomatology, this syndrome can be classified into classical and atypical RTT. Classical RTT includes a regression of development, and shows the four core symptoms of this condition:

1. **Loss of hand skills.**
2. **Loss of speech.**
3. **Stereotypic hand movements.**
4. **Motor abnormalities.**

In atypical RTT, only two out of the four core criteria are required, along with at least five of the eleven supportive criteria (see table 2). A period of regression is also present in both classical and atypical RTT. This period is characterized by either

gradual or sudden loss of hand and communication skills, loss of balance and development of hand stereotypies (reviewed in Leonard et al., 2016). Regression is also followed by a phase of recovery or stabilization.

Table 2: Diagnostic necessary and supporting criteria for Rett (adapted from Neul et al., 2010). Diagnosis should be considered when postnatal deceleration of head growth is observed, but it is not a required symptom for the diagnosis of RTT.

Required for classic RTT	***Required for atypical RTT***
1. A regression followed by recovery or stabilization 2. All main and exclusion criteria 3. Supportive criteria are not required, although are often present	1. A regression followed by recovery or stabilization 2. At least 2 out of the 4 main criteria 3. 5 out of the 11 supportive criteria

Main criteria	***Exclusion criteria***
1. Loss of purposeful hand skills 2. Loss of speech 3. Gait abnormalities (dyspraxia or absence) 4. Stereotypic hand movements	1. Brain injury secondary to trauma, neurometabolic disease, or severe infection causing neurological problems 2. Abnormal psychomotor development by the age of 6 months

Supportive criteria for atypical RTT

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

Hagberg grouped atypical cases of RTT into five different variants (reviewed in Christodoulou, 2001):

1) An apparently **classical RTT** whose onset happens before the age of 6 months, and predomination of seizures.

2) **Congenital or precocious RTT**: constitutes another variant very similar to classical cases, but in which the phase of regression is not clearly identified.

3) **Variants** in which regression develops later and in a more gradual way compared to classical RTT.

4) '**Forme fruste' RTT**: with milder clinical presentation, which is also incomplete and even protracted. Furthermore, regression does not occur until the age of 1 to 3 years, and is less severe compared to classical RTT. In addition, hand use is preserved and stereotypic hand movements are minimal or even atypical.

5) '**Preserved speech'** variant of **RTT**.

The severe functional impairments caused by classical RTT syndrome, make affected girls need substantial support in daily life, with precarious quality of life. On this basis, clarifying the main causes and internal processes impaired in RTT patients becomes essential to improve their conditions and quality of life.

Mutations in *MECP2* gene are the main cause of classical Rett syndrome

At least 95% of the cases of classical RTT are caused by a large battery of different mutations in the *Methyl-CpG-binding protein 2* gene (*MECP2* gene), that usually arise in the paternal germline. The remaining 5% are caused by mutations within the regulatory regions of *MECP2* transcription (Neul et al., 2008; Shin et al., 2013; Zoghbi, 2016; Zoghbi et al., 1999). The *MECP2* gene is located on the X_{q28} chromosome, making RTT an X-linked dominant disorder that affects heterozygous females and produces non-viable hemizygous males (Hagberg et al., 1983). Thus,

boys usually die from severe neonatal encephalopathy before their first year of age [reviewed in (Santos et al., 2009)]. Mutations in this gene include missense and nonsense alterations by deletions and small insertions (Neul et al., 2008). Smaller deletions and slight alterations in the *MECP2* gene give raise to milder phenotypes, whilst larger or complete deletions of the gene lead to a more severe symptomatology (Calfa et al., 2011; Neul et al., 2008). Mutations in *MECP2* have been associated to several neurodevelopmental disorders causing intellectual disability and autism (Gonzales & LaSalle, 2010) of which RTT is the most representative (Zoghbi et al., 1999).

Atypical phenotypes of RTT are either caused by favourably skewed X inactivation patterns or mild hypomorphic mutations in *MECP2* gene. In these cases, girls usually show only a subset of typical features of the syndrome, such as obsessive-compulsive behaviours or slight cognitive deficits. Mild Rett phenotypes have been included within atypical RTT cases, suffering from speech delay, sleep disturbances and tremulous movements. By contrast, those girls are able to retain their capacity to walk, use their hands with ease and even maintain a conversation by the age of 10-12 years. However, they still show mildly ataxic gait and mild hand stereotypies (reviewed in Leonard et al., 2016). In the case of males, slight symptomatology can be caused by slight mutations within *MECP2* gene. An example could be found in the A140V missense mutation. This variant allows boys survive, although manifesting intellectual impairment and even motor and/or psychiatric disorders (reviewed in Zoghbi, 2016). Some studies also reported rare cases of Klinefelter males (XXY) with milder RTT symptoms (Schwartzman et al., 1999).

Abnormalities in the *MECP2* gene have also been observed in some boys suffering from infantile encephalopathy, as well as in some cases with Angelman syndrome phenotype, and boys with intellectual disability (reviewed in D. D. Armstrong, 2001). Similarly, boys with *MECP2*-duplication syndrome display intellectual disability and autism, whereas girls are either asymptomatic carriers or display some

neuropsychiatric symptoms like depression and obsessive-compulsive disorder (Ramocki et al., 2010; reviewed in Zoghbi, 2016).

Although RTT is caused by mutations in the *MECP2* gene, there are other mutations that lead to RTT-like phenotype. These disorders come from a different genetic origin than classical RTT, and are considered as Rett-related disorders (Naidu & Johnston, 2011). One of them is the CDKL5 disorder (Urbanowicz et al., 2015), which is caused by mutations in *FOXG1* gene and is related to early seizure onset.

The molecular function of MeCP2

The *MECP2* gene encodes for the methyl-CpG-binding protein 2 (MeCP2). This protein has a broad distribution throughout the body, with the highest levels in the brain, lungs, and spleen (Kishi & Macklis, 2004; Luikenhuis et al., 2004). In the rodent brain, *Mecp2* is expressed specifically in mature neurons, and absent in glial cells (Akbarian et al., 2001; Jung et al., 2003; LaSalle et al., 2001). *Mecp2* expression is also time and region-specific (Kishi & Macklis, 2004; Mullaney et al., 2004; M. D. Shahbazian et al., 2002), with a very precise mechanism controlling MeCP2 levels inside the cells and tissues (Chao et al., 2007). Despite its high presence in the brain, MeCP2 is preferentially abundant in specific brain areas like the olfactory system or the cerebral cortex (for a review see R. Z. Chen et al., 2001; Coy et al., 1999; Lee et al., 2014; Lombardi et al., 2015; M. D. Shahbazian et al., 2002). This specific distribution gives rise to the idea that MeCP2 performs specialized functions, which are region-dependent. Additionally, *Mecp2* expression is also gender-specific during development, probably involved in dimorphisms in brain morphology and sexual behaviour (Kurian et al., 2007, 2008).

There are two isoforms of MeCP2 protein (MeCP2_e1 and MeCP2_e2) that only differ at the extreme N-terminus and are functionally equivalent (Fig. 2a; (Good et al., 2021; Kerr et al., 2012; Luikenhuis et al., 2004). However, the e1 isoform is the most predominant isoform in brain. MeCP2 is a nuclear protein that binds to the

methylated CpG dinucleotides present within the DNA sequence, through its methyl-CpG binding domain (MBD; Nan et al., 1993). Although it was first considered as a solely transcriptional repressor (Nan et al., 1993), MeCP2 is currently known as a regulator of gene expression, inducing and repressing gene transcription at very precise local levels (Bird, 2002; Chahrour et al., 2008; Guy et al., 2011; Shin et al., 2013; Squillaro et al., 2012). To regulate gene transcription, MeCP2 recruits typical co-repressors like Sin3A and histone deacetylases 1 and 2 (Bird, 2002; R. Z. Chen et al., 2001; Jones et al., 1998; Nan et al., 1998), or activators such as phosphorylated CREB1 (Fig. 2b; Chahrour et al., 2008). This way, MeCP2 modulates gene expression during neuronal development in the embryo, and brain circuitry refinement and maturation in the adult brain (Kishi & Macklis, 2004; Luikenhuis et al., 2004; Tsujimura et al., 2009). Among its functions, we can highlight its role in neuronal maturation, neurite complexity, synaptogenesis, chromatin organization, long-term potentiation, and synaptic plasticity (S. Cohen et al., 2011; Degano et al., 2014; Jones et al., 1998; Kishi & Macklis, 2004; Krishnan et al., 2017; H. Li et al., 2011; Mullaney et al., 2004; Nan et al., 1998).

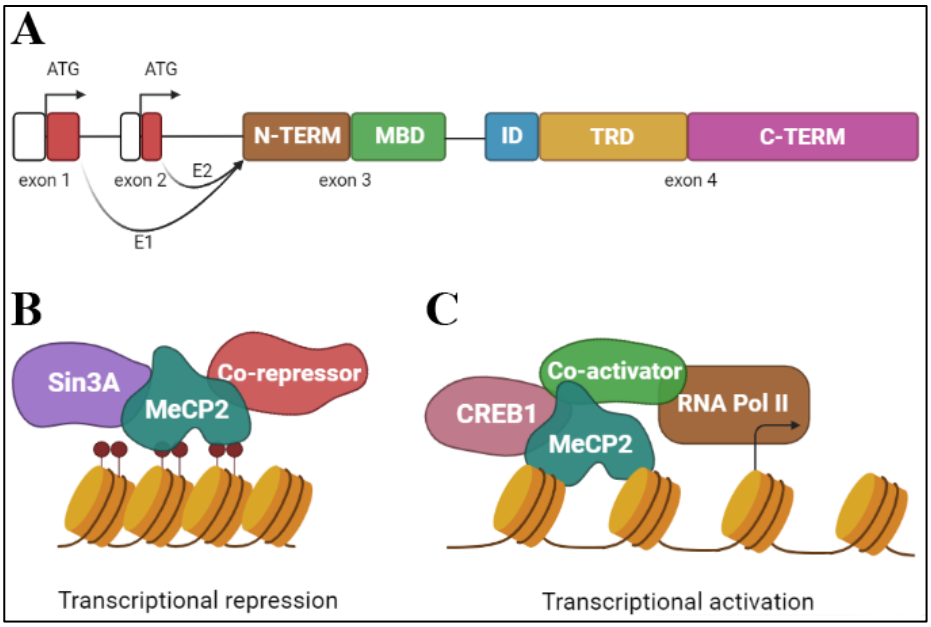


Figure 2: The molecular functions of MeCP2 protein encoded by *MECP2* gene. *a*) Schematic structure of *MECP2* gene, and the key protein domains of E1 and E2 isoforms; **b)** simplified

mechanism of action of MeCP2 and its partners in the processes of transcriptional repression and activation of genes regulated by this protein. Created with BioRender.com.

As a consequence of MeCP2 dysfunction, many different cellular systems are disrupted, such as synaptic function and plasticity, protein synthesis, mitochondrial function, management of oxidative stress, various signalling and homeostatic pathways, and both energy and lipid metabolisms (reviewed in Leonard et al., 2016).

Lower levels of MeCP2 within the Central Nervous System (CNS) are likely to be the cause of changes in brain morphology described in RTT patients (Lombardi et al., 2015). Changes include decreased dendritic branching and spine density (D. Armstrong et al., 1995; D. D. Armstrong, 2001; R. Z. Chen et al., 2001; Duncan Armstrong, 2005; Kishi & Macklis, 2004, 2010; A. M. Palmer et al., 2012; Tomassy et al., 2014), leading to a more compacted brain with 20% less weight than normal (Zoghbi, 2016). Reduction of brain weight specifically affects prefrontal cortex, posterior frontal and anterior temporal regions, with preservation in the posterior temporal and posterior occipital regions (Reiss et al., 1993; Schultz et al., 1993). RTT patients also present reduced dendritic arborisation in the pyramidal neurons of layer III and IV in frontal, motor, and inferior cortices of temporal regions. In addition, synaptic input is also reduced (reviewed in D. D. Armstrong, 2001).

Effect of lack of MeCP2 in the expression of other genes: *Creb* and *Bdnf*

Since MeCP2 is a transcriptional regulator, lack of MeCP2 affects the expression of several genes. Among them, *Creb* and *Bdnf* are of special relevance, due to their role in brain development and maintenance [for a review see (Braun et al., 2012; Kurian et al., 2007); (W. G. Chen, 2003; Degano et al., 2014; Huang & Reichardt, 2001)].

Levels of both CREB and phosphorylated CREB are decreased in cultures of forebrain neurons differentiated from stem cell lines of different *Mecp2*-mutant mice (Bu et al., 2017). In the open field test, it has been demonstrated that pharmacological activation of CREB through rolipram administration increases the ratio of time spent

in the centre over time spent in the periphery of *Mecp2*-heterozygous female mice. In addition, this drug increases time spent freezing by *Mecp2*-het females in a fear-conditioning paradigm (which is usually lower compared to WT mice) up to WT levels (Bu et al., 2017). Likewise, overexpression of CREB or pharmacological activation of CREB signalling in those forebrain neurons rescued the phenotypes in neurite growth, dendritic complexity, and mitochondrial function impaired in RTT (Bu et al., 2017). In addition, some reports have demonstrated that overexpression of *Mecp2* in transgenic mice also weakens both acute mechanical and thermal pain (R. Zhang et al., 2015). These data reveal a potential role of CREB signalling in RTT pathogenesis and suggest an analgesic role of MeCP2 in acute pain transduction through the p-CREB/miR-132 signalling cascade. In addition, CREB regulates *Bdnf* expression through a direct binding to its promoter (Y. C. Chen et al., 2014).

Transcription of *Bdnf* is highly regulated in the adult brain in an activity-dependent process (Chahrour et al., 2008; W. G. Chen, 2003; W. Li & Pozzo-Miller, 2014; Nuber et al., 2005; Tai et al., 2016; Zhou et al., 2006), due to its implication in brain development and adult neurogenesis (E. J. Huang & Reichardt, 2001). Lack of MeCP2 decreases both RNA and protein levels of BDNF in mice (Chahrour et al., 2008; Q. Chang et al., 2006; Wang et al., 2006; Zhou et al., 2006). Furthermore, restoration of BDNF levels improves breathing abnormalities (Ogier et al., 2007) and motor dysfunction (Deogracias et al., 2012) in *Mecp2*-null male mice.

Interplay between MeCP2 and epigenetics

MeCP2 binds to all methylated cytosines in the promoter of its target genes. Hence, it can be considered a mediator between epigenetics and phenotype. Epigenetics involves changes in gene expression without DNA sequence modification. Epigenetic modifications include DNA methylation, post-translational modifications of histone proteins, exchange of histone variants and the activity of non-coding RNAs, among others (Cheung et al., 2000; Luger et al., 2012; Strahl & Allis, 2000; Sweatt, 2013).

Those changes have been linked to many biological processes. In mammals, for example, DNA methylation plays a role in defining tissue-specificity during development (Géranton & Tochiki, 2015a). It occurs at CpG islands in the promoter of genes (Crow et al., 2013). Furthermore, methylation at these CpG islands is associated to gene silencing (Borrelli et al., 2008; Géranton, 2012; Géranton & Tochiki, 2015a).

In the nervous system, epigenetic processes have been implicated in the reprogramming of neuronal tissues, which are triggered by different processes: synaptic plasticity, learning, injury, neurodegenerative diseases, stress and addiction (see Torres Perez, 2017). Thus, epigenetic modifications can be correlated with neuronal and glial phenotypic plasticity, as a mechanism of adaptation to a constantly changing environment. This epigenetic control may be relevant for different processes, including neuronal maturation under specific conditions, or even pain processing after injuries (see Torres Perez, 2017).

Therapeutic strategies for Rett syndrome

Since there is no cure for Rett syndrome, medical management is essentially symptomatic and supportive. The main therapeutic approach in the recent decade is based on MeCP2 restoration. However, overexpression of MeCP2 also produces RTT-like symptomatology (Collins et al., 2004; Ricceri et al., 2013). Thus, given that the level of MeCP2 in a cell is critical, restoring MeCP2 function without producing overexpression continues to be a challenge. However, it has been demonstrated that lack of MeCP2 can be restored in adulthood in mice, upon the activation of the endogenous gene (Guy et al., 2007; Ricceri et al., 2013), suggesting that defective neurons can be repaired even in fully symptomatic adults.

Other pharmacological strategies include: targeting the neurotransmitter system (especially GABA, acetylcholine, and monoamines), promote brain growth and development (modulating the BDNF pathway), or even modulate other cellular

processes perturbed in RTT (energy metabolism, protein synthesis, etc.). As mentioned above, rolipram administration restores normal CREB levels, improving some behaviours in *Mecp2*-het females (Bu et al., 2017).

Some clinical studies with treatments based on L-carnitine have also demonstrated an improvement in the general well-being of patients with RTT. These girls showed a substantial improvement in hand function, eye contact, concentration span, vocalization, and mobility (Ellaway et al., 1999). In addition, magnesium supplementation reduced the frequency of seizures and hyperventilation, as well as stereotypic hand movements and agitated behaviours (Egger et al., 1992). Likewise, melatonin decreased sleep-onset latency and improved sleep time and efficiency (McArthur & Budden, 1998).

Recently, it has been demonstrated that trofinetide administration (a synthetic analogue of the amino-terminal tripeptide of IGF-1) improves the core symptoms of RTT, reducing neuroinflammation. This drug also promotes synaptic maturation and helps restore the synaptic and neuronal immaturities reported in RTT, supporting synaptic function (*Acadia Pharmaceuticals Announces Trofinetide New Drug Application for the Treatment of Rett Syndrome Has Been Accepted for Filing and Review by U.S. FDA | Business Wire*, n.d.)

Animal models widely used to study and better understand Rett syndrome

Since mutations in *MECP2* gene are the main cause of classical RTT (Chao et al., 2010; R. Z. Chen et al., 2001; Maunakea et al., 2013; Tomassy et al., 2014), three independent lines of *Mecp2*-deficient mice were successfully generated between 2001 and 2002 (R. Z. Chen et al., 2001; Guy et al., 2001; M. Shahbazian et al., 2002). Of these, the mouse model designed by Guy et al in 2001 (*Mecp2*$^{tm.1Bird}$) is one of the most commonly used, and the one we selected for the present study. This model was developed by using a CMV promoter-driven cre recombinase and removing

exons 3 and 4 (Guy et al., 2001). These mice manifest the typical symptomatology of the syndrome, including uncoordinated gait, breathing impairments, behavioural phenotypes, hindlimb clasping, and short life span (see Table 3). Similar to what is described in RTT patients, brains of these mice also show morphological changes, e.g., reduction in their brain size, small somas, and decreased dendritic complexity (R. Z. Chen et al., 2001; Kishi & Macklis, 2004; Tomassy et al., 2014); decreased synaptic plasticity (Asaka et al., 2006; Guy et al., 2007; Moretti et al., 2006), and compromised stability and brain function (D. Armstrong et al., 1995; Guy et al., 2001; Kishi & Macklis, 2010; Squillaro et al., 2012).

Table 3: table summarizing the RTT symptomatology that mimics the animal model designed by Guy et al., 2001. Abbreviations: *+*, observed in multiple studies; ***, decreased; *-*, not present; *ND*, not determined; *Het*, heterozygous.

	RTT SYMPTOMS	BIRD MODEL	
		***Mecp2*-null males**	***Mecp2*-het females**
MOTOR	**Limited mobility**	**+**	**+**
	Ataxic gait	**+**	**+**
	Dystonia/rigidity	**+**	**+**
	Tremor	**+**	**+**
	Stereotypies	**-**	**-**
COGNITIVE AND SOCIAL ABILITIES	**Decreased cognition**	**+**	**+**
	Social avoidance	**-**	**+**
	Anxiety	*****	*****
MORPHOLOGICAL	**Microencephaly**	**+**	**-**
	Neuronal hypotrophy	**+**	**+**
AUTONOMIC DYSFUNCTION	**Breathing abnormalities**	**+**	**+**
	Reduced lifespan	**2 mo**	**9 mo**
OTHER	**Seizures**	**+**	**-**
	Fertility	**-**	**+**

It is important to mention that *Mecp2*-mutant males are hemizygous (*Mecp2*-null) for the mutation due to the presence of a single X chromosome, so they are devoid of MeCP2. By contrast, *Mecp2*-mutant females are heterozygous for the mutation (*Mecp2*-het) due to the presence of a second X chromosome carrying the wild type (WT) allele, with the consequent reduced expression of MeCP2 protein. Therefore, male mice allow the study of consequences of the lack of MeCP2 for brain

development and function, whereas female mice better represent the delayed onset of symptomatology and mosaicism found in girls suffering the syndrome, having a higher face validity (R. Z. Chen et al., 2001; Guy et al., 2001; Leonard et al., 2016; M. Shahbazian et al., 2002; Young & Zoghbi, 2004). Consequently, onset of symptomatology arises earlier in *Mecp2*-null males (between 5 to 8 weeks of age; showing premature death around 60 days of age) than in *Mecp2*-het females (three to six months of age) making males more severely affected by the lack of MeCP2 than females (R. Z. Chen et al., 2001; Guy et al., 2001). Moreover, *Mecp2*-null males are infertile, so the breeding pairs are established with *Mecp2*-het females paired to WT males. This impedes the production of *Mecp2*-null females in this strain (Guy et al., 2001).

Overall, the three models mimic the core symptomatology of the syndrome, suggesting an equivalent role of MeCP2 in the development of both humans and rodents (Kurian et al., 2007; Matarazzo et al., 2004). Indeed, expression of this gene is similar in both species, with only subtle differences due to the specificity between species (M. Shahbazian et al., 2002). Hence, these animal models provide a valuable tool to investigate the role of MeCP2 in the development and organisation of the nervous system and, thus, better understanding RTT.

Possible explanations to the pathophysiology of Rett syndrome

Different hypotheses have arisen to explain the symptomatology of RTT. Mainly, it has been proposed that RTT could be caused by a lack of maintenance of neuronal health instead of a defective brain development (Akbarian et al., 2001; R. Z. Chen et al., 2001; Guy et al., 2001). However, cell proliferation, survival or apoptosis does not seem to be affected by deficits of *Mecp2* (Agustín-Pavón et al., 2016; Matarazzo et al., 2004; Smrt et al., 2007; Tsujimura et al., 2009). In addition, there is no evidence of significant neuronal loss (D. Armstrong et al., 1995), brain lesions, or presence of markers of degenerative disorders inside the brains of RTT patients nor

in mouse models (R. Z. Chen et al., 2001; Matarazzo & Ronnett, 2004). By contrast, it has also been proposed that symptomatology of RTT could be due to an arrest of the maturation process of neuroblasts (D. Armstrong et al., 1995; W. G. Chen, 2003; D. R. S. Cohen et al., 2003; Degano et al., 2014; Duncan Armstrong, 2005; Smrt et al., 2007). Accordingly, postnatal deletion of *Mecp2* in a mature nervous system, results in RTT-like phenotypes (Cheval et al., 2012; McGraw et al., 2011). Furthermore, the presence of higher rates of immature neurons found in the dentate gyrus and olfactory system of both RTT patients and mouse models (D. R. S. Cohen et al., 2003; Matarazzo et al., 2004; Ronnett et al., 2003; Smrt et al., 2007) suggests an important role of MeCP2 in neurogenesis and maturation of the brain, which may underlie some of the defects observed in RTT.

On this framework, for the first chapter of the present thesis we decided to explore the distribution of doublecortin, a marker for immature neurons, within the olfactory system and dentate gyrus of young adult *Mecp2*-null and *Mecp2*-het mice, to evaluate possible alterations in the process of neuronal maturation. Moreover, early life stress, in a paradigm of maternal separation, is able to promote neuronal maturation in mice (Bath et al., 2016). Since MeCP2 is an epigenetic transcriptional regulator that acts in a context-dependent manner (Sharifi & Yasui, 2021), we speculate that maternal separation may interfere in the process of neuronal maturation in mutant mice. Thus, we also decided to analyse doublecortin expression in a group of *Mecp2*-mutant mice exposed to maternal separation, to examine a possible interplay between early life stress and deficits of MeCP2. Some of the data derived from this chapter has been published elsewhere (Martínez-Rodríguez et al., 2019; Torres-Pérez et al., 2022).

Classical Rett syndrome has usually been considered as an autism spectrum disorder (ASD) due to the social isolation displayed by patients. However, recent investigations revealed substantial differences between this syndrome and autism, excluding it from the ASD group. As an example, subsequent reports revealed that,

after the adolescence, many of the patients significantly recover the ability to socialize. Furthermore, RTT girls are more likely to make eye contact and initiate social interactions (Olsson & Rett, 1985), compared to children suffering from autism. Therefore, social deficits described in RTT seem to be a consequence of loss of speech and motor impairment experimented by these girls, rather than autistic features as previously thought. However, little is known about social behaviours in *Mecp2*-deficient mice. Overall, these mice show slow movements, hypo activity, and amimia (Olsson & Rett, 1985). Concerning social interaction, some studies have observed reduced social approach behaviours in *Mecp2*$^{308/y}$ and conditional *Mecp2*-knockout mice (Gemelli et al., 2006; Moretti et al., 2005). Conversely, *Mecp2*$^{1/ox}$ mice exhibit increased social approach compared to controls, and usually show more interest in novel stranger mice, and spend more time in closer contact with them (Schaevitz et al., 2010). On the other side, an increased aggression in mice lacking MeCP2 in Sim-1 expressing neurons in the hypothalamus has been reported (Fyffe et al., 2008).

On this basis, the nonapeptidergic system is involved in the control of social behaviours in mammals. Thus, understanding possible dysfunctions within this system would help to ameliorate social deficits in RTT girls. Therefore, for the second chapter we explored the social brain of young-adult *Mecp2*-null and *Mecp2*-het females. This social brain is composed by a network that controls social behaviours in both humans and mice. Hence, it is important to consider that this social brain starts with the olfactory system in mice. Thus, alterations found within the olfactory system of some patients could also be interfering in social behaviours in *Mecp2*-mutant mice. Considering these data and the misunderstanding around social deficits described in RTT, we also deemed it of special interest to analyse some of the normative social behaviours in our *Mecp2*-null mice, in a paradigm of Resident-Intruder test. Data from this chapter has been published elsewhere (Martínez-Rodríguez et al., 2020)

In accordance with the above, previous studies have revealed that *Mecp2*-mutant mice show lower levels of anxiety, since mice spent more time in the open arms of the elevated plus maze (Abellán-Álvaro et al., 2021). However, some researchers suggested that *Mecp2*-mutant mice could be avoiding the closed arms, since they may experience more pain compared to wild type mice, whenever their whiskers touch the walls (Flores Gutiérrez et al., 2020). Thus, we must consider other aspects from the symptomatology of RTT that could be interfering in the data obtained. Likewise, deficits in communication, as well as motor impairment, should also be considered when interpreting results from other areas of interest, like perception of pain. Abnormal responses to painful stimuli have commonly been reported in cases of RTT. Verbal and non-verbal communication of pain has commonly been used to measure pain in patients. Among them, facial expression constitutes a powerful way to communicate pain (Arif-Rahu & Grap, 2010; Kappesser, 2019). However, depending on the age or even condition of each person, facial responses may vary or even be absent, even in the presence of painful states. That is the case of RTT patients. Additionally, their motor disturbances and poor language skills prevent them from communicating pain. Therefore, it remains unclear whether these girls actually experience lower pain sensation or a problem in the ability to communicate pain by themselves. Thus, for chapter 3 we decided to explore nociception in young and old *Mecp2*-het female mice, measuring mechanosensitivity and thermosensitivity of those mice in the Von Frey and Hot Plate tests, respectively. We also explored some other motor features in those mice, such as their gait and clasping behaviour, as well as their weight, to include possible factors that could also influence their nociception or expression of pain.

MAIN OBJECTIVES

The specific aims of the present doctoral thesis are:

1. To explore cell survival of SVZ-derived cells within the olfactory bulbs and striatum in young *Mecp2*-null males.
2. To describe the distribution of doublecortin, a marker of immature neurons, in the brain of young-adult *Mecp2*-null and *Mecp2*-het mice.
3. To investigate the effect of early life stress, induced by a paradigm of maternal separation, in the density of doublecortin neurons.
4. To analyse distribution of vasopressin and oxytocin, two nonapeptides involved in regulating social behaviours in mice, in the social brain of young-adult *Mepc2*-mutant mice.
5. To analyse NADPH expression as an indirect modulator of aggressive behaviours in male mice.
6. To study social and aggressive behaviours in young-adult *Mecp2*-null males.
7. To examine nociception, in terms of mechanosensitivity in a longitudinal study of young-adult and old *Mecp2*-het female mice.
8. To explore how other symptoms such as gait, weight or clasping behaviour may interfere in pain expression in mice.
9. To explore thermal sensitivity in young pre-symptomatic and old symptomatic *Mecp2*-het females.

10. To examine the activation patterns within different nuclei involved in nociception in old *Mecp2*-het female mice.

CHAPTER 1. EFFECT OF *MECP2* DEFICIENCY IN POSTNATAL NEUROGENESIS

INTRODUCTION

In this chapter, we mainly explored doublecortin immunoreactivity, a well stablished marker for immature neurons, in the brain of young adult *Mecp2*-heterozygous female and *Mecp2*-null male mice and their wild type littermates. Since it has been suggested that early life stress can interfere in neuronal maturation, we also analysed doublecortin expression in a group of animals submitted to a paradigm of maternal separation. On the same line, apoptotic processes do not seem to be upregulated in MeCP2 deficient mice (Tsujimura et al., 2009). Additionally, preliminary data from the lab suggest that cell proliferation is not altered in the subventricular zone of *Mecp2*-deficient mice (Z. Chen et al., 2017, Agustín-Pavón et al., 2016). Thus, to clarify whether possible differences in cell survival could be interfering in the number of immature neurons, we also analysed the number of BrdU positive cells in the olfactory bulbs of a group of *Mecp2*-null and wild type male mice. Part of the results presented here have been published in two peer-reviewed papers (Martínez-Rodríguez et al., 2020; Torres-Pérez et al., 2022).

Rett syndrome as an alteration of neuronal maturation

Up to now, three different possible hypotheses arise to explain the symptomatology of RTT: (i) **alterations in the normal brain development**, (ii) **affected brain survival leading to higher rates of apoptotic processes**, or a (iii) **defective brain maturation**.

It has been proposed that RTT could be caused by a lack of maintenance of neuronal health instead of a defective brain development (Akbarian et al., 2001; R. Z. Chen et al., 2001; Guy et al., 2001). This hypothesis is supported by the more compacted and smaller size of brains found in RTT patients (D. Armstrong et al., 1995; D. D. Armstrong, 2001; Duncan Armstrong, 2005). On this basis, it has been suggested

that neuronal survival could be compromised, leading to higher rates of apoptotic processes and neuronal death than normal. However, reported changes in brain morphology seem to be due to smaller and more densely packed neurons (D. Armstrong et al., 1995) with reduced dendritic arborization (A. M. Palmer et al., 2012). In addition, there is no significant evidence of neuronal loss (D. Armstrong et al., 1995), brain lesions, or presence of markers of degenerative disorders inside the brains of RTT patients nor in mouse models (R. Z. Chen et al., 2001; Matarazzo & Ronnett, 2004).

Additionally, MeCP2 does not seem to affect proliferation or apoptosis (Tsujimura et al., 2009). Also, previous results from our lab, together with findings from other groups, suggest that proliferation, survival, and differentiation in the subventricular zone (SVZ) and subgranular zone of the dentate gyrus (DG), are not significantly affected by the absence of MeCP2 (Agustín-Pavón et al., 2016; Matarazzo et al., 2004; Smrt et al., 2007). Hence, it has been generally concluded that no recognizable degenerative, demyelinating, or gross malformative process explains the RTT phenotype.

By contrast, based on reduced brain and neuronal size and dendritic branching, together with discoveries of immature neurons in the DG and olfactory system (D. R. S. Cohen et al., 2003; Matarazzo et al., 2004; Ronnett et al., 2003; Smrt et al., 2007), it has also been proposed that symptomatology of RTT could be due to an arrest of neuroblasts process of maturation (D. Armstrong et al., 1995; W. G. Chen, 2003; D. R. S. Cohen et al., 2003; Degano et al., 2014; Duncan Armstrong, 2005; Smrt et al., 2007). On this framework, olfactory epithelium of mouse models and patients show a delayed maturation of their olfactory receptor neurons, that leads to a reduced size of the olfactory glomeruli (Ronnett et al., 2003).

In addition, the approximate correspondence in real time between RTT human and mouse phenotype argues in favour of no defects in developmental neurogenesis,

but rather an effect in maintenance of neuronal functionality (Guy et al., 2001). Accordingly, MeCP2 protein plays an important role in neuronal maturation and differentiation (Kishi & Macklis, 2004). In rodent and human brains, the levels of MeCP2 protein are faint during brain embryonic development and reach the highest levels in more mature structures (Cassel et al., 2004; Mullaney et al., 2004; M. D. Shahbazian et al., 2002). Additionally, the wide variety of neurological and neuropsychiatric symptoms caused by either lack or excess of MeCP2 protein is related to its important expression in the mature neurons of the central nervous system (LaSalle et al., 2001). Finally, postnatal deletion of *Mecp2* in a mature nervous system, results in RTT-like phenotypes (Cheval et al., 2012; McGraw et al., 2011). Taken together, these data suggest an important role of MeCP2 in neurogenesis and maturation of the brain, which may underlie some of the defects observed in RTT.

The olfactory system and hippocampus as models to study differentiation and maturation

For our investigation, we focused on analysing the functional neuroanatomy of the olfactory system in our *Mepc2*-mutant mice. Due to the high levels of MeCP2 protein found in the olfactory bulb (OB), this structure has been considered as a good model to study mutations of *MECP2* gene and their implications in the brain of RTT patients (Cassel et al., 2004; D. R. S. Cohen et al., 2003; Degano et al., 2014; Gage, 2002; Lee et al., 2014; Matarazzo & Ronnett, 2004; Ronnett et al., 2003). Indeed, MeCP2 has also been implicated in neuronal maturation and refinement of olfactory circuits. In addition, the olfactory system is of special relevance for social communication, and a very good model for the study of developmental diseases. Furthermore, the olfactory system is impaired in autism, and some reports suggest possible alterations in patients with RTT. Thus, since adult neurogenesis occurs in both the olfactory system and the DG in rodents (Gage, 2002), these two areas provide an

excellent model for studying generation and maturation of neurons in the adult brain (D. R. S. Cohen et al., 2003; Matarazzo et al., 2004; Ronnett et al., 2003).

In the olfactory system, odorants are detected by the Olfactory Sensory Neurons (OSNs) located in the olfactory epithelium. Chemical information is then sent through the olfactory nerve fibres to the mitral and tufted cells in the glomeruli of the olfactory bulbs. Odour information will be next sent to the different brain areas compounding the olfactory cortex, such as the piriform cortex (Pir), amygdala (Amy), olfactory tubercle (Tu) and parahippocampal gyrus.

The olfactory system is one of the areas in which neurogenesis is persistent during the whole life span. Thus, cells are continuously being generated in the SVZ and migrate through the Rostral Migratory Stream (RMS) to the OB, where they will differentiate into mature neurons and integrate in the pre-existing circuitry (Shipley et al., 2004; Yang et al., 2004). At the age of 2 weeks, the olfactory system becomes completely mature (Hinds & Hinds, 1976), coinciding with a time of increment in *Mecp2* expression within this region (D. R. S. Cohen et al., 2003). MeCP2 is especially abundant in the olfactory epithelium and OB (Cassel et al., 2004; Ronnett et al., 2003), playing relevant roles during the whole process of development in both embryonic and adult olfactory system (Kishi & Macklis, 2004; Kurian et al., 2007; Luikenhuis et al., 2004; Matarazzo et al., 2004).

In the main olfactory epithelium, MeCP2 seems to be necessary for maturation and differentiation of OSNs (Degano et al., 2009; A. Palmer et al., 2008). Involvement of MeCP2 in neuronal maturation is consistent with previous discoveries of high percentages of immature neurons in the olfactory system of *Mecp2*-null mice (Matarazzo et al., 2004) and olfactory biopsies obtained from RTT patients (Ronnett et al., 2003). Those immature neurons show dysmorphic and shrunken morphologies with abnormal and reduced dendritic arborization as well as un-fasciculated axons (Ronnett et al., 2003). Additionally, MeCP2 is necessary for

activity-dependent transcriptional responses to odorant stimulation in the OSNs (Degano et al., 2014; Lee et al., 2014; Matarazzo et al., 2004), including processes of circuitry refinement, which seem to be altered in the absence of MeCP2 (Degano et al., 2009, 2014), and changes in adhesion molecules involved in axonal convergence and fasciculation (Degano et al., 2009). Impairments in axon refinement in *MECP2*-mutant mice and patients suffering this syndrome persist into adulthood (Lee et al., 2014).

Within the OB, MeCP2 is needed for glomerular organization during early postnatal stages (Degano et al., 2009; A. Palmer et al., 2008). MeCP2 deficiency, thus, leads to heterogeneous and supernumerary reduced glomeruli also observed in post-mortem brains of RTT patients (D. Armstrong et al., 1995; Neul et al., 2010), which failed to be refined (Degano et al., 2009, 2014; Matarazzo et al., 2004).

The Pir is a trilaminar paleocortex with a highly cellularized layer II containing packed cell bodies of mature projecting excitatory neurons, as well as a small number of immature neurons (Nacher et al., 2001). Most part of these immature neurons are generated during embryonic development (M. Á. Gómez-Climent et al., 2008; Rubio et al., 2016), although a small fraction of them are supposed to be generated during adulthood (Bernier et al., 2002; Bonfanti & Nacher, 2012; Pekcec et al., 2006; Shapiro et al., 2007). Those immature neurons do not seem to form synaptic contacts (M. Á. Gómez-Climent et al., 2008; Nacher et al., 2001) and very recent evidence suggest that they progressively turn into glutamatergic neurons with age (Rotheneichner et al., 2018) and probably integrate in the existing circuitry of Pir (Varea et al., 2009). For this reason, it has been suggested that those neurons may act as a necessary reservoir for processes of brain plasticity (Nacher et al., 2001). Importantly, a recent study suggests that these cells are present in the cortex of humans (Coviello et al., 2022).

The Pir, together with the anterior olfactory nucleus (AON) and the Tu, constitute the olfactory cortex in the rodent olfactory system. In the case of the Tu, it is a trilaminar region consisting of the most ventral molecular layer (layer I), a medial dense cell layer (layer II) and the most internal multiform layer (layer III). Tu also contains dense cluster of granule cells known as the Islands of Calleja (ICj) and the ventral pallidum (reviewed in Wesson & Wilson, 2011). As a component of the ventral striatum, Tu is intimately interconnected with several reward, affective and motivation-related brain centres. These anatomical features, together with previous results from our group revealing that female mice bearing lesions into the Tu displayed no attraction to male odours (Agustín-Pavón et al., 2014), suggest a likely role of the olfactory tubercle in regulating odour-hedonics behaviours (FitzGerald et al., 2014; Gadziola et al., 2015), that might seem to be altered in autism (Rozenkrantz et al., 2015).

The Dentate Gyrus of the hippocampus (DG) is a neurogenic area that consists of three layers, which, from the inside out, are: the molecular layer, granular layer and the hilus. Precursors located in the subgranular zone of the DG are continuously generating new neurons in the adult rodent brain (reviewed in Gage, 2002), although it is still in controversy in the case of human beings (Boldrini et al., 2018; Sorrells et al., 2018).

On this basis, there is a population of immature neurons typically located in the subgranular zone. However, in *Mecp2*-null mice those immature neurons send horizontal and short processes parallel to the hilar boundary, which is characteristic for neurons at an early stage of differentiation (Esposito et al., 2005; Smrt et al., 2007), suggesting a possible delayed onset on transitioning to mature stages and resulting in adult *Mecp2*-deficient brains retaining characteristics of immature brains (Smrt et al., 2007).

Differences found in the hippocampus of *MECP2*-deficient brains are consistent with defective pathfinding and deficits in spatial and emotional tasks observed in mouse models and girls suffering this syndrome (Gemelli et al., 2006; Moretti et al., 2006; Pelka et al., 2006). Molecular events involved in establishment of long-term potentiation and depression also seem to be impaired in mature hippocampal neurons (Asaka et al., 2006), contributing to malfunction of this structure.

Doublecortin as a marker for immature neurons

Doublecortin (DCX) is a microtubule-associated protein expressed in migrating neuroblasts and differentiating neurons (F. Francis et al., 1999; Gleeson et al., 1998), and hence it is frequently used as a neurogenic marker (Saaltink et al., 2012). In the adult rodent brain, DCX expression is restricted to the neurogenic areas of the SVZ, RMS, OB, and DG (Nacher et al., 2001; Saaltink et al., 2012). In addition, some DCX-immunoreactive cells with neuronal morphology are found in the striatum next to the SVZ (Nacher et al., 2001). Finally, as mentioned above, there is an interesting population of DCX-ir neurons in layer II of the Pir, which is mostly generated during embryonic development, persists into adulthood and declines with ageing (M. Á. Gómez-Climent et al., 2008; Rubio et al., 2016). These cells have been recently shown to mature and integrate as glutamatergic neurons (Rotheneichner et al., 2018). In fact, in rodents and in other mammalian species the DCX-positive cells express the transcription factor Tbr-1 (Luzzati et al., 2009), which is characteristic of the pallial glutamatergic projection neurons (Guillemot et al., 2006). They may constitute a source of neural plasticity, which may contribute to some forms of olfactory learning (but see Á. Gómez-Climent et al., 2011).

Immunohistochemistry for DCX allows analysing immature neurons in the brain. For this reason, it has widely been used to study both embryonic and adult neurogenesis, as well as neuronal maturation in adult rodents (Brown et al., 2003; Gleeson et al., 1999; Nacher et al., 2001; Rao & Shetty, 2004). DCX is an

intracytoplasmic microtubule-associated protein (F. Francis et al., 1999; Gleeson et al., 1999; M. Á. Gómez-Climent et al., 2008) located in the periphery of the soma (F. Francis et al., 1999; Gleeson et al., 1999) of newborn neurons in both embryonic and adult brain. Although DCX expression appears strongly diminished in the adult brain (F. Francis et al., 1999), it is still present in neuroblasts generated in the typical adult-neurogenic areas like the SVZ and the subgranular zone of the DG (Brown et al., 2003; Gleeson et al., 1999; Nacher et al., 2001; Rao & Shetty, 2004), probably involved in neurite development and neuronal migration (F. Francis et al., 1999). DCX-ir neuroblasts are also present in non-proliferative areas as well, enabling microtubule reorganization and synaptogenesis (Nacher et al., 2001). Those non-proliferative areas include the main and accessory OBs (MOB and AOB, respectively), Pir layers II and III, Tu, striatum (St), endopiriform nucleus (En) and corpus callosum (CC; Brown et al., 2003; Gleeson et al., 1999; Nacher et al., 2001; Rao & Shetty, 2004). Additionally, DCX-labelled neuroblasts can also be found in the adult RMS, migrating to the OB (Brown et al., 2003; Gleeson et al., 1999; Nacher et al., 2001; Rao & Shetty, 2004), consistent with the proposed role of DCX in neuronal migration. DCX appears to be restricted to the neuronal lineage, being expressed in neuronal precursors and migrating neuroblasts (F. Francis et al., 1999; Gleeson et al., 1999; Walker et al., 2007). In addition, downregulation of DCX occurs at the same time those cells begin to express a neuronal nuclear protein called NeuN, commonly used as a marker for mature neurons (Brown et al., 2003). For these reasons, DCX has commonly been considered as a good marker for immature neurons (Brown et al., 2003; Rao & Shetty, 2004; Saaltink et al., 2012).

Early life stress promotes neuronal maturation

MeCP2 protein has been involved in the control of neuronal activity-dependent gene expression (W. G. Chen et al., 2003; Martinowich et al., 2003) as well as in experience-dependent epigenetic programming (Murgatroyd et al., 2009; Stroud et al., 2017). This epigenetic transcriptional regulator acts in a context-dependent

manner (Sharifi & Yasui, 2021). As an epigenetic reader, MeCP2 controls critical periods of postnatal development, which are periods of high plasticity and sensitivity to the environment (Picard & Fagiolini, 2019). Adverse events occurring during this period are engraved in 'permanent' epigenetic marks, with MeCP2 being a master regulator of these processes (Murgatroyd et al., 2009). MeCP2-dosage is carefully regulated against specific environmental factors, changing the epigenetic imprinting of neurons and thus, playing important key roles during post-natal critical periods in response to those environmental inputs. In this scenario, it is plausible that decreased functionality of MeCP2 early in life, as that seen in RTT patients, will affect their epigenetic programming.

Previous studies have demonstrated that early life stress (ELS), in the form of maternal separation (MS) can switch from growth to maturation at both behavioural and neuronal levels (Bath et al., 2016). ELS may promote an earlier silencing of typical biomarkers associated with cell proliferation and differentiation, in favour of neuronal maturation. In fact, ELS not only promotes neuronal maturation, but also enhances cell death and loss of dendrites and spines, decreasing cell proliferation and synaptic density (reviewed in Bath et al., 2016).

Giving support to this, Bath et al. proved that MS promotes neuronal maturation in the hippocampus of male mice, as an adaptative mechanism for survival (Bath et al., 2016). Recent publications from our lab have also demonstrated that MS reduces depressive and anxious behaviours in both *Mecp2*-het and WT females, as they spent more time in the open arm of the elevated plus maze (Abellán-Álvaro et al., 2021). Moreover, those behavioural changes correlate with decreased neuronal activation at the hypothalamic paraventricular nucleus, modifying the normal response to stressful situations. Thus, analysing the interplay between MS and deficits in MeCP2 is of special relevance to better understand the environmental stimuli that may affect this condition.

In this framework, we sought to investigate the effect of MeCP2 deficiency in the distribution and density of DCX-positive neurons in the young adult mouse brain, focusing on the olfactory system. To do so, we performed an immunohistochemical detection of DCX in young adult *Mecp2*-heterozygous female (*Mecp2*-het) and *Mecp2*-null male and their male and female WT littermates. Given the previously suggested role of MeCP2 in neuronal maturation, we hypothesised that *Mecp2*-deficient animals may display an increased density of immature neurons, and hence an increase in DCX-positive neurons with respect to their WT controls. However, although cell proliferation and apoptosis do not seem to be altered in those mice (Z. Chen et al., 2017; Tsujimura et al., 2009), little is known about possible impairments in cell survival. Thus, we analysed cell survival of postnatal SVZ-generated neurons by means of a BrdU experiment. Finally, we sought to investigate whether MS in *Mecp2* deficient mice hinders neuronal maturation during adulthood, in the aftermath of ELS. For this experiment, we used *Mecp2*-mutant and wild type mice that were or were not submitted to MS, a classical rodent paradigm to study ELS (Lehmann & Feldon, 2000; Macrí et al., 2004) that is known to affect neurogenesis (Bath et al., 2016; Koehl et al., 2012; Suri et al., 2013).

MATERIALS AND METHODS

Animals

For this chapter, we used 70 young mice of the strain *Mecp2*$^{tm1.1Bird/J}$ and their WT siblings, distributed in three experiments. Experiments were aimed at investigating: the **effect of lack of MeCP2 on cell survival** (Experiment 1), the **effect of MeCP2 deficiency in the density of immature neurons** (Experiment 2, a subset of the data published in Martínez-Rodríguez et al., 2019), and the **interaction of early-life stress and MeCP2 deficiency on the density of immature neurons** (Experiment 3). Sex, age at sacrifice, and number of each group of animals are shown in Table 4. Brains from these mice were sliced in five parallel series, and two of the series derived from Experiment 2 were used in Chapter 2 (see below). For Experiment 3, we worked with a subset of samples derived from a maternal separation study, and behavioural and anatomical data from the females of this experiment has been published elsewhere (Abellán-Álvaro et al., 2021; Torres-Pérez et al., 2022).

Table 4: Number of animals used for each experiment classified by sex, genotype and condition, and age of their sacrifice. Abbreviations: *BrdU*, bromodeoxyuridine; *DCX*, doublecortin; *het*, heterozygous; *MS*, maternal separation; *NeuN*, neuronal nuclear protein; *OB*, olfactory bulbs; *WT*, wild type.

Experiment	Subjects		Total	Age of sacrifice	Markers
	Females	Males			
1. Effect of lack of MeCP2 in cell survival in the OB		5 WT 5 *Mecp2*-null	10	5-weeks	BrdU
2. Effect of MeCP2 deficiency on the density of immature neurons	6 WT 5 *Mecp2*-het	7 WT 5 *Mecp2*-null	23	8-weeks	DCX NeuN
3. Interaction between early-life stress and MeCP2 deficiency on the density of immature neurons	4 Naïve-WT 3 Naïve-het 8 MS-3h-WT 6 MS-3h-het	3 Naïve-WT 4 Naïve-null 6 MS-3h-WT 3 MS-3h-null	37	6-weeks	DCX NeuN

All mice were purchased at The Jackson Laboratory and kindly donated by Dr Mónica Santos from CNC-Coimbra and Dr Oliver Stork from Otto-von-Guericke University,

Magdeburg. Mice were housed in standard laboratory cages in groups of 2–5 animals, with controlled humidity and temperature (22 °C), a 12:12-h inverted light/dark cycle, and water and food available ad libitum. All the procedures were carried out in strict accordance with the EU directive 2010/63/EU. The protocols were approved by the local veterinary and the Ethics in Animal Experimentation Committee of the University of Valencia.

Genotyping

For genotyping, we used the Phire^{TM} Tissue Direct PCR Master Mix from thermo scientific (Ref: F-120S) and applied the protocol supplied by the Jackson Laboratory for this strain after the extraction of the DNA from the ear biopsies of the mice at weaning.

For DNA extraction, we (i) prepared a mix of 20 µl of dilution buffer and 0.5 µl of DNA release additive and added it to each tissue sample, being sure that ear snips were completely submerged. (ii) Next, the mixture was spined and (iii) incubated for 5 minutes at room temperature (RT, approximately 25 °C), followed by 2 minutes at 98^{o}C. (iv) Supernatant was taken and (v) 1 µl of sample was added to a mixture of 7.5 µl H_2O, 10 µl of 2x Phire Tissue Direct PCR Master Mix, and 0.5 µl of each primer (p9875, p9877, and poMR7172) diluted at 20µM. For the PCR protocol, we followed the thermal conditions described in table 5.

Next, we prepared a 2% TAE agarose gel with 5 µl of GreenSafe Premium (Nzytech, REF: MB13201) per 100 ml agarose, and injected 75% of the finished PCR reaction volume into well (15 µl). Finally, we run the gel at 100V for 30 to 45 minutes, and took pictures with a fluorescent lamp (see Figure 3).

Table 5: thermal conditions followed of PCR reaction for the amplification of DNA of the samples.

1	**Heat Lid 110.0 ºC**
2	Temp. 98.0 ºC for 5’ 0’’
3	Start Cycle x10
4	Temp. 98.0 ºC for 5’’
5	Temp. 65.0 ºC for 5’’
6	Temp. 72.0 ºC for 20’’
7	Close Cycle
8	Start Cycle x20
9	Temp. 98.0 ºC for 5’’
10	Temp. 65.0 ºC for 5’’ (-0.5 ºC, +0``)/Cycle
11	Temp. 72.0 ºC for 20’’
12	Close Cycle
13	Start Cycle x10
14	Temp. 98.0 ºC for 5’’
15	Temp. 55.0 ºC for 5’’
16	Temp. 72.0 ºC for 20’’
17	Close Cycle
18	Maintain at 15 ºC forever

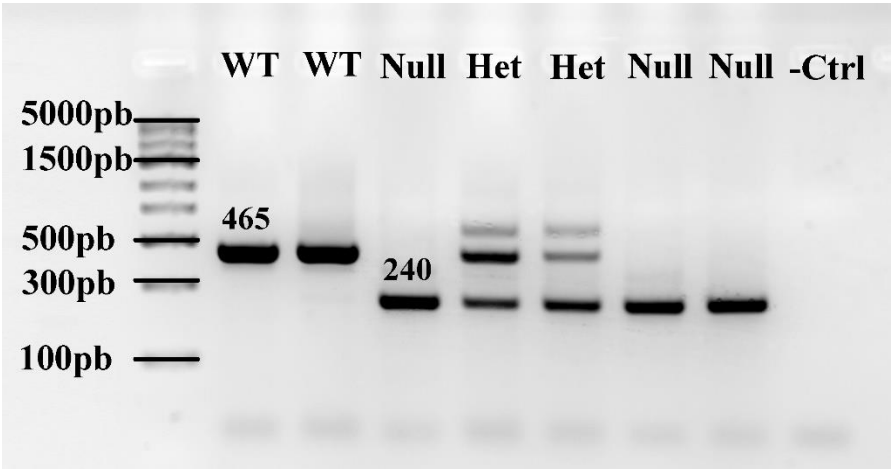


Figure 3: representative picture of genotyped ear samples obtained from wild type (WT), *Mecp2*-null males (KO) and *Mecp2*-heterozygous females (Het) mice.

5-Bromodeoxyuridine (BrdU) injections

Mice from the experiment of neuronal survival were administered with BrdU (Sigma, St Louis, MO) in 0,9% sodium chloride, intraperitoneal, once a day for three consecutive days, at a dose of 50 mg/kg of body weight. Mice were sacrificed 12 days after the last injection of BrdU to check for cell survival in the OB and St.

Histology

Animals for anatomical studies were deeply anaesthetized using a mixture of ketamine (75 mg/Kg) and medetomidine (1 mg/Kg) and transcardially perfused with saline solution followed by 4% formaldehyde in 0.1M phosphate buffer (PB) pH 7.4. Brains were carefully removed from the skull, postfixed in the same fixative overnight and placed into 30% sucrose (in 0.01M phosphate-buffered saline, pH 7.6; PBS) until they sank. The brains were then frozen and cut in five parallel series of 40-µm-thick coronal sections with a freezing microtome. Free-floating sections were stored in the freezer in phosphate-buffered 30% sucrose (0.1M pH 7.4) for future processing.

Immunohistochemical techniques

BrdU and DCX permanent immunostaining with DAB

For the analysis of cell survival and neuronal maturation, we obtained permanent immunostained preparations for BrdU and DCX in one out of the five parallel series of their corresponding group.

For BrdU-immunodetection only, we first performed an antigen retrieval procedure by incubating the sections with 2M HCl at 40 ^{o}C for 30 minutes. Next, brain slices were incubated with: (i) 1% H_2O_2 in 0.05M TRIS buffered (TB) saline (TBS, 0.9% NaCl in TB) for 30 min at RT to block endogenous peroxidase activity; (ii) 2% normal serum and 0.3% Triton-X100 in 0.05M TBS pH 7.4 for 1 h at RT, to block nonspecific binding. (iii) Brain slices were then incubated overnight at 4 °C with the corresponding

primary antibody (see Table 6) diluted in 0.05M TBS pH 7.4, with 2% normal serum and 0.3% Triton-X100. (iv) The next day, sections were incubated for 2 h at RT with biotinylated secondary antibody diluted in the same buffer (see Table 6). (iv) Afterwards, sections were incubated for 90 min at RT in avidin-biotin-peroxidase complex (ABC Elite kit; Vector Labs, PK-6200) in TBS with 0.3% Triton-X100. Sections were thoroughly washed in TBS (3 × 10 min) between each step, except between step ii and iii. To reveal peroxidase activity, sections were incubated for 5 min in 3,3'-diaminobenzidine (DAB)-SigmaFAST (Sigma) in TB. All the samples of the same experiment were processed at the same time and by the same experimenter.

Table 6: Primary and secondary antibodies used for BrdU, DCX and NeuN experiments.

Experiment		Primary Antibody		Secondary Antibody	
BrdU	DAB	Rat anti-BrdU	1:2000 Abcam Ab6326	Biotinylated Goat anti-Rat IgG	1:200 Vector Labs BA9400
DCX	DAB	Goat anti-DCX polyclonal IgG	1:500 Santa Cruz Biotech. Inc sc-8066	Biotinylated Horse anti-Goat IgG	1:200 Vector Labs BA9500
	Fluor.		1:250 Santa Cruz Biotech. Inc sc-8066	Alexa Fluor 568 Donkey anti- Goat IgG	1:200 ab175474
NeuN	Fluor.	Mouse anti-NeuN IgG	1:2500 Millipore MAB377	Alexa Fluor 488 Donkey anti- Mouse IgG	1:250 A21202

Double immunofluorescence of DCX and NeuN

In the males used for Experiment 2 and all the samples from Experiment 3, we employed immunofluorescence for simultaneous immunolabelling of DCX and NeuN.

Sections were incubated sequentially in: (i) 1% sodium borohydride ($NaBH_4$) in TBS at RT for 30 min (min); (ii) 0.05M TBS pH 7.6 with 0.3% Triton X-100, and 4% normal serum at RT for 1 h; (iii) next, sections were incubated with TBS with 0.2% Triton X-100, 4% normal serum for 48 h at 4°C with the mix of primary antibodies (see table 6); (iv) after incubation with primary antibodies, sections were incubated with

fluorescent-labelled secondary antibodies for 90 min at RT in TBS with 0.2% Triton X-100 (see table 6). Between each step, sections were washed in TBS (except between step ii and iii). Sections were finally incubated in DAPI (4',6-diamino-2-fenilindol, 600nM) for 1 minute, rinsed thoroughly in TB, mounted onto gelatinized slides and cover-slipped with fluorescence mounting medium (Dako, Glosrup, Denmark; or FluorSave TM Reagent). Immunofluorescent staining of the samples to be directly compared (i.e., WT males vs *Mecp2*-null or WT females vs *Mecp2*-het) was performed at the same time, and all the materials were processed by the same experimenter.

Immunohistochemical analysis and quantification

For quantification of immunoreactive nuclei or cells, an experimenter blind to genotype and sex of mice took pictures of both hemispheres of previously selected levels of Bregma [following (Franklin & Paxinos, 2013)] depending on the aims of each experiment (see Table 7).

Table 7: Nuclei and Bregma levels selected for the quantification of BrdU, DCX and NeuN positive cells in *Mecp2*-mutant and WT mice. Abbreviations: *DG*, dentate gyrus of the hippocampus; *dSt*, dorsal striatum; *OB*, olfactory bulbs; *Pir*, piriform cortex; *St*, striatum; *Tu*, olfactory tubercle; *vSt*, ventral striatum.

BrdU-ir somata

Nucleus	Approximate mm to Bregma
OB	+3.2, +3.1, +3
St	+1.5, +1.1, +0.7
Pir	+1, 0, -1, -2

DCX-ir and DCX/NeuN-if somata

Nucleus	Approximate mm to Bregma
OB	+3.2, +3.1, +3
dSt	+1.5, +1.1, +0.7
vSt	+1.5, +1.1, +0.7
Tu	+1.7, +1.5, +1.3, +1.1
Pir	+1, 0, -1, -2
DG	-1.4, -2.2, -2.8, -3.2
DAPI-stained somata	
Pir	+1, 0, -1, -2

Pictures were taken with a digital Leica DFC495 camera attached to a microscope equipped with both conventional light and fluorescent lamps (Leica Leitz DMRB) and software LAS v4.3. We adjusted the most accurate conditions of exposition, gamma and saturation for each brain region. For each experiment, pictures from both genotypes/conditions were taken under the same scan settings. Immunofluorescent pictures were obtained using the green channel for Alexa Fluor 488 and then changing to the red channel for Alexa Fluor 568, and blue channel for DAPI staining. In cases in which co-localization analysis was required, images with different channels were taken at exactly the same location. All the subsequent steps were performed using ImageJ free software (NIH).

For convenience, from now on we will use the suffix -ir for samples stained with the ABC/DAB procedure, and -if for samples labelled with immunofluorescence.

Analysis and quantification of BrdU immunoreactive cells

To analyse neuronal survival, we first took pictures with the conventional light of BrdU-ir cells in the OBs, and dorsal and ventral striatum (dSt, vSt). A person who was blind to the genotype of the animals manually counted labelled nuclei in two a priori defined region of interest (ROI), one for the granular layer (GrOB) and one for the glomerular layer (GlOB; Figure 4a). In the case of St, BrdU-ir cells were also manually counted, but without any ROI. Cell density of BrdU-ir cells was calculated as number of cells per mm^2 of the ROI.

Analysis and quantification of DCX-immunoreactive cells

We used the conventional light of the microscope to take pictures of DCX-ir cells in the GrOB, dSt, vSt, Tu, Pir, and DG.

In the GrOB, due to the high density of cells and fibres, we calculated the area fraction covered by DCX-ir in DAB-stained material by converting the image to greyscale by splitting the RGB image into the three channels and selecting the green

channel—since it provides the better contrast for DAB staining—, and binarizing by establishing 90% of the grey histogram mode as a threshold level. We normalised these data by dividing the area fraction covered by DCX-ir elements in the OB by the area fraction covered by DCX-ir elements (mostly glial cells) in the anterior olfactory nucleus, and calculated the average area fraction of all Bregma levels analysed, hence obtaining a single measure per animal.

For Pir and St, cells were manually counted individual cells using the Multipoint plugin of the Image-J image analysis software (NIH), and cell density was calculated as number of cells per mm^2 of the ROI. In the Tu and DG, due to the low and high density of cells, respectively, counts were performed with a manual counter at higher magnification directly observing the samples with the microscope, and cell density is presented in number of cells per coronal section.

Analysis and quantification of double-labelled DCX/NeuN-immunofluorescent cells

For quantification of immunofluorescent labelling, we obtained images at the same Bregma levels as for DCX-ir analysis (see Table 7) with the 488 and 568 nm filters. Next, we merged the photos of both channels using Image-J, and quantified single DCX-if and double DCX/NeuN-if cells with the Multipoint plugin.

To analyse the immunofluorescence in the granular layer of the OB, we split the RGB image, selected the red channel, inverted the greyscale resulting image and measured the optical density. Quantification of DCX/NeuN-if somata in the GlOB, Pir, dSt, vSt, Tu and DG was performed as described for DCX-ir quantification using the multipoint plugin of the ImageJ. We also calculated the density of DCX-if cells as number of nuclei per mm^2 of the ROI. Double DCX/NeuN-if transitioning neurons were calculated as a percentage of the total number of DCX-if cells in each region.

For the maternal separation experiment, we also calculated the percentage of immature DCX-if cells classified as tangled (see Fig. 7c, arrow) and complex (see Fig. 7c, inset) neurons according to their morphology (as described in Benedetti et al.,

2020; Gómez-Climent et al., 2008). Tangled neurons are considered as more immature than complex ones, with a tiny round nucleus and almost none or few short prolongations. By contrast, complex neurons show larger somas with abundant and long dendritic and axonal elongations.

Analysis and quantification of DAPI staining

We obtained photographs in the same levels of the Pir as described in Table 7 with the Leica Leitz DMRB microscope with excitation wavelength of 405 nm. DAPI-labelled nuclei were counted manually by a researcher who was blind to the sex and genotype of the animals with the multipoint plugin and the GRID tool of Image-J. We counted DAPI-labelled neuronal-like, regularly-shaped nuclei of around 10 µm of diameter, in 12 squares of 3×10^3 μm^2 in each slice.

Behavioural procedures

Early-life stress: maternal separation

From PND3 to PND21 pups were separated from the dam as a group, and kept in a new cage filled with sawdust and warming blanket for 3 h per day, after which pups were returned to their home cage. As controls, we used standard care animals, that were maintained undisturbed with their dams in the home cage until weaning. The behavioural profile of the whole group of females (Abellán-Álvaro et al., 2021) and males (Torres-Pérez et al., 2022) has been recently reported.

Statistical analysis

Data were analysed using the software IBM SPSS Statistics 22.0. We first checked the data for normality (Shapiro-Wilk test) and homoscedasticity (Levene's test). Next, data were evaluated using Student's t tests. For MS experiment we also analysed the data using a two-way analysis of variance (ANOVA) with group (naïve or MS) and genotype (WT or *Mecp2*-null) as between-subject sources of variance,

followed by post hoc Bonferroni correction for multiple tests. Significance level was always set at $p<0.05$.

RESULTS

Experiment 1.1 Effect of lack of MeCP2 in cell survival in the OB

Cell survival is not significantly affected in the olfactory structures nor in the striatum in Mecp2*-null mice*

First, we checked whether lack of MeCP2 affected survival of SVZ-generated cells in young males. We found no differences between BrdU-ir density in the granular (Student's t test, t=0.23, p=0.82) or glomerular layer (Student's t test, t=0.35, p=0.73; Figs. 4a, c, c'). As expected, in the Pir, we found only 1-2 labelled nuclei per field of observation, except on two animals, one *Mecp2*-null and one WT, in which we found >5 nuclei (Fig. 4b). This is consistent with the previously described data by (Rubio et al., 2016), who found that DCX-ir population in the Pir is established during embryonic development.

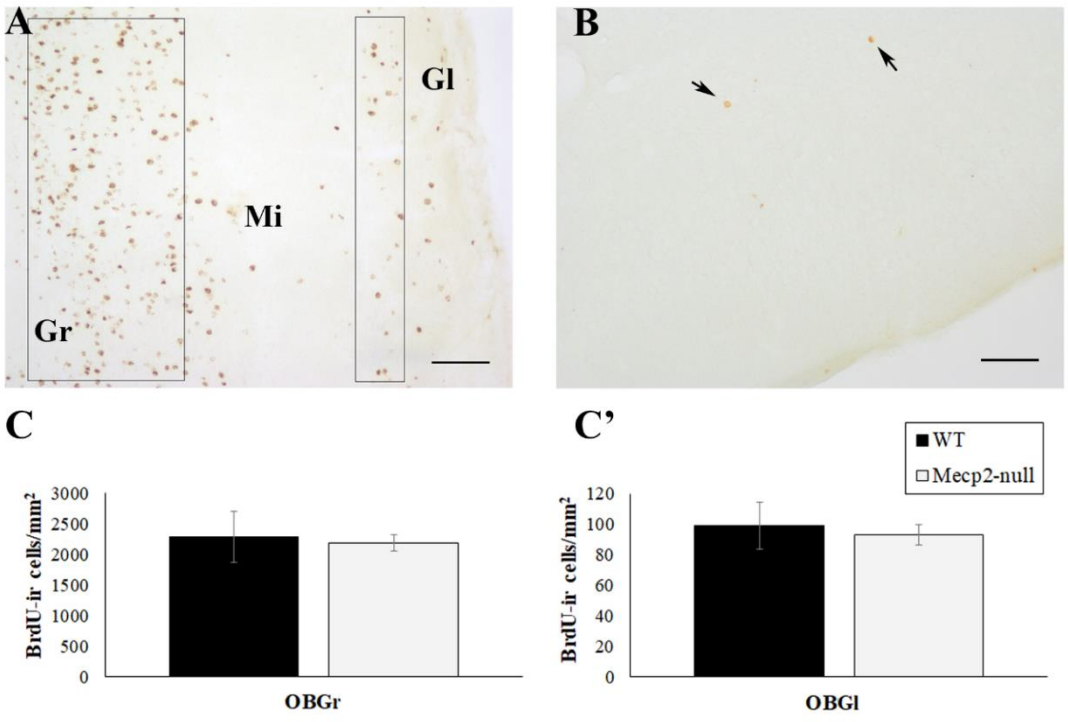


Figure 4: cell survival as analysed by BrdU detection 12 days post-injection. **a)** BrdU-immunolabeled cells in the granular and glomerular layers of the OB. Rectangles represent the two ROI employed to count the cells. **b)** Only few scattered BrdU-ir cells were found in

the Pir. **c**, **c')** Bar charts of the number of BrdU-ir nuclei per mm^2 found in the glomerular and granular layer of the OB respectively, in WT (black bars) and *Mecp2*-null (grey bars) males. Abbreviations: *Gl*, glomerular layer; *Gr*, granular layer; *Mi*, mitral layer; *Pir*, piriform cortex. The scale bar is 100µm. Data are expressed as Mean±S.E.M

Likewise, no significant effect of genotype was found in dSt (Student's t test, t=2.209, p=0.09), nor in vSt (Student's t test, t=-0.988, p=0.35; Fig. 5). These data suggest that lack of MeCP2 does not affect SVZ-generated cells in young mice.

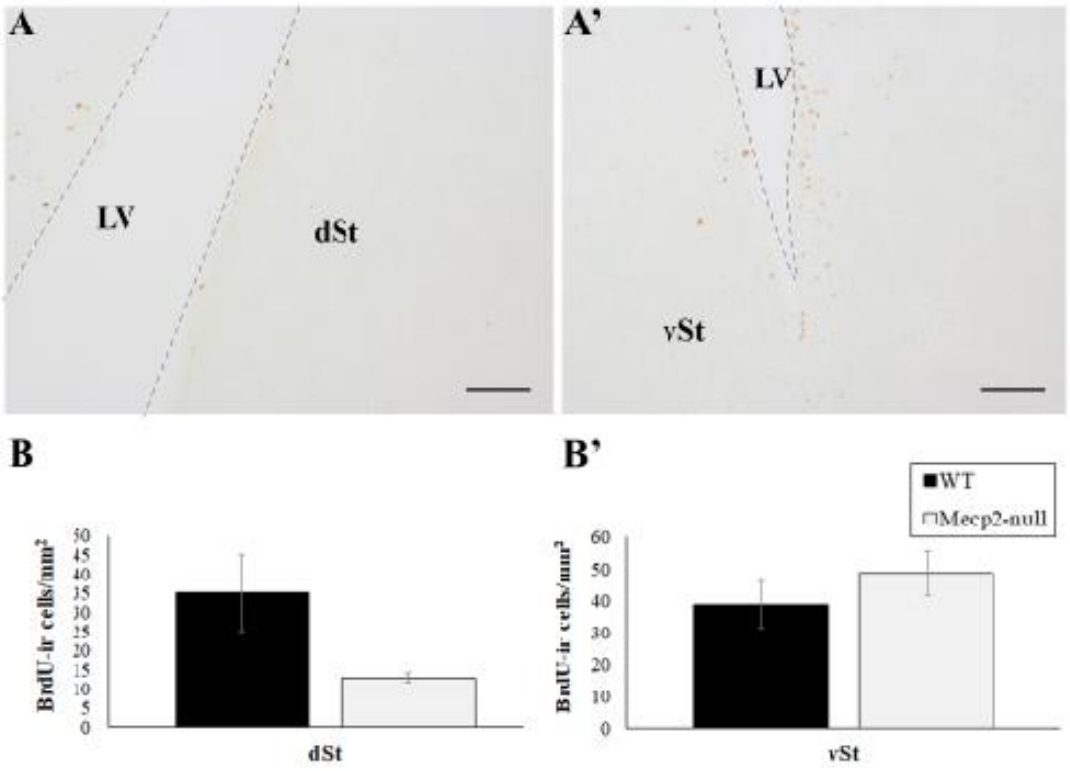


Figure 5: BrdU analysis in dSt and vSt. a, a') Pictures of BrdU-immunostaining in dSt and vSt, respectively. **b, b')** Bar charts representing the number of BrdU-ir nuclei per mm^2 in slices at Bregma level 1.5mm, 1.1mm and 0.7mm in both dSt and vSt, respectively, in WT (black bars) and *Mecp2*-null (grey bars) male mice. Abbreviations: *dSt*, Dorsal striatum; *LV*, Lateral ventricle; *vSt*, Ventral striatum. The scale bar is 100µm. Data are expressed as Mean±S.E.M.

Experiment 1.2 Effect of MeCP2 deficiency on the density of immature neurons

Distribution of DCX-ir cell bodies is not affected by sex or genotype

The distribution of DCX-ir somata in our DAB-stained samples was consistent with that previously described in the rat (Nacher et al., 2001) and mouse (Saaltink et al., 2012) brains. No differences in distribution were apparent between DAB-stained or fluorescence-labelled samples, thus both methods are comparable.

Overall, we found abundant DCX-ir in the SVZ of the lateral ventricles (Fig. 6a) and RMS (Figs. 6d, d', 7a). Some of the cells next to SVZ were integrated in the striatal parenchyma both at the dorsomedial part of the striatum (Fig. 7a, inset) and the ventral striatum (accumbens core and lateral septum) next to the ventral tip of the ventricle (Fig. 6b, b'). Abundant DCX-ir neuronal somata were also present in the subgranular zone of the DG of the hippocampus (Fig. 6c) and granular (Gr) and periglomerular (PGl) layers of the OB (Figs. 6d, d', 7a, b), in the piriform cortex (Figs. 6e, e', 7c) and dorsal and ventral endopiriform nuclei. Importantly, we discovered a previously unnoticed, to our knowledge, small population of DCX-ir cells in layer I of the Tu (Figs. 6f, f', 7d). These cells displayed abundant processes and sometimes were densely packed in groups of 5–10 cells, especially in the mutant animals (Figs. 6f', 7d). Although these DCX-expressing cells were quite scarce, their presence has been confirmed in another two strains of mice (see supplementary information in Martínez-Rodríguez et al., 2019; data from the lab). Further, around 80% of these DCX-if cells co-express the polysialylated form of the neural cell adhesion molecule (PSA-NCAM), a plasticity marker described to co-locate with DCX in the Pir immature population of layer II (Rubio et al., 2016), and some of them co-expressed the neuronal nuclear antigen (NeuN), a neuronal marker (see supplementary information in Martínez-Rodríguez et al., 2019). These data suggest that DCX-if cells in the Tu might be, although scarce, biologically significant, immature neurons.

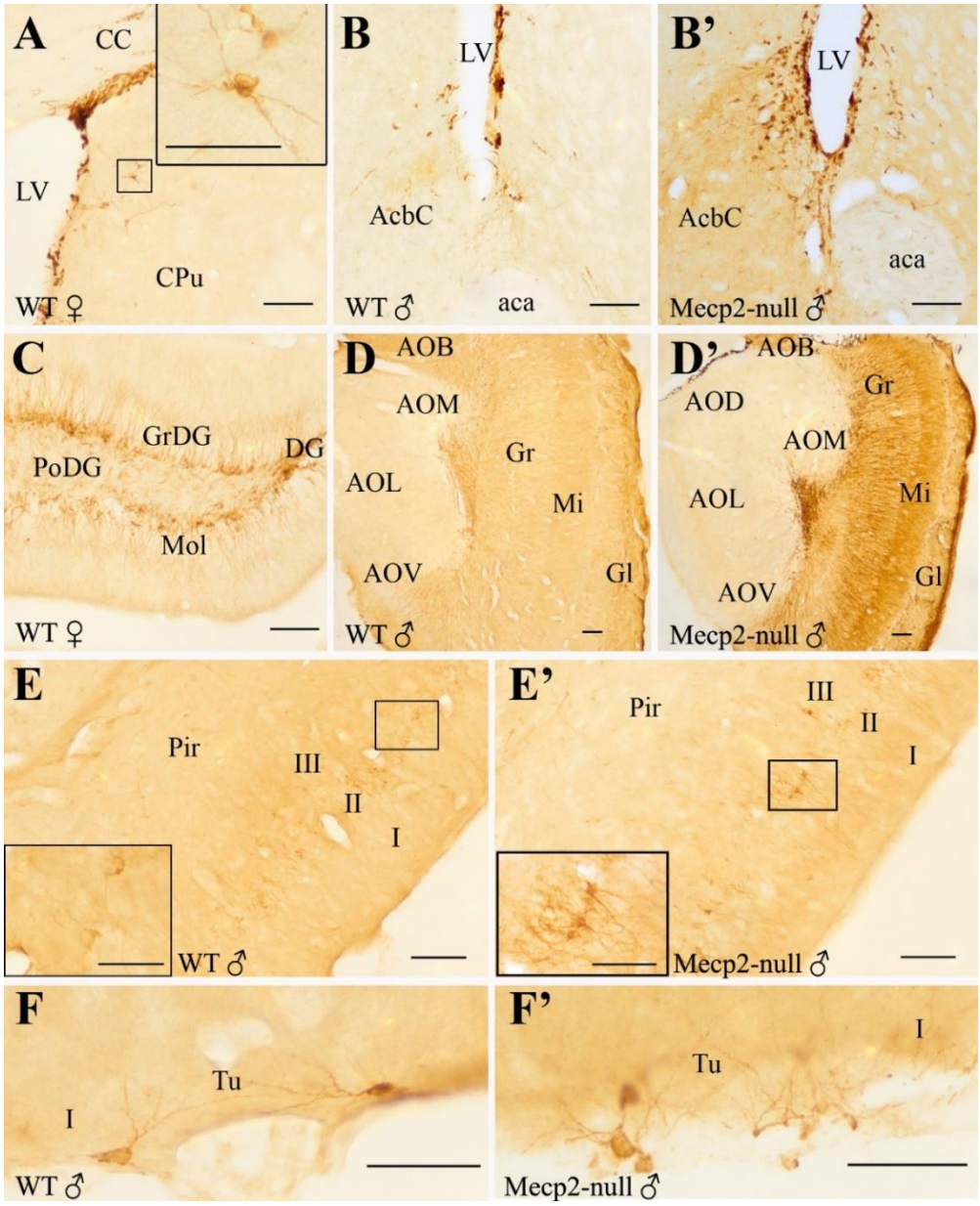


Figure 6: Representative photomicrographs of the distribution of DCX-ir in the brain of *Mecp2*-mutant and WT mice. **a)** Example of DCX-ir in the SVZ and dSt (inset) in a WT female. **b)** DCX-ir in the ventral part of the SVZ and vST in a WT male. **b')** DCX-ir in the vSt of a *Mecp2*-null male. **c)** DCX-ir in the DG of a WT female. **d)** DCX-ir in the OB of a WT male. **d')** DCX-ir in the OB of a *Mecp2*-null male. **e)** DCX-ir in the Pir of a WT male. **e')** DCX-ir in the Pir of *Mecp2*-null male. **f)** DCX-ir in layer I of Tu on a WT male. **f')** DCX-ir in layer I of Tu on a *Mecp2*-null male. Scale bar: 100 µm. Abbreviations: *Aca,* anterior commissure; *AcbC,* core of the nucleus accumbens; *AOB,* accessory olfactory bulb; *AOL,* anterior olfactory nucleus, lateral; *AOM,* anterior olfactory nucleus, medial; *AOV,* anterior olfactory nucleus, ventral; *CC,* corpus callosum; *CPu,* caudatus putamen; *DG,* dentate gyrus of the hippocampus; *Gl* glomerular cell layer of the olfactory bulb; *Gr,* granular layer of the olfactory bulb; *GrDG,* granular layer

of the dentate gyrus; *LV,* lateral ventricle; *Mi,* mitral cell layer of the olfactory bulb; *Mol,* molecular layer of the dentate gyrus; *PoDG,* polymorphic-oriens layer of the dentate gyrus; *Pir,* piriform cortex; *Tu* olfactory tubercle; *WT*, wild type.

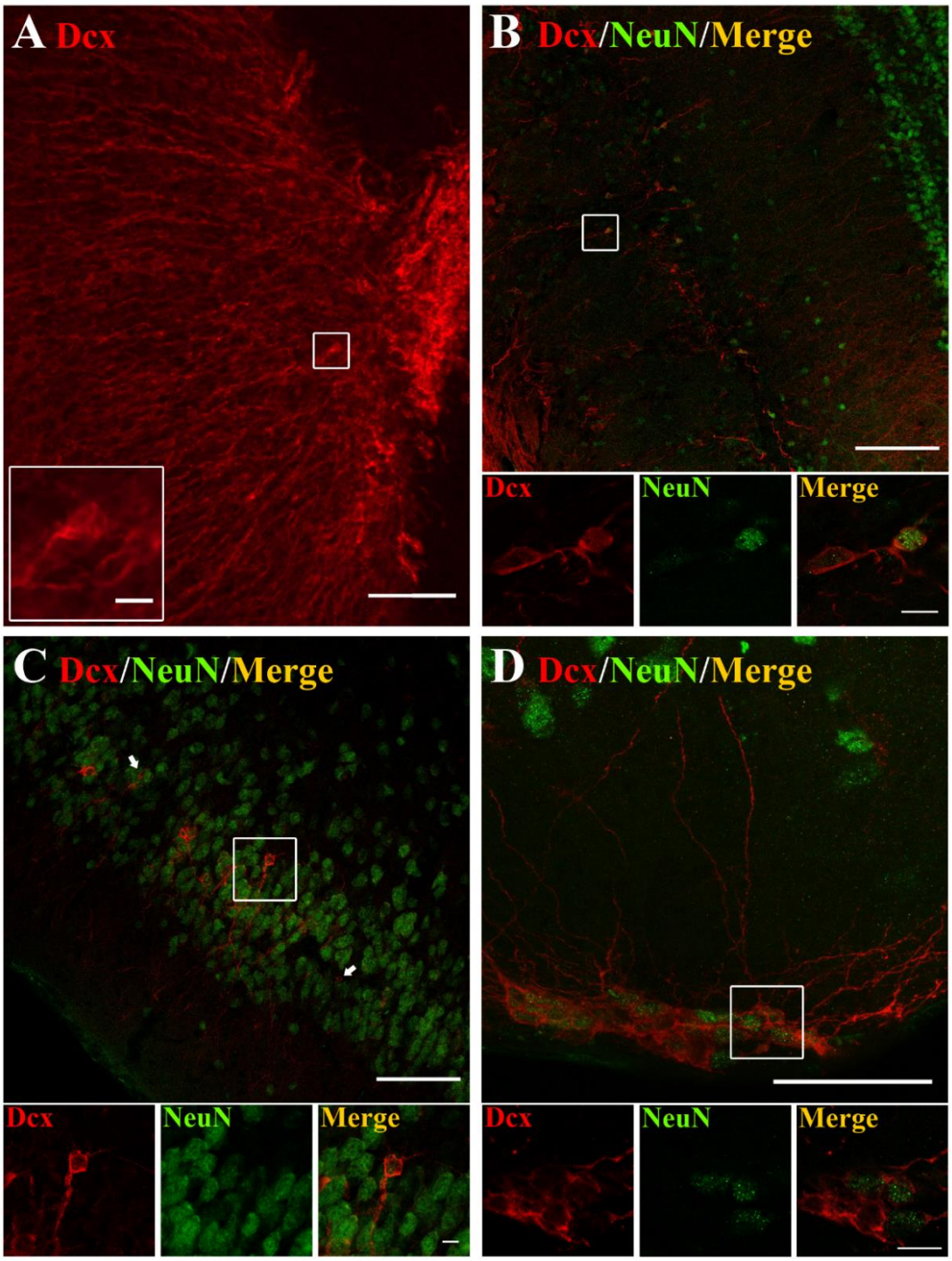


Figure 7: Representative images of regions of the olfactory system of *Mecp2*-null and WT male mice co-labelled by immunofluorescence for DCX (red) and NeuN (green). a) Example of DCX-if fibres and somata (inset) found in the olfactory bulb of a WT male. **b)** Olfactory

bulb of a WT male, showing an example of a transitioning neuron in the periglomerular zone co-labelled for DCX and NeuN in the inset. White arrows point to DCX^+ tangled neurons. **c)** Example of DCX-if in the piriform cortex of a *Mecp2*-null, showing a $DCX^+/NeuN^-$ cell in the inset. **d)** Olfactory tubercle from a *Mecp2*-null male showing a cluster of DCX-if cells in layer I. Three "transitioning cells", immature neurons colabelled by DCX and NeuN, and four $DCX^+/NeuN^-$ cells are displayed in the inset. Scale bar 100 µm, in insets 10 µm.

DCX-ir cell density is increased in olfactory structures in Mecp2*-null male mice*

We first quantified the density of DCX-ir in our DAB-stained material, and then confirmed some of the significant differences found, or their lack thereof, in our immunofluorescent samples. Area fraction covered by DCX-ir in the OB was not significantly different between females of both genotypes (Student's t test, t=-1.1, p=0.325, Fig. 8a), or between *Mecp2*-null males and WT (Student's t test, t=-2.23, p=0.06, Fig. 9a). Accordingly, in the samples labelled with immunofluorescence, we found no significant differences between *Mecp2*-null and their WT littermates in optic density of DCX-if in the granular layer (Student's t test, t=-0.809, p=0.44; Fig. 9a') or in the number of DCX-if cells in the periglomerular cell layer (Student's t test, t=-0.724, p=0.49). Thus, globally, DCX expression in the OB seems not affected by lack or deficiency of MeCP2.

Similarly, we found no significant differences between genotypes in the Tu of females (Student's t test, t=-2.00, p=0.08; Fig. 8b). By contrast, there was a significant increase of both DCX-ir and DCX-if cells in the Tu of *Mecp2*-null males with respect to WT (Student's t test, t=-4.65, p=0.001; and t=-2.861, p=0.02, respectively; Figs. 9b, b').

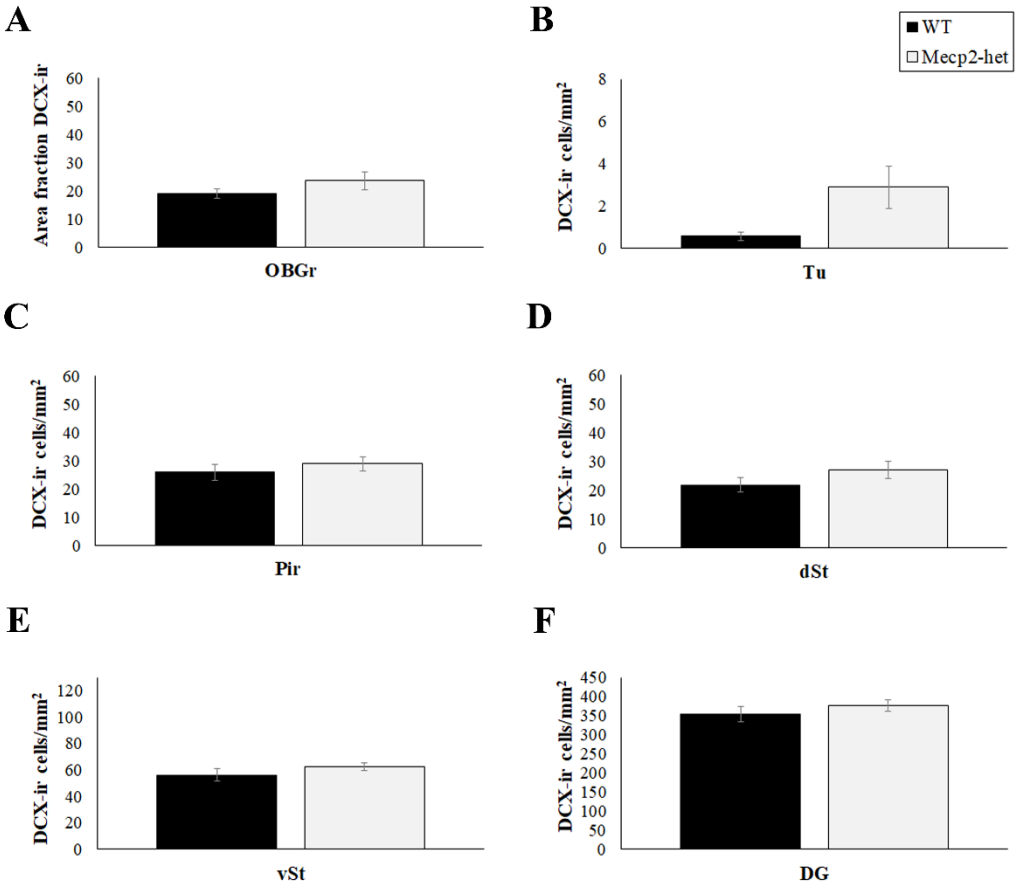


Figure 8: Quantitative analysis of DCX-ir cell density in wild type (black bars) and *Mecp2*-het (grey bars) female mice. DCX expression is not significantly affected in females. Abbreviations: *DG*, dentate gyrus; *dSt,* dorsal striatum; *OBGr,* granular layer of the olfactory bulb; *Pir,* piriform cortex; *Tu,* olfactory tubercle; *vSt,* ventral striatum. Values are presented as mean±SEM

In the Pir of females, we found no significant differences between genotypes (Student's t test, t=-0.82, p=0.43, Fig. 8c), but both DCX-ir and DCX-if cell density were significantly higher in the Pir of *Mecp2*-null males than in their WT littermates (Student's t test, t=-4.20, p=0.002; and t=-4.1, p=0.003, respectively, Figs. 9c, c'). Since brain size of *Mecp2*-mutant animals has been reported to be smaller than that of their WT siblings (Kishi & Macklis, 2004), corresponding to smaller cells that are more densely packed (R. Z. Chen et al., 2001; Kishi & Macklis, 2004), we estimated the cell density and width of layer II in the Pir in a DAPI-labelled parallel section of the analysed samples. In addition, we calculated the average diameter of somata of

DCX-ir cells. The statistical analyses revealed no significant differences in any of those measures between genotypes, neither in males nor in females (Table 8). Thus, the significant increase in DCX-ir cells in the Pir in *Mecp2*-null animals is not likely to be due to a brain size effect in our sample. In sum, we found a significant increase in DCX-expressing cells in olfactory areas in *Mecp2*-null, but not in *Mecp2*-het animals, as compared with their WT littermates.

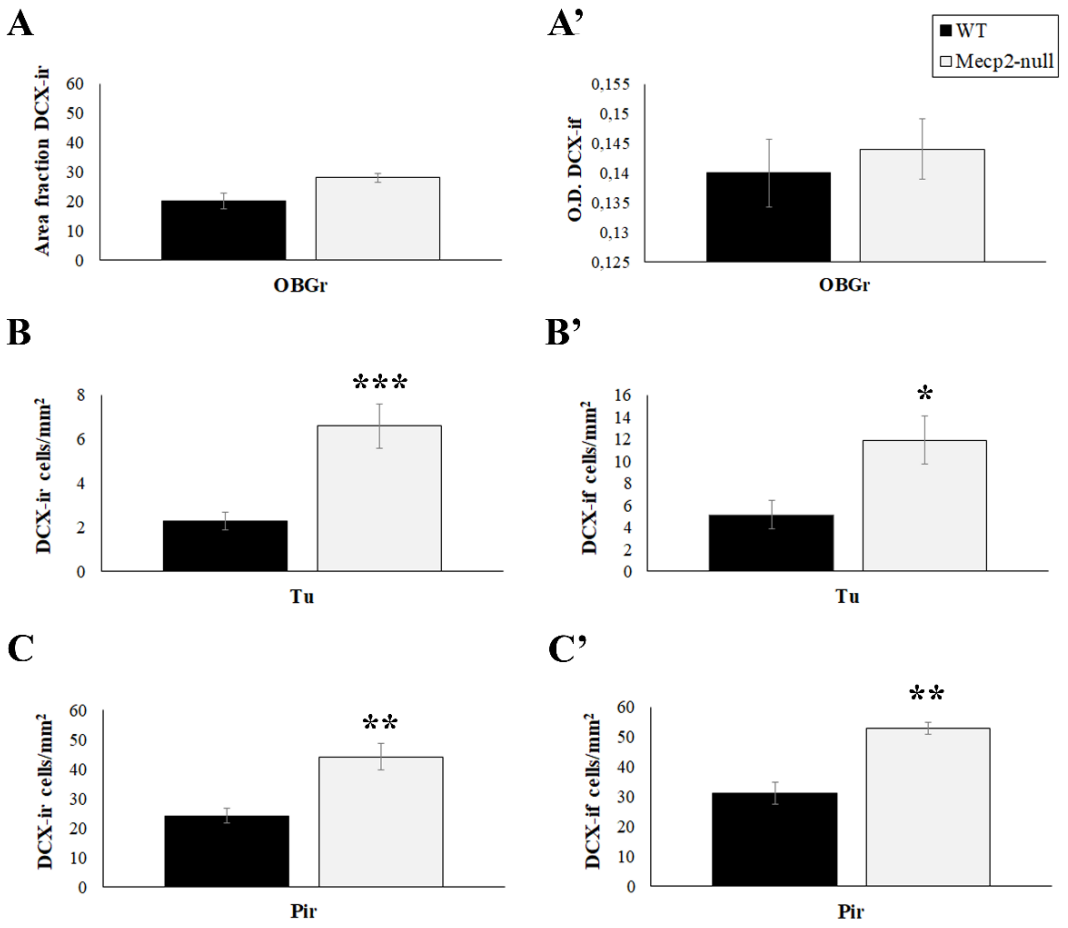


Figure 9: Quantitative analysis of DCX-ir and DCX-if cell density in the olfactory system of wild type (black bars) and *Mecp2*-null (grey bars) male mice. We found a significant increment in the population of immature neurons in Pir and Tu of *Mecp2*-null males compared to the WT. Abbreviations: *OBGr,* granular layer of the olfactory bulb; *Pir,* piriform cortex; *Tu,* olfactory tubercle. Values are presented as mean±SEM. *p<0.05; **p<0.01; *Mecp2* vs WT.

Table 8: Quantitative analysis of cell density, width of layer II and diameter of DCX-ir somata in the piriform cortex of wild type and *Mecp2*-deficient mice. No significant differences were found in the analysis.

Cell density (average of both hemispheres, nuclei/mm^2)			
Females	**WT**	2826.3 ± 108.0	*p*=0.66
	***Mecp2*-het**	2899.0 ± 116.6	
Males	**WT**	2464.7 ± 56.9	*p*=0.56
	***Mecp2*-null**	2514.4 ± 57.5	
Width of layer II (average, µm)			
Females	**WT**	128.7 ± 8.2	*p*=0.38
	***Mecp2*-het**	121.2 ± 4.8	
Males	**WT**	129.6 ± 2.9	*p*=0.87
	***Mecp2*-null**	128.8 ± 3.8	
Diameter of the soma (average, µm)			
Females	**WT**	12.2 ± 0.3	*p*=0.21
	***Mecp2*-het**	11.6 ± 0.2	
Males	**WT**	11.7 ± 0.1	*p*=0.68
	***Mecp2*-null**	11.9 ± 0.4	

Values are presented as mean±SEM

The percentage of immature neurons co-expressing DCX/NeuN is not differentially affected in olfactory structures in Mecp2*-null male mice*

Since our results showed a significant increase in total DCX-expressing cells in olfactory structures in *Mecp2*−null males with respect to their WT controls, we next calculated the percentage of the DCX-if cells that were also expressing NeuN, i.e., "transitioning neurons", in Pir and Tu in our sample of male mice (see Fig. 7c, inset). Data showed that the percentage of DCX/NeuN-if "transitioning neurons" were not significantly affected by genotype in Tu (Student's t test, t=1.294, p=0.23; Fig. 10a), or the Pir (Student's t test, t=2.019, p=0.85; Fig. 10b) of males. Regarding the latter result, the total number of double-labelled cells in the Tu was variable, and in fact there were no double labelled cells in the Tu in three out of seven WT and in one out of five *Mecp2*-null mice. The total number of double labelled DCX/NeuN-if cells in the Tu was not significantly different between genotypes (0.57 ± 0.3 cells/section in the WT vs 0.43 ± 0.21 cells/section in the *Mecp2*-null mice, p=0.74). In the Pir, where neurons are generated during embryonic development, and in the Tu, lack of MeCP2 increased DCX cells while not affecting transitioning neurons.

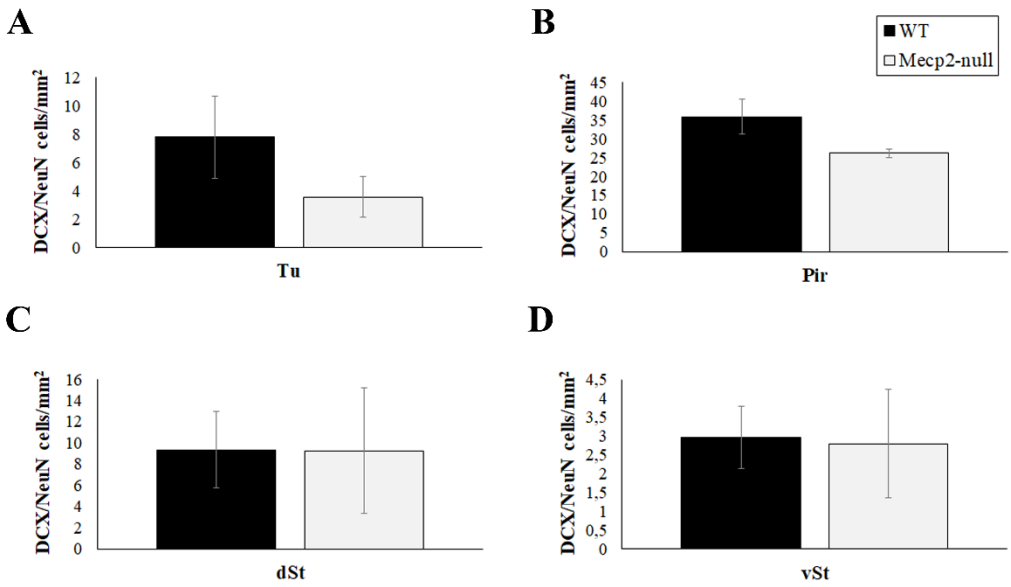


Figure 10: Quantitative analysis of double DCX/NeuN-if cell density in the olfactory system of wild type (black bars) and *Mecp2*-null (grey bars) male mice. No significant differences were found. Abbreviations: *dSt,* dorsal striatum; *Pir,* piriform cortex; *Tu,* olfactory tubercle; *vSt,* ventral striatum. Values are presented as mean±SEM

DCX-if cells are affected by deficiency of MeCP2 in the striatum, but not in the hippocampus

Regarding DCX-ir cells in females, Student's t test revealed no significant differences between genotypes in dSt (Student's t test, t=-1.32, p=0.22; Fig. 8d), vSt (Student's t test, t=-1.44, p=0.19; Fig. 8e), and DG (Student's t test, t=-0.72, p=0.49; Fig. 8f).

In the case of males, our analysis showed no significant differences between genotypes in density of DCX-ir cells in dSt (Student's t test, t=-2.23, p=0.67; Table 9) or vSt (Student's t test, t=-1.89, p=0.09; Table 9) although we found a trend in this latter one. However, analysis of DCX-if in striatum revealed significant differences between genotypes in both dSt and vSt (Student's t test dSt, t=-2.497, p=0.04; vSt, t=-2.996, p=0.02; Table 9). Thus, we found inconsistent statistical results between samples, although the direction of data is the same. In addition, percentages of transitioning neurons were not different between the *Mecp2*-null and WT males in the striatum (p>0.05; 10c, d). Finally, in the hippocampus, the Student's t test found

no significant differences between genotypes in males (Student's t test, t=-0.38, p=0.76; Table 9).

Table 9: Quantitative analysis of DCX-ir and DCX-if cell density in the striatum and hippocampus of wild type and *Mecp2*-null male mice. We found a significant increase in the number of DCX-if cells in dSt and vSt of *Mecp2*-null males. Although we found inconsistent statistical results between DAB and fluorescent samples, the direction of the data is the same. No significant differences between genotypes were found in the DG. Abbreviations: *DG,* dentate gyrus; *dSt,* dorsal striatum; *vSt,* ventral striatum.

Males	DCX-ir (DAB)	DCX-if
dSt (average of both hemispheres, nuclei/mm^2)		
WT	44.45 ± 7.3	29.92 ± 2.6
***Mecp2*-null**	48.78 ± 5.3	**47.03 ± 8***
vSt (average of both hemispheres, nuclei/mm^2)		
WT	81.75 ± 9.1	111.51 ± 7.5
***Mecp2*-null**	110 ± 12.3	**147.08 ± 8.5***
DG (average of both hemispheres, nuclei/mm^2)		
WT	386.6 ± 23.4	
***Mecp2*-null**	398.7 ± 29.1	

Values are presented as mean±SEM *p<0.05 *Mecp2* vs WT.

Experiment 1.3 Interaction between early-life stress and MeCP2 deficiency on the density of immature neurons

Maternal separation increases weight of wild type male mice, with no significant effect in Mecp2*-null males nor in WT and Mecp2-het females*

Next, we analysed the effect of maternal separation on population of DCX-if cells. In the case of females, the ANOVA showed a significant effect of genotype in weight of females ($F_{(1,53)}$=12.372, p=0.001). In this case, both WT-MS ($F_{(1,53)}$=5.248, p=0.026) and WT-naïve ($F_{(1,53)}$=7.142, p=0.010) females were significantly heavier than their respective *Mecp2*-het counterparts. However, MS does not seem to affect weight of female mice, since we found no significant effect of condition.

In the case of males, the ANOVA also revealed a significant effect of genotype ($F_{(1,50)}$=137.009, p<0.001), as well as significant interaction between genotype and group ($F_{(1,50)}$=4.140, p=0.047). Subsequent pairwise comparisons with the

Bonferroni correction revealed that WT-MS males were significantly heavier than their WT-naïve counterparts ($F_{(1,50)}$=6.057, p=0.017), but no effects were found in *Mecp2*-null animals (p>0.5; Fig. 11). Thus, maternal separation seems to increase weight only in WT males exposed to MS-3h, with no effect in the *Mecp2*-null males, which are already significantly thinner than WT males.

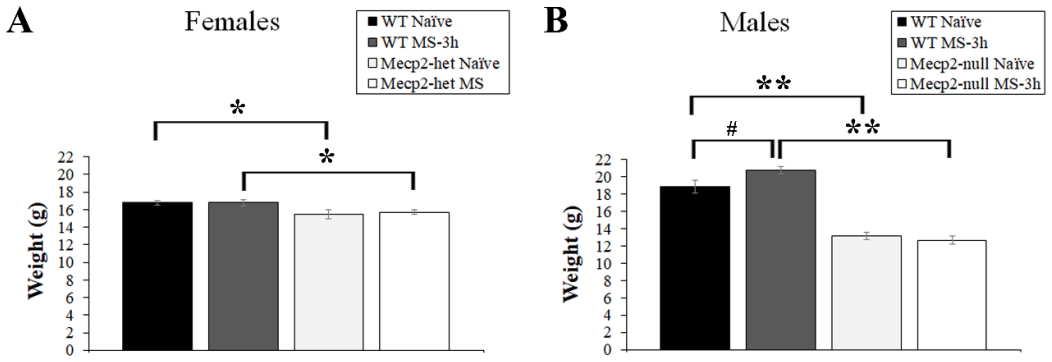


Figure 11: graphic comparison of weight between WT and *Mecp2*-mutant a) females and b) males in both conditions: naïve and affected by MS-3h. Overall, lack of MeCP2 decreases the weight of both female and male mice. In addition, maternal separation exacerbates this decrease only in the case of WT males. Values are presented as mean±SEM. *p<0.05, **p<0.01, WT vs *Mecp2*-mutant; #p<0.05, *naïve* vs MS-3h.

Maternal separation differentially affects the number of DCX$^+$ neurons males and females

In the case of females, a two-way ANOVA revealed statistically significant effect of maternal separation in the OBGr ($F_{(1,15)}$=30.069, p<0.001), with a reduction in the O.D of DCX-if in both WT-MS ($F_{(1,15)}$=17.300, p=0.001), and *Mecp2*-het-MS ($F_{(1,15)}$=12.981, p=0.003; Fig. 12a) compared to the groups of naïve females. We found no significant differences between genotypes in this nucleus. Likewise, no significant effect of genotype or condition was found in the case of OBGl, vSt, or dSt (p>0.05; Fig. 12b, c, d) of females. Analogous to that, no significant differences between genotypes or conditions were found in the OBGr or OBGl of males (p>0.05; Fig. 12a'). However, in terms of condition, we saw an effect of maternal separation in both dSt ($F_{(1,14)}$=11.215, p=0.005) and vSt ($F_{(1,14)}$=8.779, p=0.01) of males. In dSt,

MS-3h increases the number of DCX-if cells in *Mecp2*-null-MS males ($F_{(1,14)}$=7.078, p=0.019, Fig. 12c') compared to *Mecp2*-null-naïve. We also observed a trend in the case of WT-MS males ($F_{(1,14)}$=4.279, p=0.058; Fig. 12c') vs WT-naïve. In the case of vSt, MS increases the population of DCX^+ cells only in *Mecp2*-null males-MS ($F_{(1,14)}$=16.478, p=0.001; Fig. 12d') but not in the WT-MS, compared to their respective naïve group.

In addition, we found that MS-3h does not significantly affect the total number of DCX-if cells in the Pir of WT-MS and *Mecp2*-het-MS females compared to the naïve groups. However, we observed a trend towards higher levels of these DCX-if cells in the groups exposed to MS (p=0.068). DCX^+ cells in the piriform cortex have previously been distinguish between tangled (more immature) and complex neurons (in transition to maturity; Rotheneichner et al., 2018). Thus, we also analysed these two types of neurons separately in the Pir of our mice. As a result, our data revealed that, although total population of DCX^+ in Pir was not affected by genotype or condition (p>0.05; Fig. 13a), MS-3h affects and decreases the number of DCX-if tangled cells in both genotypes ($F_{(1,17)}$=5.975, p=0.026; Fig. 13b). In the case of complex DCX-if cells in Pir, we found no significant differences caused by genotype or condition (Fig. 13c).

We found the opposite effect of genotype and condition (MS-3h vs naïve) in the Tu of female mice ($F_{(1,16)}$=6.656, p=0.020). Our data showed that *Mecp2*-het-MS females have significantly more DCX-if cells in the Tu ($F_{(1,16)}$=4.928, p=0.04; Fig. 14a) compared to *Mecp2*-het-naïve. This increase was not present in the WT-MS females. Additionally, we also found an effect of genotype in the group of animals exposed to MS-3h, with an increased population of DCX^+ cells in the Tu of *Mecp2*-het-MS compared to the WT-MS female ($F_{(1,16)}$=16.658, p=0.001; Fig. 14a).

In the case of males, results of experiment 1.2 in young-adult males (8 weeks of age) reveal an increased population of DCX^+ cells in the Pir and Tu of *Mecp2*-null males

compared to WT, suggesting an impairment in neuronal maturation caused by lack of *Mecp2*. This hypothesis is also supported by some results from the present maternal separation experiment. As an example, the two-way ANOVA revealed an effect of genotype in both Pir and Tu nuclei. Thus, within the group of MS ($F_{(1,13)}$=6.059, p=0.029), higher levels of DCX^+ cells are present in the Pir of *Mecp2*-null-MS males ($F_{(1,13)}$=7.322, p=0.018, Fig. 13a') compared to WT-MS. However, *Mecp2*-null-naïve males does not show higher levels of DCX-if neurons in Pir compared to the WT-naïve males. Additionally, MS-3h increases the total number of immature neurons in *Mecp2*-null-MS males ($F_{(1,13)}$=10.055, p=0.007, Fig 13a'), compared to *Mecp2*-null-naïve.

When we analysed tangled and complex neurons in the Pir of our males exposed to MS, we found that population of tangled neurons in *Mecp2*-null-MS males is increased compared to the WT-MS ($F_{(1,13)}$=6.335, p=0.03, Fig. 13b'). Likewise, MS increases number of tangled ($F_{(1,13)}$=6.129, p=0.03, fig 13b'), and complex neurons ($F_{(1,13)}$=5.381, p=0.04, fig 13c') in *Mecp2*-null-MS males compared to *Mecp2*-naïve. Thus, the increased total number of DCX^+ cells in Pir of *Mecp2*-null-MS males is caused by higher levels of both tangled and complex immature neurons. No significant effect of MS was found between the groups of WT-MS and WT-naïve males.

In addition, we found higher levels of DCX-if cells in the Tu of *Mecp2*-null-naïve males ($F_{(1,11)}$=6.346, p=0.029, Fig. 14a') compared to the WT-naïve, and in *Mecp2*-null-MS males ($F_{(1,11)}$=7.336, p=0.020, Fig. 14a') compared to the WT-MS. However, our analysis revealed no significant effect of condition within this nucleus in our males.

Finally, no significant effect of genotype or condition was found in the DG of females (p>0.05; Fig. 14b). Conversely, we observed an effect of genotype in the DG of males ($F_{(1,14)}$=4.846, p=0.046) specific of the maternal separation group, with higher density of DCX-ir cells in the *Mecp2*-null-MS group as compared with WT-MS (p =

0.008; Fig. 14b'), but not in the *Mecp2*-null-naïve compared to WT-naïve. However, we found no significant effect of condition in DG of males.

Overall, our data revealed that maternal separation differentially affects the number of DCX^+ immature neurons in males and females in a region-dependent way. In the case of females, MS-3h reduces the number of DCX^+ neurons in the OBGr of both genotypes, as well as the number of tangled neurons in the Pir of WT females. By contrast, MS-3h increases the number of DCX^+ in the Tu of *Mecp2*-het-MS females, with no effect in the WT group. Conversely, in the case of males, both maternal separation and lack of *Mecp2* increase the number of DCX-if cells in a region-dependent manner: lack of *Mecp2* increases the number of DCX-if cells in Pir, Tu, dSt and DG; whilst maternal separation increases this population in the St and Pir of *Mecp2*-null males.

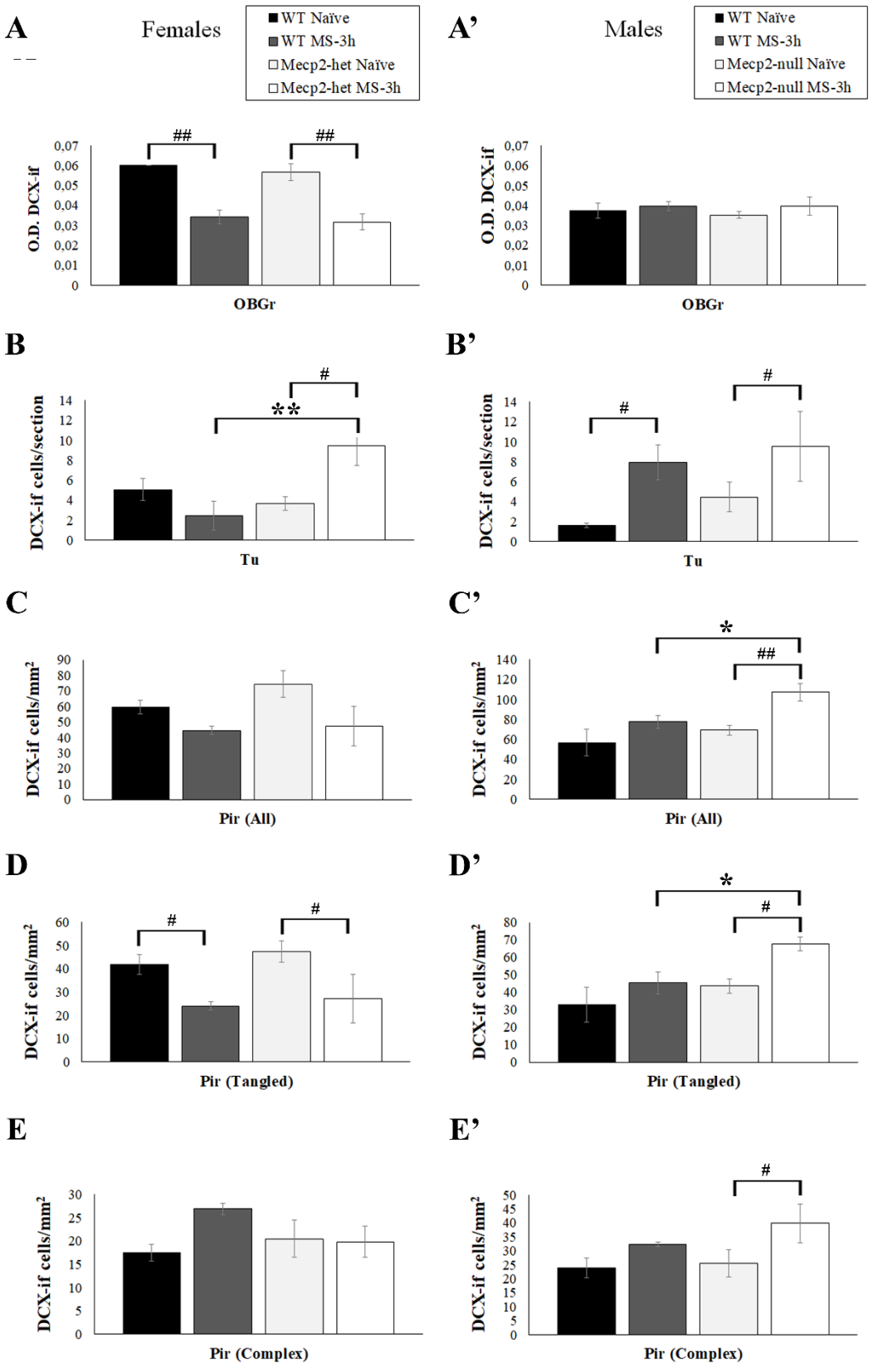

A
Females
WT Naïve
WT MS-3h
Mecp2-het Naïve
Mecp2-het MS-3h
O.D. DCX-if
OBGr
A'
Males
WT Naïve
WT MS-3h
Mecp2-null Naïve
Mecp2-null MS-3h
O.D. DCX-if
OBGr
B
DCX-if cells/section
Tu
B'
DCX-if cells/section
Tu
C
DCX-if cells/mm²
Pir (All)
C'
DCX-if cells/mm²
Pir (All)
D
DCX-if cells/mm²
Pir (Tangled)
D'
DCX-if cells/mm²
Pir (Tangled)
E
DCX-if cells/mm²
Pir (Complex)
E'
DCX-if cells/mm²
Pir (Complex)

Figure 12: Quantitative analysis of DCX-if cell density in different nuclei of the olfactory system of wild type (naïve, black bars; MS-3h, dark grey bars) and *Mecp2*-mutant (naïve, light grey bars; MS-3h, with bars) female and male mice. Maternal separation decreases the number of immature neurons in the OBGr of both *Mecp2*-het and WT females, without an effect in males. In the case of Tu, lack of *Mecp2* increases the level of DCX-if cells in MS- females and MS exacerbates this increase in the Tu of *Mecp2*-het females. In the case of males, MS increases the number of DCX-if cells in both genotypes. MS also decreases the number of tangled immature neurons in the Pir of *Mecp2*-het females, without affecting the total number of DCX^+ cells or the complex ones. Conversely, MS-3h increases the total number of DCX^+ cells in *Mecp2*-null males, affecting both tangled and complex neurons. Abbreviations: *OBGr,* granular layer of the olfactory bulb; *Pir,* piriform cortex; *Tu,* olfactory tubercle. Values are presented as mean±SEM. *p<0.05, *p<0.01, WT vs *Mecp2*-mutant; #p<0.05, ##p<0.01, MS-3h vs *naïve*.

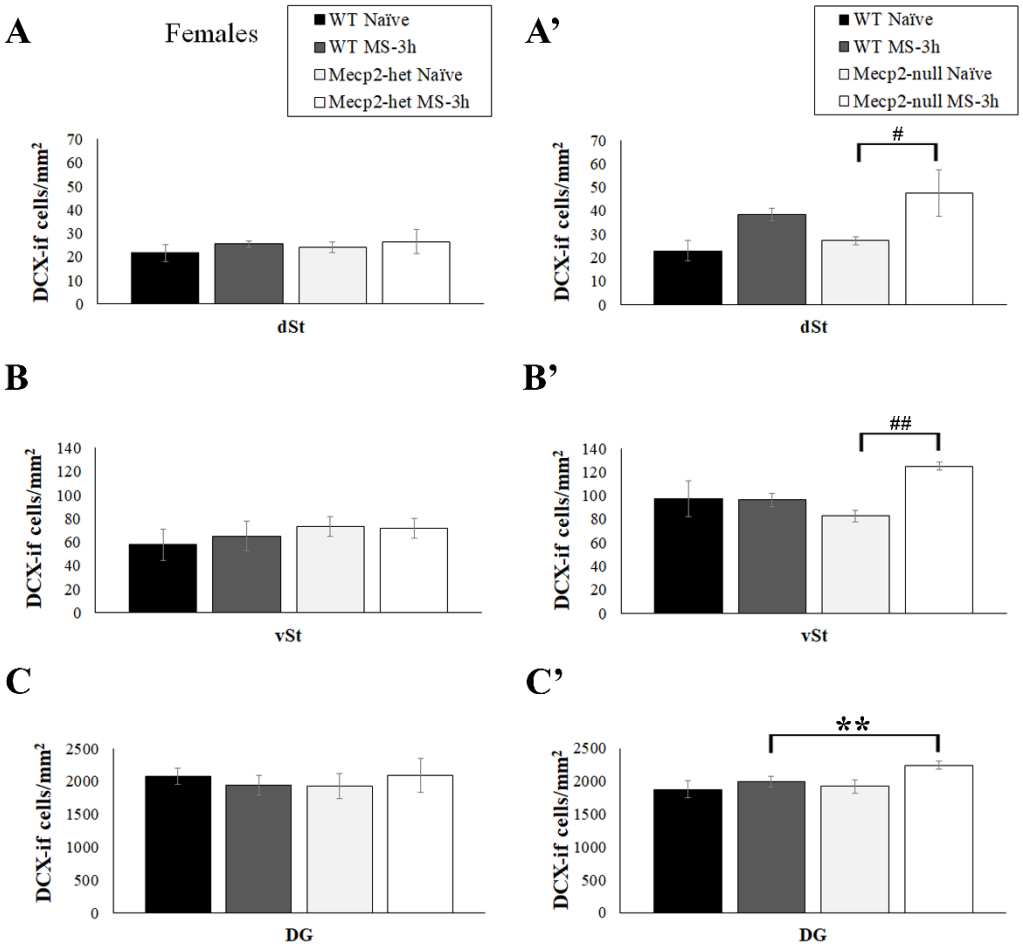


Figure 13: Quantitative analysis of DCX-if cell density in the striatum and hippocampus of wild type (naïve, black bars; MS-3h, dark grey bars) and *Mecp2*-mutant (naïve, light grey bars; MS-3h, with bars) female and male mice. MS does not affect population of DCX^+ cells in striatum or DG of females, nor the DG of males. Conversely, MS increases this population

in the dSt and vSt of MS-*Mecp2*-null males compared to Naïve-*Mecp2*-null males. In addition, MS exacerbates the consequences of lack of MeCP2 in the DG of males, increasing the number of DCX-if cells in MS-*mecp2*-null males compared to MS-WT males. Abbreviations: *DG,* dentate gyrus; *dSt,* dorsal striatum; *vSt,* ventral striatum. Values are presented as mean±SEM. **p<0.01, WT vs *Mecp2*-mutant; #p<0.05, ##p<0.01, MS-3h vs *naïve*.

DISCUSSION

In this chapter, we investigated: (i) cell survival of SVZ-generated neurons, (ii) the distribution and density of immature, DCX-expressing neurons, in young adult *Mecp2*-het and *Mecp2*-null mice, as compared to their WT littermates, and (iii) the effect of early-life stress in this population of immature neurons in *Mecp2*-null and wild type males exposed to maternal separation as compared to their naïve littermates. Overall, we did not find differences in the number of BrdU-ir cells between genotypes in males, suggesting that deficiency of MeCP2 does not interfere in cell survival. Together with previous results on proliferation in SVZ (Agustín-Pavón et al., 2016), our data point to a lack of significant effects of *Mecp2* deficiency on the first stages of postnatal neurogenesis.

Thus, we next checked for a marker of neuronal immaturity, and we found no recognisable qualitative differences in the distribution of DCX^+ somata between WT and mutant mice. On the other hand, and in agreement with a previously suggested role of MeCP2 in neuronal maturation, we found significant increases of DCX^+ cells, immature neurons, in olfactory areas in *Mecp2*-null males, specifically in the Pir and a newly identified population of DCX cells in the Tu, but not in heterozygous females. In these olfactory areas, the density of double-labelled DCX/NeuN, "transitioning neurons", was not affected by lack of MeCP2. Further, we found no differences in total expression of DCX in the olfactory bulbs nor in the number of transitioning neurons. However, density of DCX-ir neurons in the hippocampus and striatum was not affected by deficiency or lack of MeCP2.

Finally, early-life stress affects body weight of males but not females, increasing the weight of WT-MS males compared to the WT-naïve. Moreover, MS-3h decreases the number of DCX^+ cells in OBGr in both *Mecp2*-het-MS and WT-MS females. This condition also decreases the number of tangled neurons, without affecting the total number of DCX-if cells in de Pir of both genotypes in females. These results may

suggest an acceleration in the process of neuronal maturation in females. Conversely, maternal separation increases the number of DCX^+ cells in the Tu of *Mecp2*-het-MS females compared to the *Mecp2*-het-naïve. On the other side, *Mecp2*-null males exposed to MS-3h present higher levels of DCX^+ neurons in dSt, vSt and Pir (increasing both tangled and complex neurons). By contrast, number of immature neurons in DG, Tu or OB was not affected by maternal separation in males, although we found higher levels of DCX^+ cells in the DG of *Mecp2*-null-MS males compared to the WT-MS males.

Lack of MeCP2 does not affect cell proliferation or survival in the olfactory system

The olfactory system provides an excellent model to study neurodevelopmental disorders, and as such, it has been studied in both RTT patients and genetically modified mice, with most of the available data obtained in olfactory sensory neurons (OSN) and OB. Thus, early qualitative observations from patient nasal biopsies suggested that a bigger surface area of their nasal epithelium contained olfactory sensory epithelium as compared to healthy controls. In addition, OSN were dysmorphic and increased numbers of immature OSN were found in RTT patients (Ronnett et al., 2003). In *Mecp2*-null mice, there is an early postnatal disorganisation in the olfactory epithelium, with more immature OSN at postnatal week 2 and less at postnatal week 7 than in WT (Matarazzo et al., 2004). Two possible hypotheses arise from these data: in a scenario of MeCP2 deficit, (i) there is a deficit in mature OSN leading to a compensatory increase in neurogenesis—at least in the olfactory epithelium, or (ii) there is a deficit in terminal differentiation of neurons.

Some pieces of evidence support the latter possibility. First, adult neurogenesis experiments showed no differences in incorporation of cells into the olfactory epithelium at postnatal weeks 1, 2 and 7, but only a transient increase at postnatal week 4 (Matarazzo et al., 2004). In addition, although phosphorylation at a specific residue of MeCP2 is key in regulating proliferation and neural differentiation of

neural progenitors in culture (H. Li et al., 2014), proliferation of *Mecp2*-null neural progenitors did not differ from WT in vitro or in vivo (Smrt et al., 2007). Further, *Mecp2* mutation did not affect the ability of neurospheres derived from *Mecp2*-null or *Mecp2*-heterozygous mouse embryos to proliferate (Kishi & Macklis, 2004). By contrast, a study with mesenchymal cells from two patients showed a decrease in cell proliferation, increased senescence markers and reduction of neuronal markers upon an assay of neuronal differentiation (Squillaro et al., 2012). Finally, a study using a mouse model of the *MECP2* duplication syndrome found an increased number of quiescent neural stem cells and neuroblasts ($BrdU^+/DCX^+$) in the dentate gyrus of these mice as compared to WT, but again no differences in proliferation (Z. Chen et al., 2017), suggesting that MeCP2 does not affect proliferation. Additionally, MeCP2 does not seem to affect proliferation or apoptosis (Tsujimura et al., 2009). In this framework, previous results from our lab also suggest that proliferation in the SVZ is not significantly affected in *Mecp2*-null males (Agustín-Pavón et al., 2016).

It has also been proposed that symptomatology of RTT could be caused by lack of maintenance of neuronal health rather than a defective brain development (Akbarian et al., 2001; R. Z. Chen et al., 2001; Guy et al., 2001). On this basis, some authors suggested that neuronal survival could be affected, leading to higher rates of apoptotic processes than normal. This idea was also supported by morphology of brains from RTT patients that seem to be smaller and more compacted that normal brains (D. Armstrong et al., 1995; D. D. Armstrong, 2001; Duncan Armstrong, 2005), suggesting that this reduction could be due to a loss of neurons. However, there is no significant evidence of neuronal loss in RTT brains (D. Armstrong et al., 1995), and changes in brain morphology seem to be due to the presence of smaller and more densely packed neurons than normal (D. Armstrong et al., 1995) with reduced dendritic arborization (A. M. Palmer et al., 2012).

In the present study, we investigated the survival of cells generated in the adult SVZ by means of BrdU injection and analysis in young animals. We did not find significant

differences in the number of BrdU-labelled cells between genotypes in the OB, suggesting that neurons generated in the postnatal SVZ are able to survive in spite of lack of MeCP2, at least for twelve days. Moreover, BrdU-ir cells in the St surrounding the SVZ were not different between *Mecp2*-null and WT young males. These data are consistent with findings from other laboratories showing that proliferation, survival, and differentiation of neural progenitors were not impaired in postnatal hippocampal neurogenesis (Smrt et al., 2007). However, a double immunostaining with BrdU and a neuronal marker (DCX or NeuN) would help us to differentiate populations of $BrdU^+$ cells and, therefore, analyse proliferation in neurons. Future experiments studying proliferation and survival of different kind of cells should be performed to test for potential undiscovered effects of lack of MeCP2 during embryonic development.

Mecp2 deficiency impairs neuronal maturation in the olfactory system in a region-specific way

A deficit in maturation caused by lack of MeCP2 is consistent with our data showing increased density of DCX-ir cells at the Pir, in which DCX-ir cells are generated during embryonic development (Rubio et al., 2016), and at the Tu of *Mecp2*-null mice. However, we did not find significant differences in the DG of the hippocampus, although these postnatally generated cells are also continuously maturing, or in the striatum. Data in the DG are consistent with previously published results showing no differences in total DCX-ir in the hippocampus of a different *Mecp2* KO mouse model (Smrt et al., 2007). In this latter study, the authors found that the number of "transitioning neurons" ($DCX^+/NeuN^+$) was increased in the hippocampus of *Mecp2*-mutant mice, suggesting that immature neurons display a delay in losing DCX expression. Both results are in agreement with a dysfunctional maturation process, albeit with different outcome.

There are several possibilities that might explain the region-specific effects of lack of MeCP2. First, the importance of MeCP2 as a regulator and/or the players with

which it interacts might be different in each brain area. In fact, a region-specific effect of lack of MeCP2 has been reported in other studies (Santos et al., 2010; E. S. Smith et al., 2019). In this framework, the factors regulating maturation differ between embryonic-born cells of the Pir, adult-born cells from the SVZ, and adult-born cells of the DG (Hagg, 2005). Consequently, the effect of lack of MeCP2 differs between regions, i.e., it affects density of DCX-expressing cells of embryonic origin and the maturation rate in the postnatally generated cells. We currently do not know whether the small but potentially important population of DCX-ir cells in layer I of the Tu, which was increased in Mecp2-null mice, arise during embryonic development, like Pir cells, or from adult generated progenitors in the SVZ. An embryonic origin is supported by data revealing that protracted neurogenesis in the Tu from progenitors arising from the SVZ and travelling through the ventral migratory stream is restricted to early postnatal days (de Marchis et al., 2004). Second, it is known that both stress and corticosterone levels impact the population of DCX-ir in the Pir (Nacher et al., 2004), and those factors are dysregulated in *Mecp2*-mutant animals (Braun et al., 2012; McGill et al., 2006). Third, region-dependent patterns of MeCP2 expression are found in the rodent brain. For example, MeCP2 is expressed in most (around 60%) neurons in the anterior olfactory nucleus of the rat at birth and remains stable throughout life (Cassel et al., 2004), whereas MeCP2-positive cells in striatum and the DG of the hippocampus appear in reduced numbers (Cassel et al., 2004; Mullaney et al., 2004). Also in the human brain, the dorsal striatum and the hippocampus show weaker staining for MeCP2 (M. D. Shahbazian et al., 2002), and these regions displayed no effect of genotype on the total density of DCX-expressing cells (but see the study by Smrt et al., 2007 where they found increased number of “transitioning neurons” in the DG). Finally, putative olfactory deficits due to abnormalities in the olfactory epithelium commented above could influence the rate of maturation in brain olfactory regions. Similarly, structural abnormalities in olfactory structures could cause olfactory

impairment, however, it has been demonstrated that *Mecp2*-null mice are not anosmic (Martínez-Rodríguez et al., 2020; Moretti et al., 2005).

By contrast to *Mecp2*-null males, *Mecp2*-het females did not show any difference in DCX-ir density as compared to their WT controls in our study. Since these females are heterozygous, and, therefore, they possess a functional *Mecp2* allele, their phenotype is milder than that of the *Mecp2*-null males (Guy et al., 2001). Still, these females are a valuable model to study the effects of MeCP2 deficiency (Samaco et al., 2013) and their use should not be neglected, especially because RTT affects mainly females. Indeed, unpublished results from our laboratory (Sevilla-Ferrer et al., 2021), reveal that the increase in DCX-ir cells in Pir is apparent in symptomatic stages of *Mecp2*-het females (by age of 6 months), thus, suggesting that the deficits in maturation in this region are linked to the onset of pathology.

Early-life stress differently affects weight and population of immature neurons in males and females

Our results suggest that maternal separation differently affects the process of neuronal maturation, in terms of increasing/decreasing the number of immature neurons in males and females. Thus, modifications in the population of immature neurons occur in a region, genotype, and sex-specific way. As an example, in the case of both WT and *Mecp2*-het females, maternal separation reduces the number of immature neurons within the granular layer of the olfactory bulbs, and population of immature tangled neurons, in layer II of Pir. However, population of complex neurons and total number of DCX^+ are not affected by MS-3h in the Pir of young females. Lack of differences in the number of complex neurons could be due to a promotion in both tangled and complex neurons, leading to a conversion of tangled cells into complex neurons that will in turn transition to mature neurons. This way, the population of tangled neurons would decrease, without affecting the number of complex cells. Although no significant differences were found in the total number of DCX-if cells in Pir, we could observe a trend ($p=0.068$) towards a decrease in this

population in both genotypes of females exposed to maternal separation. However, it should be emphasized that, due to the poor condition of the tissue, two *Mecp2*-het-naïve females were excluded for this analysis. Thus, the lower n of *Mecp2*-mutant females could be interfering in the results obtained. This reduction of immature neurons observed in our young females, is in agreement with a previous report in rats showing that stress or corticosterone treatment are able to affect immature neurons of the Pir (Nacher et al., 2004). More recently, it has been shown that manipulations with dopaminergic drugs alter the ratio of maturation of these cells (Coviello et al., 2020), and it is known that *Mecp2*-deficient mice display abnormal levels of monoamines (Santos et al., 2010), maybe affecting to the rate of maturation of these neurons. Importantly, although these type of DCX-expressing embryonic-born cells are restricted to the Pir in rodents, they display a widespread distribution in the primate (including human) neocortex and amygdala (la Rosa et al., 2020). Thus, potential maturation deficits in this population in RTT could contribute to cognitive impairment.

However, in the case of Tu, MS-3h increases, rather than decrease, the number of DCX^+ cells in the *Mecp2*-het-MS females, compared to *Mecp2*-het-naïve. In this framework, two different possible explanations arise: (i) although deficits of *Mecp2* does not seem to affect population of immature neurons in young females (6 weeks of age), MS-3h may exacerbate the impairments caused by mutations in gene *Mecp2*. In our young and young-adult males, we observed that lack of *Mecp2* increases the number of immature neurons within the layer I of Tu. Symptomatology in males is significantly worse compared to females of the same age, particularly since a majority of females remain asymptomatic until the $3^{rd}/4^{th}$ month of age. Moreover, unpublished results from our lab suggest that the increase of DCX^+ neurons in the olfactory cortex could be linked to symptom onset in *Mecp2*-mutant mice, in particular in females (Sevilla-Ferrer et al., 2021). On this basis, differences in severity of the symptoms may lead to a different effect over this immature

population. (ii) Maternal separation could be promoting proliferation in the Tu of *Mecp2*-het females. Population of DCX^+ cells in the Tu appear in two different states: (i) forming clusters of DCX-if cells or (ii) in a single state. Individual DCX^+ cells are bigger, with rounded nuclei and large projections. In addition, separated immature cells are usually attached to blood vessels and located close to layer II of Tu. However, DCX^+ cells in clusters are smaller and densely packed. In addition, unpublished results from our lab demonstrate that those clusters are surrounded by $Ki67^+$ cells (a widely used marker for cell proliferation), with some cells even co-expressing both markers (Esteve-Pérez et al., 2022). Additionally, 4 weeks old pre-adolescent wild type female mice have a significantly higher number of Ki67-ir cells in the Tu than post-pubertal 8 weeks females, which, on the contrary, have almost double DCX^+ cells than pre-pubertal females. Thus, maternal separation may promote proliferation of these cells, leading to an increase of DCX-if neurons in Tu. However, this population of immature neurons in Tu remains practically undescribed, so one should be careful with this interpretation. In addition, there is a lot of controversy in the literature about whether maternal separation increases or decreases neurogenesis (Hulshof et al., 2011; Leslie et al., 2011; Lievajová et al., 2011; Račeková et al., 2009; Reshetnikov et al., 2020; Sachs et al., 2013; Sung et al., 2010; Q. Zhang et al., 2021). Likewise, this would not explain why we did not find differences within the group of WT females, or how deficits on *Mecp2* may interfere in the interplay between MS and post-natal neurogenesis. Further experiments would be necessary to unravel this possible explanation.

As previously mentioned, neuronal maturation experiment revealed an increase of immature neurons in the Pir, Tu and DG of *Mecp2*-null-MS males, without effect in the St. Those results are consistent with the higher levels of DCX-if cells found in the Tu and Pir (in particular tangled neurons) of *Mecp2*-null-naïve males compared to the WT-naïve. These results support the hypothesis of lack of *Mecp2* decelerating neuronal maturation in these nuclei. By contrast to the continuous adult

neurogenesis that occurs in the DG (Bonfanti & Peretto, 2011), DCX-expressing neurons of the Pir are born during embryonic development (M. Á. Gómez-Climent et al., 2008; Rubio et al., 2016), and decrease gradually as the animal ages, by maturing and integrating in the Pir as excitatory neurons (Rotheneichner et al., 2018). Thus, lack of MeCP2 seems to affect specifically this process of postnatal neuronal maturation, rather than proliferative neurogenesis. Furthermore, maternal separation undercovers an increase in the density of immature neurons at other regions such as the dSt and vSt in adolescent mice. Thus, MS seems to exacerbate the number of DCX-ir cells at the Pir, thus suggesting that ELS can interfere with neuronal maturation in a region-specific manner. Interestingly, the increase on DCX-ir cells at the Pir is more explicit in the *Mecp2*-null mice. Therefore, lack of MeCP2 seems to increase the susceptibility to an early life stressor in male mice in terms of neuronal maturation at the Pir, which could be associated with the intellectual disabilities reported in humans lacking functional *MECP2* (Kudo et al., 2002; Orrico et al., 2000).

Furthermore, recent data from our lab revealed expression changes of reelin (Torres-Pérez et al., 2022). Reelin is a protein implicated in both maturation and migration of neurons (Carceller et al., 2016; Tissir & Goffinet, 2003), and its expression is known to be under *Mecp2's* epigenetic control (Sánchez-Lafuente et al., 2022). Accordingly, lack of *Mecp2* leads to reduced levels of this protein at the outermost layer of the Pir, the Tu, and both the polymorph and the granular layers of the DG. Additionally, the exposure to MS resulted in its reduced expression at these areas, regardless of the genotype of the mice. Therefore, MS seems to reduce neuronal maturation/migration, in agreement to that previously reported at the hippocampus of various animal models of chronic stress (Brymer et al., 2018).

Similarly, we observed that MS differently affects weight of males and females. We observed that MS-3h increases weight of WT-MS males. By contrast, MS does not seem to affect weight of *Mecp2*-null males or WT/*Mecp2*-het females. Thus, weight

is only increased in the group of WT males exposed to MS. Differences between sexes are consistent with previous results of our lab suggesting that MS also has a different effect on anxiety-like behaviours depending on the sex of mice: it further decreases anxiety-like responses of females in the EPM (Abellán-Álvaro et al., 2021), but does not affect these responses in males (Torres-Pérez et al., 2022). On this basis, variances in *Mecp2*-dosage depending on genotype and sex, as well as differences in onset and, therefore, severity of symptomatology, may lead to different degrees of impairments caused by deficits of MeCP2.

Limitations, conclusions and future directions

The present study is a promising starting point to investigate the consequences of MeCP2 deficiency in the development and function of the olfactory system and the so-called adult non-proliferative neurogenesis (König et al., 2016). We know that children with autism spectrum disorders, some of which are linked to mutations in *MECP2* (Gonzales & LaSalle, 2010), present an aberrant behavioural response to emotional odours, with longer sniff duration for unpleasant odours vs pleasant as compared to typically developing children. This aberrant response is correlated with the severity of autistic symptoms (Rozenkrantz et al., 2015). Thus, it would be interesting to test whether increased immature neurons in Pir or Tu might be contributing to some of the aberrant functional responses. Of note, the distribution of DCX-ir cells in the primate (including human) brain is quite more extensive than that shown in rodents, and includes the associative cortices and the amygdala (Bloch et al., 2011; Cai et al., 2009; Y. W. J. Liu et al., 2008; X. M. Zhang et al., 2009). Given the importance of these structures in cognitive capacities and affective behaviours, a deficient neuronal maturation in these brain areas resulting from the *MECP2* mutations is likely to be involved in the deficits that characterise RTT and autism spectrum disorders.

Importantly, we show that population of immature neurons is susceptible to environmental manipulations, highlighting the importance of early non-pharmacological interventions, such as environmental enrichment, to promote neuronal maturation. Future research should test the likely impairment in maturation of DCX-expressing cells in older, symptomatic *Mecp2*-het females, and whether early environmental or pharmacological interventions could ameliorate this deficit. It would also be interesting to test the effect of MS in older females, since, at least in the hippocampus of rats, effects of ELS have been found to be biphasic, with an increase in neurogenesis in young adults followed by a decrease in middle age animals (Suri et al., 2013). These future studies could provide new avenues for treatments boosting structural plasticity.

CHAPTER 2. EFFECT OF *MECP2* DEFICIENCY IN THE SOCIAL BRAIN NETWORK: THE NONAPEPTIDERGIC SYSTEM

INTRODUCTION

Classical Rett syndrome has usually been considered as an autism spectrum disorder due to the social isolation displayed by patients. However, recent investigations revealed substantial differences between this syndrome and autism, excluding it from the ASD group. Nonetheless, understanding possible dysfunctions within the social brain network would help to ameliorate social alterations in RTT girls.

Socio-sexual brain of Rett patients

Social behaviours are controlled by a brain network called the socio-sexual brain (Newman, 1999). This network involves a complex circuit in which several nuclei interact to modulate social cognition in humans, which is dependent on similar neural circuitry as in the rodent, but with added complexity in cortical regulation (Donaldson & Young, 2008; J. L. Goodson, 2013).

In rodents, this social brain starts with the olfactory system, as a dominant modality for the perception of social information, receiving information from olfactory pheromones (Crawley, 2012). Volatile and non-volatile social odours are perceived by the main and accessory olfactory bulbs, respectively. Both regions are of critical relevance in rodents for the production of normative social behaviours (Petrulis, 2009). Information from both olfactory systems goes through mitral and tufted neurons to the amygdala, in a process gated and regulated by levels of oxytocin (OT) and arginine-vasopressin (AVP). These two neuropeptides or neurotransmitters play a key role mediating social behaviours in both humans and rodents. In the case of OT, its release promotes social olfactory exploration in mice, facilitating social recognition (Oettl et al., 2016). Connections between the MOB/AOB and the Amy

are critical for the production of normative social behaviours in rodents (Petrulis, 2009).

The Amy serves as a "gateway" in the processing of sensory information (Zalla & Sperduti, 2013), including the information received from the MOB and AOB that travels to the medial amygdala (MeA). Activation of MeA is regulated by oxytocinergic projections from the supraoptic nucleus of the hypothalamus, and produces social behaviours. These behavioural effects of MeA are mediated through downstream projections of the brainstem and the hypothalamus (Keshavarzi et al., 2014). In the modulation of social behaviours evoked by MeA, different nuclei are involved, such as the basolateral amygdala (BLA), which also projects to the nucleus accumbens (Acb) and the ventral pallidum (VP) and receives information from the ventral tegmental area (VTA; LaLumiere, 2014).

Additionally, relevant projections between the primary sensory and perceptual cortical areas to the Amy enable to process social information and to assess an effective valence to the stimuli (Zalla & Sperduti, 2013). The Amy, strongly influenced by the hypothalamus, interplays with the hippocampus and connects with the mesolimbic dopamine system (MLDS) to create a reward circuitry to promote social stimuli. There are also projections from MLDS to the Amy, hypothalamus, hippocampus and habenula, to associate each specific social sensory stimuli with the appropriate reinforcement cues within the striatum.

Thus, decision between approaching and avoiding behaviours depends on different brain nuclei activation and neuromodulation. General projections from the MeA to the ventromedial nucleus of the hypothalamus (VMH) innervate the bed nucleus of the stria terminalis (BST), promoting social behaviours. However, activation of a discrete population of VMH induces aggressive behaviours towards same sex conspecifics (D. Lin et al., 2011). In the case of the lateral habenula (LHb), it is bidirectionally modulated in response to either rewarding or aversive social stimuli.

Likewise, olfactory and associative centres of the Amy project directly to the ventromedial striato-pallidum (vmStP; Pardo-Bellver et al., 2012), which is considered as the core centre of the circuitry of motivated behaviours. Indeed, a specific region within the vmStP is crucial for deciding whether attacking or expressing affiliative, parental, or sexual behaviours toward conspecifics (Otero-Garcia et al., 2014). This nucleus has also been proposed as an important part of the neural network of the socio-sexual brain, constituting a node for motivation in the context of chemosensory-guided social interactions (Otero-Garcia et al., 2014).

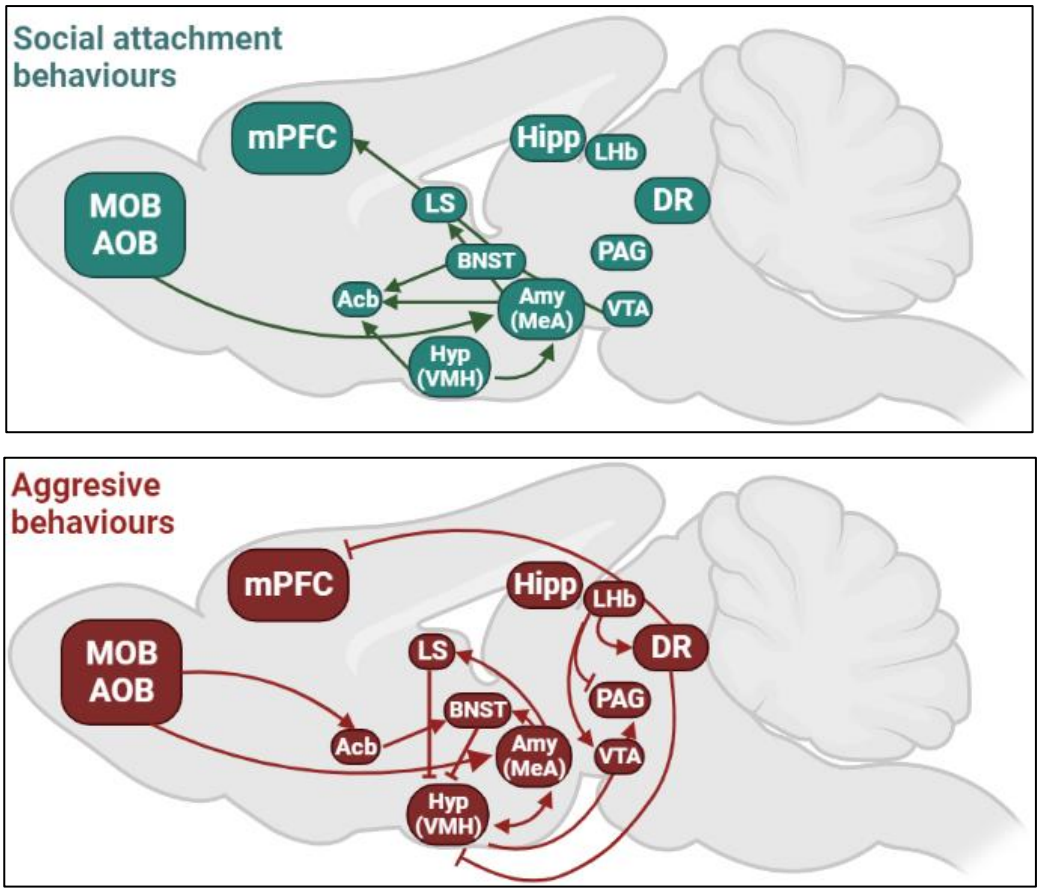


Figure 14: schematic representation of social network in the rodent brain involved in social attachment behaviours (in green) and aversive behaviours (red). Abbreviations: *Acb,* accumbens nucleus; *Amy,* amygdala; *AOB*, accessory olfactory bulb; *BNST*, bed nucleus of the stria terminalis; *DR*, dorsal raphe; *Hipp*, hippocampus; *Hyp*, hypothalamus; *LHb*, lateral habenula; *LS*, lateral septum; *MeA*, medial amygdala; *MOB*, main olfactory bulb; *mPFC*,

medial prefrontal cortex; *PAG*, periaqueductal gray; *VMH,* ventromedial hypothalamus; *VTA*, ventral tegmental area. Adapted from (Ko, 2017) and created with BioRender.com.

Arginine vasopressin and oxytocin: two nonapeptides closely implicated in social behaviours

Arginine vasopressin and oxytocin are two related nonapeptides mainly synthesized in the hypothalamic paraventricular (Pa) and supraoptic nuclei (SON). In addition, AVP is present in the suprachiasmatic nucleus (SCh), and AVP-containing neurons in the BST and medial amygdala (Me), which display a marked sexual dimorphism in favour of males (Otero-Garcia et al., 2014, 2016; Rood et al., 2013). Central projections of the nonapeptide-synthesising groups to brain nodes of the social brain network possess relevant roles in the control of social, sexual and parental behaviours in mammals (J. Goodson, 2008). Interestingly, some of the target regions of AVP neurons, such as the lateral septum (LS), vmStP, posterodorsal medial amygdala (MePD) or LHb, display significantly higher density of AVP-immunoreactive fibres in males than in females (Otero-Garcia et al., 2014).

This sexually-dimorphic AVP innervation is dependent on testosterone (Otero-Garcia et al., 2014; Rood et al., 2013), which aromatized to estradiol acts on estradiol receptor α (ERα; Scordalakes & Rissman, 2004). In turn, transcriptional regulators, such as MeCP2, are involved in the regulation of both AVP and Erα (Forbes-Lorman et al., 2012; Murgatroyd et al., 2009; Westberry et al., 2010) suggesting a complex interplay between gonadal hormones and epigenetics. This interplay would contribute to the shaping and functioning of the key social brain nuclei (Newman, 1999) for the development and expression of social behaviour (Auger et al., 2011; Romano et al., 2016).

There are many other neuromodulators involved in the regulation and promotion of specific social behaviours. As an example, dopamine signalling is increased in the St in response to novel social investigation (D. L. Robinson et al., 2002), and decreases as the animal habituates to the conspecific (D. L. Robinson et al., 2002), mediating

social recognition in rodents. In addition, dopamine release in the Acb promotes investigation of a novel conspecific in mice (Gunaydin et al., 2014). Likewise, different hypothalamic nuclei, such as the paraventricular nucleus (PVN) and SON, evoke responses to social stimuli through the regulation of AVP and/or OT release. OT interacts with serotonin within the Acb, mediating social reward (Dölen et al., 2013). Moreover, OT release in the MeA has been implicated in regulating social bonding (Gur et al., 2014), whilst activation of OT-ergic neurons in the hypothalamus induces social preference (through the receptors in Pir; Choe et al., 2015), social exploration and recognition in rats (through the olfactory cortex; Oettl et al., 2016), and social bonding in voles. Likewise, AVP is relevant for the mediation of social recognition (Gabor et al., 2012) and social motivation (Wersinger et al., 2004).

Given the central role of nonapeptides in the control of social behaviour, it is not surprising that alterations in vasopressinergic (AVP-ergic) and oxytocinergic (OT-ergic) systems have been reported in neurodevelopmental disorders causing alterations in social behaviour. Many of the mutations identified in ASD, alter the connectivity within the limbic components of the social circuit and higher cortical processing areas (such as the prefrontal cortex and cerebellum). This leads to a disruption in the production of social behaviour in mutant mouse models. Thus, nonapeptidergic systems are affected in autistic spectrum and psychiatric disorders in both humans and rodents (Domes et al., 2007; S. M. Francis et al., 2014; Freeman et al., 2018; Lukas & Neumann, 2013; Menon et al., 2018; Miller et al., 2013; Modahl et al., 1998; Waterhouse et al., 1996; Winslow & Insel, 2004). In the case of RTT, the neurotransmitter system is altered in patients and mouse models, with a dysregulation in the levels of acetylcholine, dopamine, serotonin, glutamate, substance P and nerve growth factors (Santos et al., 2010), as a consequence of deficits of MeCP2. However, AVP-ergic and OT-ergic systems have not been analysed in mouse models of *MECP2*-related syndromes. In this framework, investigating

possible alterations within the socio-sexual brain of patients of RTT may help to improve altered social conducts in those girls.

On this basis, our first aim was to analyse the consequences of MeCP2 deficit on AVP and OT expression in the brain of young adult male and female mice. Knowing that the main AVP-ergic innervation within the socio-sexual brain is the testosterone-dependent, together with the anecdotal observation reported in Guy et al about *Mecp2*-null males having internal testicles and being infertile, we hypothesised that *Mecp2*-null males may have lower levels of circulating testosterone. Thus, we next analysed other features known to be affected by the levels of testosterone. First, we analysed the density of nitrergic cells, by using NADPH-diaphorase histochemistry, a marker of activity of nitric oxide synthase (NOS). This marker is known to be increased by castration (Singh et al., 2000). Second, we hypothesised that aggressive behaviour of *Mecp2*-null males would be decreased, and tested this hypothesis by analysing the agonistic behavioural profile of *Mecp2*-null males and their WT littermates in the resident-intruder test. Data from this chapter has been published elsewhere in Martínez-Rodríguez et al., 2020.

MATERIALS AND METHODS

Animals

For this chapter, we employed samples from the same 23 young female and male mice of the strain *Mecp2*$^{tm1.1Bird/J}$ and their WT siblings used in Experiment 2 of Chapter 1. Further, we used 45 male mice for Experiment 2. Sex, age at sacrifice and number of each experimental group of animals are shown table 10. Data shown here is a subset of a larger study published elsewhere (Martínez-Rodríguez et al., 2020).

Mice were housed in groups of 2–5 animals in standard laboratory cages with controlled humidity and temperature (22 °C), a 12:12-h light/dark cycle, and water and food available ad libitum. All the procedures were carried out in strict accordance with the EU directive 2010/63/EU. The protocols were approved by the local veterinary and the Ethics in Animal Experimentation Committee of the University of Valencia.

Table 10: Number of animals used for each experiment classified by sex and genotype, together with their age of sacrifice.

Experiment	Subjects		Total	Age of sacrifice	Markers
	Female	Male			
1. Effect of MeCP2 deficiency in nonapeptidergic systems	WT: 6 *Mecp2*-het: 5	WT: 7 *Mecp2*-null: 5	23	8-weeks	AVP OT NADPH-diaphorase
2. Effect of lack of MeCP2 in aggressive behaviour		WT: 32 *Mecp2*-null: 13	45	8-weeks	

Genotyping and histology

We followed the same genotyping, perfusion and sectioning protocols as described in chapter 1.

Immunohistochemical techniques

Double immunofluorescence for arginine-vasopressin and oxytocin with DAPI labelling

We employed combined immunofluorescence for simultaneous immunolabelling of vasopressin and oxytocin, following the protocol described in (Martínez-Rodríguez et al., 2020) and for DCX/NeuN in Chapter 1. Antibodies used in this case are listed in Table 11.

To reveal the cytoarchitecture of the brain in the same sections, prior to mounting, sections were counterstained in DAPI for 45s at RT. Sections were finally rinsed thoroughly in TB, mounted onto gelatinized slides and cover-slipped with fluorescence mounting medium (Dako, Glosrup, Denmark; or FluorSave TM Reagent).

Table 11: Primary and secondary antibodies selected for each immunodetection.

Experiment		Primary Antibody		Secondary Antibody	
AVP	DAB	Rabbit anti-AVP IgG	1:10.000 Chemicon AB1565	Biotinylated Goat anti-Rabbit IgG	1:200 Vector Labs BA-1000
	Fluor.	Rabbit anti-AVP IgG	1:2500 Millipore AB1565	Alexa Fluor 488 Goat anti-Rabbit IgG	1:250 Jackson ImmunoResearch 111-545-003
OT	Fluor.	Mouse anti-OT monoclonal IgG	1:200 Dr. Harold Gainer, NIH, PS38	Rhodamine Red X Goat anti-Mouse IgG	1:250 Invitrogen R6393

Permanent arginine-vasopressin immunohistochemistry with NADPH-diaphorase staining

We obtained permanent immunostained preparations for AVP combined with NADPH-diaphorase in one out of five parallel series, using the indirect avidin–biotin complex/DAB-staining procedure. We followed the same protocol as described for permanent immunohistochemistry in chapter 1, but using the antibodies specified in Table 11. After AVP-ir staining, we performed the NADPH-diaphorase

histochemistry. Sections were incubated at 37ºC for 1-2 h in dark conditions in 10 ml of 0.1M phosphate buffer containing 2.5 mg/ml of nitro-blue tetrazolium (Sigma), 2.5 mg/ml of β-NADPH (Sigma) and 300 µl of 0.3% Triton-X-100 as described in (Otero-Garcia et al., 2014). Staining was checked periodically, and the reaction was stopped by rinsing the sections in TBS. Brain slices were finally mounted using 0.2% gelatine in TB, dehydrated with alcohol, cleared with xylol and cover-slipped with Entellan (Merck Millipore, Burlington, MA, USA).

Immunohistochemical analysis and quantification

Analysis and quantification of AVP and OT cells: single and double staining

Somata labelled with vasopressin-immunofluorescence (AVP-if) and oxytocin-immunofluorescence (OT-if) were analysed in different brain areas at previously selected Bregma levels (see Table 12). Those nuclei include the posterointermediate part of the medial division of the bed nucleus of the stria terminalis (BSTMPI) and the anterior and posterodorsal parts of the medial amygdaloid nucleus (MeA, MePD). We also analysed AVP-if and OT-if cells in the hypothalamic region between the anterodorsal preoptic nucleus and the nucleus of the anterior commissure (AC/ADP), as well as other hypothalamic nuclei, such as the paraventricular hypothalamic nucleus (Pa), suprachiasmatic nucleus (SCh), supraoptic nucleus (SON), and the retrochiasmatic region of the supraoptic nucleus (SOR). Because some nuclei are heterogeneous in the density of AVP-if and OT-if along the rostro-caudal axis, extra levels of Bregma were selected for those, and we calculated the average density of AVP-if and OT-if cells for each nucleus.

For histology analysis, an observer blind to the experimental conditions took pictures from both hemispheres at specific objective magnification and manually counted the number of AVP-if and OT-if cells with the multipoint plugin of the ImageJ software. We also analysed co-localization of AVP and OT in the AC/ADP and Pa, using ImageJ software. Pictures were taken with same equipment and conditions

as described in chapter 1. All the subsequent steps were performed using ImageJ free software (NIH).

Table 11: Nuclei and Bregma levels (following Paxinos and Franklin, 2013) selected for quantification of different proteins of interest in *Mecp2*-mutant and WT mice. Abbreviations: *AC/ADP*, nucleus of the anterior commissure/anterodorsal preoptic nucleus; *AcbC,* nucleus accumbens, core; *AcbSh,* nucleus accumbens, shell; *BST,* bed nucleus of the stria terminalis; *BSTMPI,* bed nucleus of the stria terminalis, medial division, posterointermediate part; *Ce,* central amygdaloid nucleus; *dEn,* dorsal endopiriform cortex; *dlPAG*, dorsolateral periaqueductal grey; *DMH,* dorsomedial hypothalamic nucleus; *DR,* dorsal raphe nucleus; *DTg,* dorsal tegmental nucleus; *LHb,* lateral habenular nucleus; *LS,* lateral septum; *MeA,* medial amygdaloid nucleus, anterior part; *MCx*, motor cortex; *MePD,* medial amygdaloid nucleus, posterodorsal part; *Pa,* paraventricular hypothalamic nucleus; *SCh,* suprachiasmatic nucleus; *SON,* supraoptic nucleus; *SOR,* retrochiasmatic part of the supraoptic nucleus; *Tu*, olfactory tubercle; *vHip,* ventral hippocampus; *vlPAG,* ventrolateral periaqueductal grey; *vmStP,* ventromedial striatopallidum.

AVP/OT-if fibres and somata

Nucleus	AVP	OT	Approximate mm to Bregma
dEn	Fibres	-	-2.92
vHip	Fibres	-	-2.92
AcbC	-	Fibres	+1.10
AcbSh	-	Fibres	+1.18, +1.10, +0.98
vmStP	Fibres	Fibres	+1.10
LS	Fibres	Fibres	+0.38, +0.14
BSTMPI	Fibres, somata	Fibres, somata	-0.22
MeA	Fibres, somata	Fibres, somata	-1.22
MePD	Fibres, somata	Fibres, somata	-1.34
Ce	-	Fibres	-1.34
LHb	Fibres	Fibres	-1.46
AC/ADP	Somata	Somata	-0.22
Pa	Somata	Somata	-0.58, -0.70, -0.94
SCh	Somata	-	-0.82
SON	Somata	Somata	-0.82
SOR	Somata	Somata	-1.34
DMH	Fibres	Fibres	-1.46
dlPAG	Fibres	-	-4.24, -4.48, -4.72
vlPAG	Fibres	Fibres	-4.24, -4.48, -4.72
DR	Fibres	-	-4.72

NADPH-ir somata

Nucleus	Approximate mm to Bregma
AcbC	+1.50, +0.86, +0.26
AcbSh	+1.50, +0.86, +0.26
BST	-0.22
MePD	-1.35
MCx	+1.70, +1.10, +0.74
dlPAG	-4.24, -4.48, -4.72
dSt	+1.50, +0.86, +0.26
DTg	-5.02
Tu	+1.50, +0.86, +0.26

Analysis and quantification of AVP and OT immunoreactive terminal fields

We took pictures of AVP-ergic and oxytocinergic OT-ergic innervation in different brain regions of the socio-sexual brain such as: the dorsal endopiriform cortex (dEn), ventral hippocampus (vHip), the core and shell of the nucleus accumbens (AcbC, AcbSh), vmStP, LS, BSTMPI, MeA, MePD, central amygdaloid nucleus (Ce), LHb, dorsomedial hypothalamic nucleus (DMH), and the dorsolateral and ventrolateral periaqueductal grey (dlPAG, vlPAG).

Analysis was performed following the protocol described in (Menon et al., 2018): we drew a grid with the plugin of the ImageJ and counted the number of crossings of the fibres with the grid bars with the multi-point tool of the Image J. In the case of the AVP-ergic fibres in the LHb, due to intricate labelling found, we counted the total number of squares containing labelled puncta.

Analysis and quantification of NADPH-diaphorase stained somata

An experimenter blind to genotype and sex of mice manually counted the number of NADPH-diaphorase stained cells with the multipoint plugin of the Image J. To do so, we selected different Bregma levels of several cortical and subcortical areas such as the motor cortex (MCx), AcbC, AcbSh, dSt, Tu, BST, and the MePD. We also quantified somata in two mesencephalic regions such as the dorsal tegmental nucleus (DTg) and dlPAG (see table 12).

Behavioural procedures

Resident intruder test (RI)

Resident-intruder test was performed between 10 a.m. and 5 p.m. Animals were isolated in their home cages for at least 1 week with no bedding changes. On the day of testing, an intruder male mouse (WT or *Mecp2*-null male of 2–4 months old) was introduced in the home cage of the test animal and the behaviour was registered for 5 min. After 1 week, the test was repeated, but now with the test animal as intruder. An observer blind to the experimental conditions manually scored several behavioural parameters using the plugging event recorder of SMART 2.5 (Panlab, Barcelona, Spain). The parameters were number and total time in seconds of attack from resident to intruder, number of times that the resident chased the intruder, time that the resident spent investigating the intruder (sniffing the face, body or anogenital zone), number of times that the resident escaped from the intruder, and time spent by the resident self-grooming.

Statistical analysis

For the immunohistochemical techniques, data were analysed using the software IBM SPSS Statistics 22.0. We first checked the data for normality (Shapiro–Wilk test) and homoscedasticity (Levene's test). Next, we evaluated the differences between gender/genotypes/condition using Student's t test or Mann–Whitney U test when appropriate. For the resident-intruder test, we also used Chi-square test to compare the number of resident mice displaying aggressive behaviours (attack and chase). Significance was set at $p < 0.05$.

RESULTS

Immunofluorescent samples obtained from our *Mecp2*-mutant and WT mice males and females allowed us to analyse the distribution of nonapeptidergic somata and fibres. Number of AVP and OT immunofluorescent fibres and somata (AVP-if, OT-if), as well as their co-localization, were also analysed in those samples. We found statistically significant differences between genotypes in the case of males, but not in the case of females. Thus, permanent immunostaining for AVP (AVP-ir) with NADPH-diaphorase was performed only in males to corroborate results obtained in the analysis from AVP-if, and analyse some regions with scarce AVP fibres that were not visible with fluorescent techniques (vHip, dEn, dlPAG, MePD, vlPAG, and DR). Further, we analysed the density of nitrergic cells and agonistic behaviours in the RI-test, regulated by testosterone, in males only.

Experiment 2.1 Effect of MeCP2 deficiency in nonapeptidergic systems

Distribution of nonapeptidergic cells and fibres is not affected by MeCP2 deficiency

Qualitatively, the distribution of AVP-if and OT-if somata and fibres in both *Mecp2*-mutant and WT mice males and females matched with previous reports analysing nonapeptidergic systems in WT mice of two different strains (Otero-Garcia et al., 2014; Rood et al., 2013). We found abundant AVP-if cells in the hypothalamic nuclei Pa, SCh, SON, and the retrochiasmatic part of the supraoptic nucleus (SOR; Fig. 15), and few scattered AVP-if cells in other areas of the brain, such as the nucleus of the anterior commissure/anterodorsal preoptic nucleus region (AC/ADP), BSTMPI, MeA, and MePD (Fig. 16). Similarly, the population of OT-if cells was abundant in AC/ADP, Pa, SON, and SOR, and sparse in BSTMPI, MeA, and MePD (see examples in Figs. 15, 16). Additionally, we observed some co-localization of both neuropeptides in AC/ADP and Pa, as previously described by (Otero-Garcia et al., 2016). In general, we did not find qualitative differences in the distribution of nonapeptidergic somata between males and females.

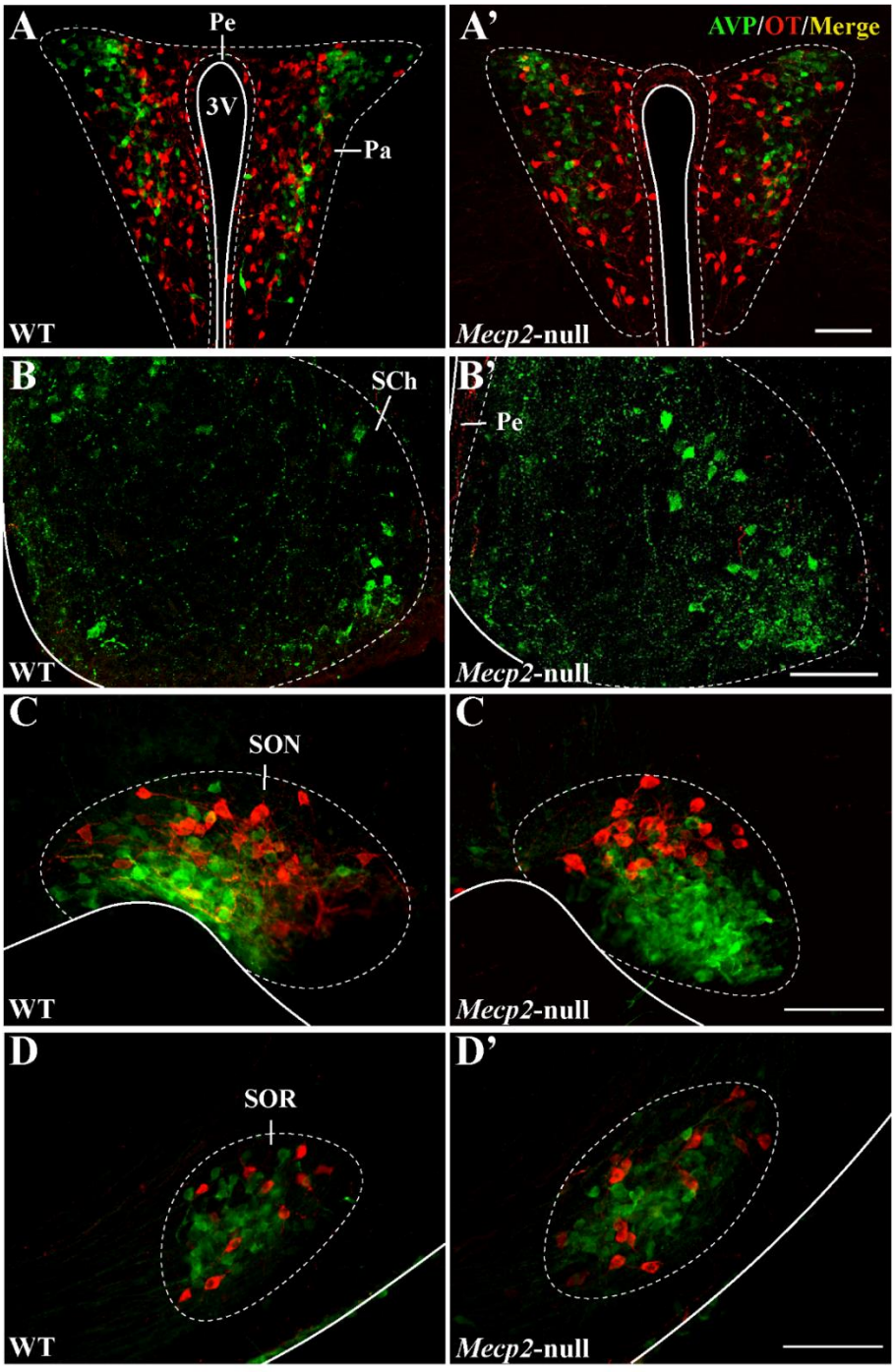


Figure 15: Representative pictures of AVP- (green) and OT-ergic (red) somata in four hypothalamic regions of WT and *Mecp2*-null males. AVP and OT-if somata in hypothalamic **a**, **a')** paraventricular, **b)** suprachiasmatic, **c)** supraoptic and **d**, **d')** the retroquiasmatic part of the supraoptic nucleus. The distribution and density of AVP and OT-if somata in *Mecp2*-null or *Mecp2*-het mice do not differ from WT mice. Scale bar 100 µm. Abbreviations: *3V,* 3rd ventricle; *Pa,* paraventricular hypothalamic nucleus; *Pe,* periventricular hypothalamic

nucleus; *SCh,* suprachiasmatic nucleus; *SON,* supraoptic nucleus; *SOR,* retroquiasmatic part of the supraoptic nucleus.

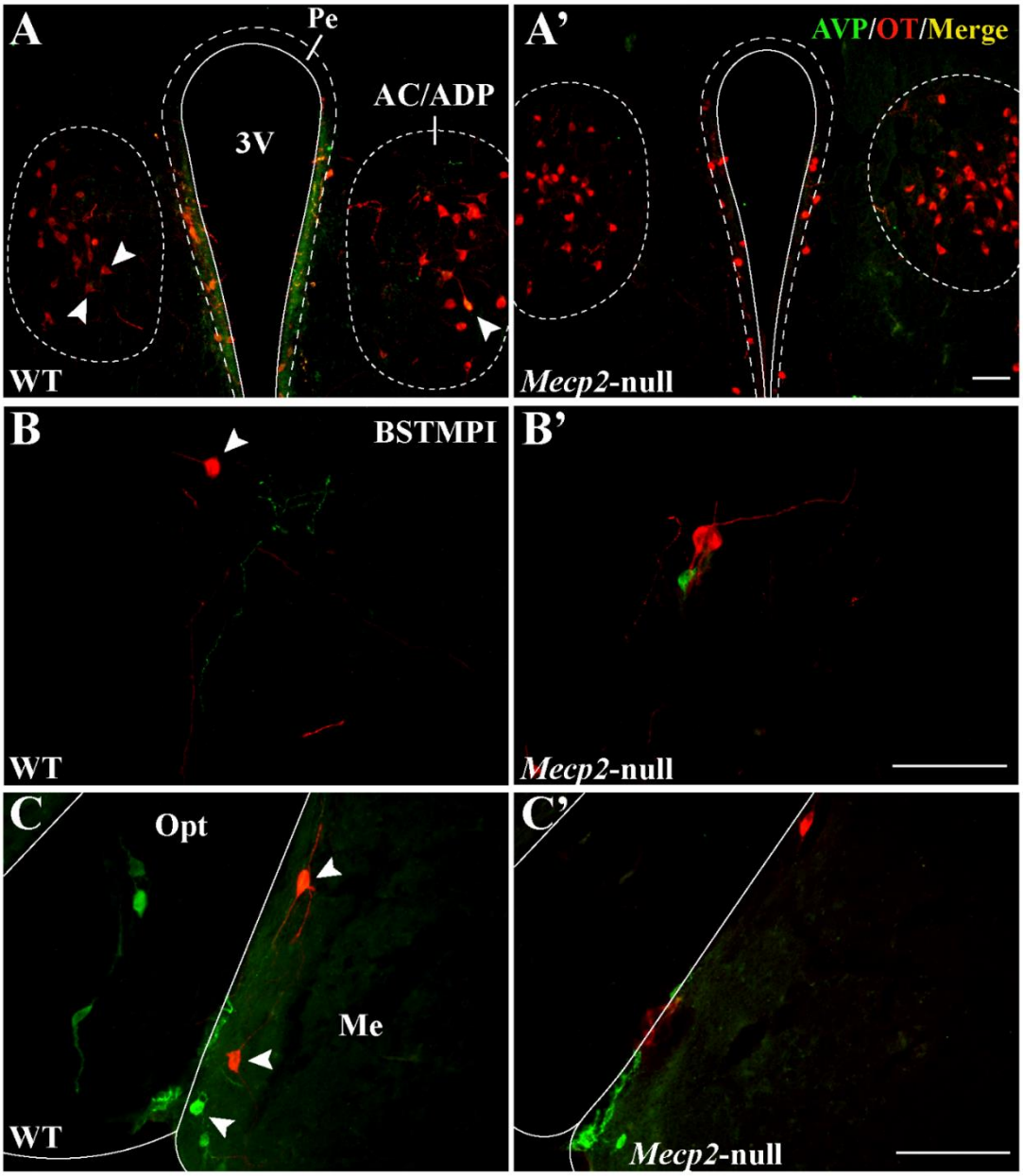


Figure 16: Representative pictures of AVP- (green) and OT-ergic (red) immunofluorescence in three brain regions of WT and *Mecp2*-null males. AVP and OT-if in **a**, **a')** nucleus of the anterior commissure/anterodorsal preoptic nucleus region, **b**, **b')** bed nucleus of the stria terminalis, medial division, and **c**, **c')** medial amygdala. The distribution and density of AVP and OT-if somata in *Mecp2*-null or *Mecp2*-het mice do not differ from WT mice. Scale bar 100 µm. Abbreviations: *3V*, 3rd ventricle; *AC/ADP,* nucleus of the anterior commissure/anterodorsal preoptic nucleus region; *BSTMPI,* bed nucleus of the stria

terminalis, medial division; *Me,* medial amygdala; *Opt,* optic tract; *Pe*, periventricular hypothalamic nucleus.

Regarding AVP innervation of the telencephalon, we found moderate to dense terminal fields in the LS, vmStP, BST (Fig. 17) and Me, and scarce AVP-ir fibres in the ventral hippocampus in WT males (Fig. 18), whereas this innervation was reduced in *Mecp2*-null males and both groups of females (see below).

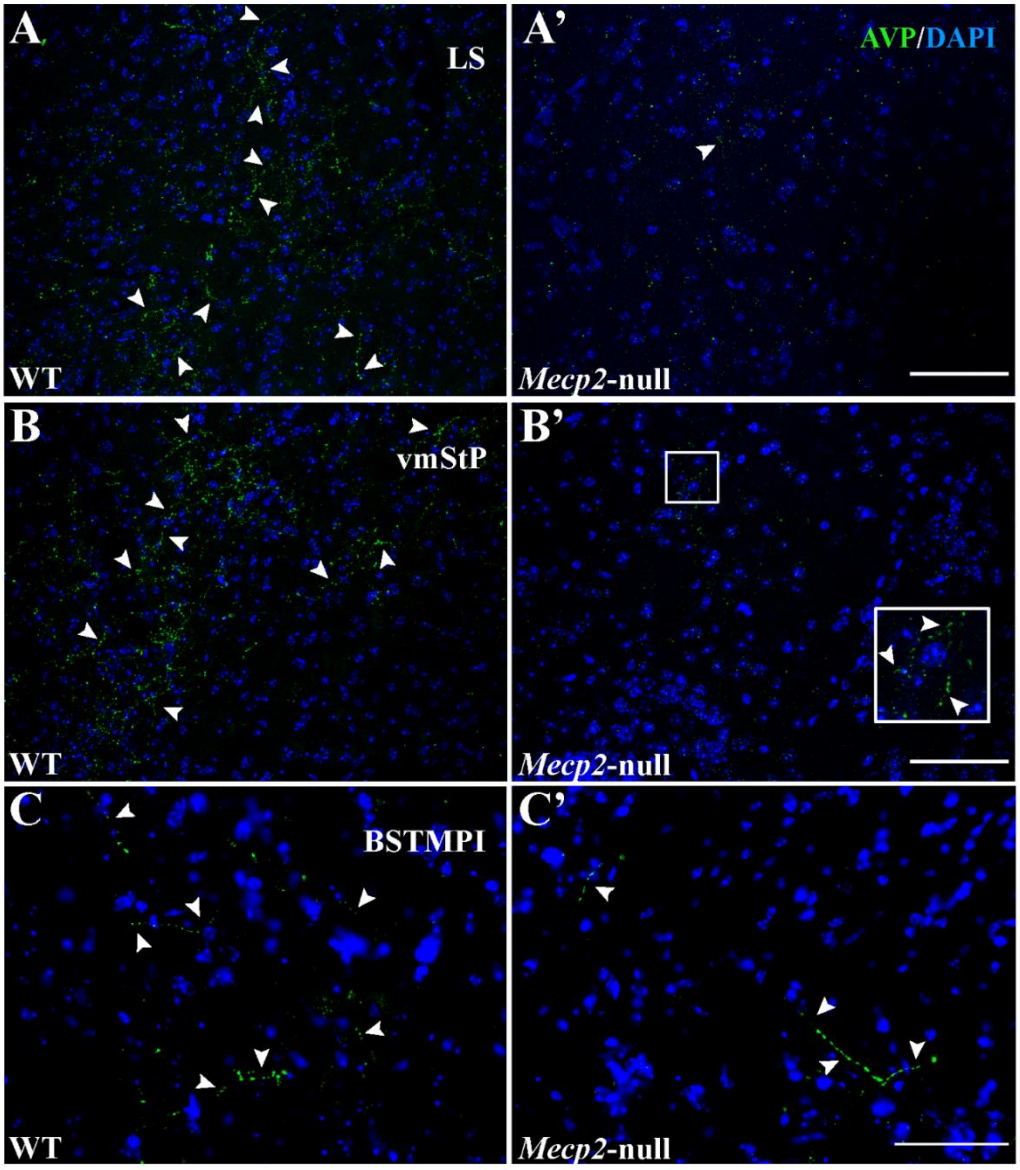


Figure 17: Representative pictures of AVP-immunofluorescence (green) and DAPI labelling (blue) in three brain regions of WT and *Mecp2*-null males. Arrows point to AVP-if fibres. Testosterone-dependent AVP-ergic innervation in the, **a')** lateral septum, **b**, **b')**

ventromedial striato-pallidum, and **c**, **c')** bed nucleus of the stria terminalis, was significantly reduced in *Mecp2*-null males as compared to WT males. Scale bar 100 µm. Abbreviations: *BSTMPI,* bed nucleus of the stria terminalis, medial division; *LS,* lateral septum; *vmStP,* ventromedial striato-pallidum.

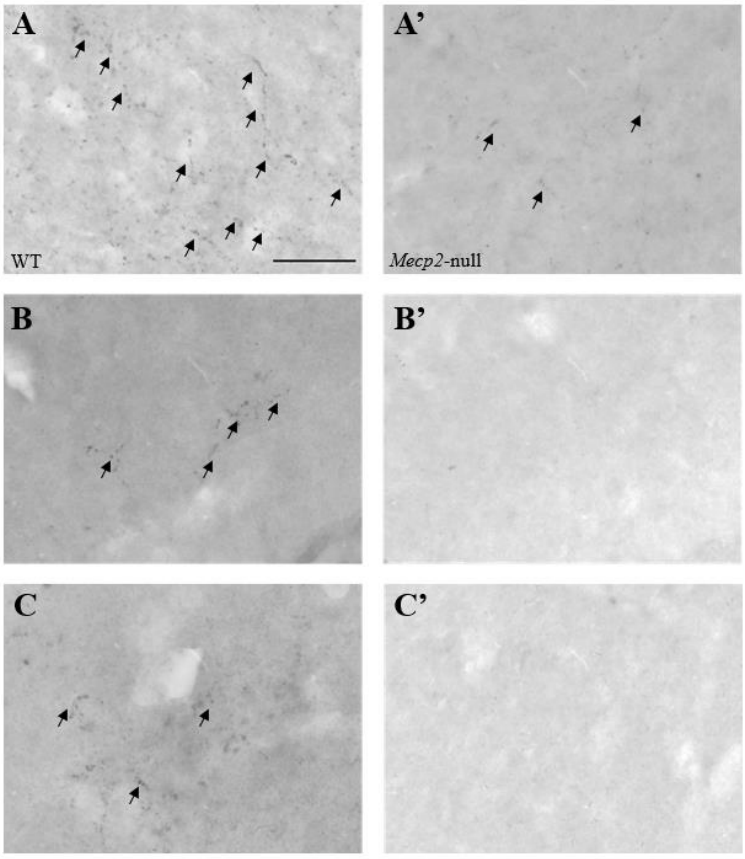


Figure 18: Representative images of AVP-immunostaining with DAB in the **a**, **a`)** MePD, **b**, **b')** vHip and **c**, **c')** dEn of WT (left column) and *Mecp2*-null males (right column). Arrows point to AVP-ir fibres. The density of AVP-ir fibres was significantly reduced in these areas in *Mecp2*-null males as compared to WT siblings (see main text). Scale bar, 50 µm.

In the diencephalon, we found abundant AVP-if in the periventricular and lateral compartments at preoptic and anterior levels, as well as in the DMH and LHb (Fig. 19a, a'), being the latter only significant in WT males (see below). In the mesencephalon, we found AVP-ir in the periaqueductal grey (PAG) and dorsal raphe (DR). As for OT-ergic in the telencephalon, we found only a few scattered OT-if fibres in the AcbC and AcbSh. Modest OT-ergic innervation was also present in the vmStP, whereas only a few OT-if terminal fields were present in LS. In the case of BSTMPI,

MeA, MePD, central amygdala (Ce) and LHb (Fig. 19b, b'), we observed a scarce OT-ergic innervation. Conversely, there was an abundant OT-ergic innervation in the vlPAG and DR.

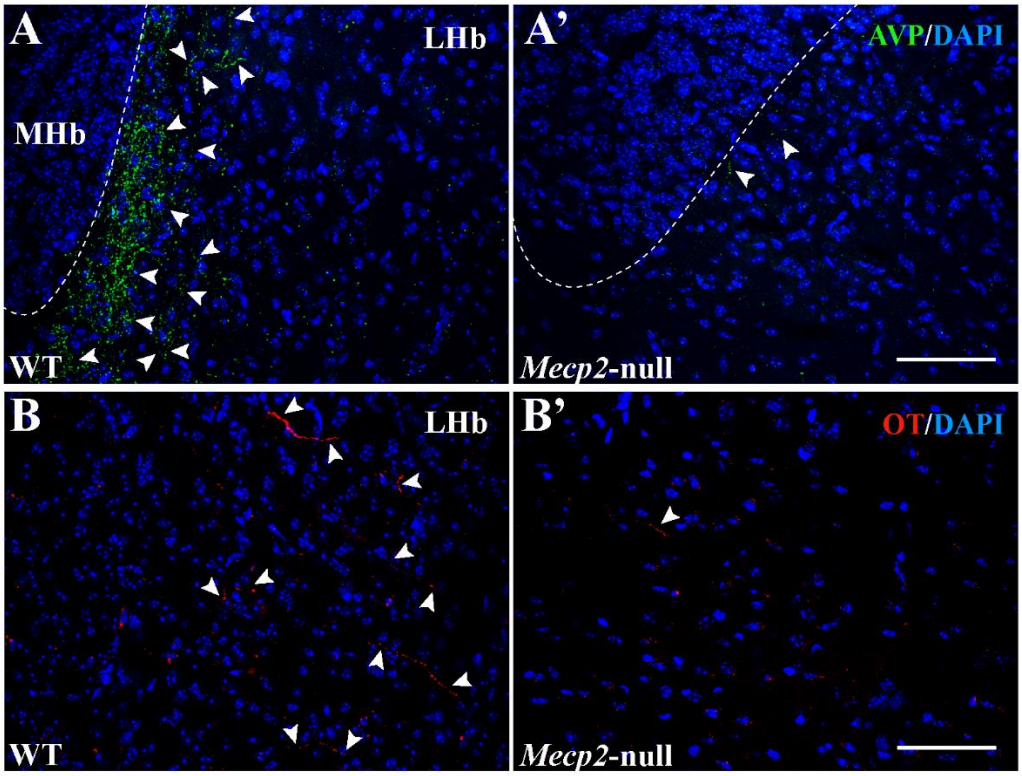


Figure 19: Representative pictures of AVP- (green) and OT-ergic (red) immunofluorescence and DAPI labelling (blue) in the lateral habenula of WT and *Mecp2*-null males. Arrows point to immunofluorescent APV-ergic and OT-ergic fibres. Both **a**, **a')** AVP and **b**, **b')** OT-if innervation was significantly reduced in *Mecp2*-null as compared to WT mice. Scale bar 100 µm. Abbreviations: *LHb,* lateral habenular nucleus; *MHb,* medial habenular nucleus.

Density of nonapeptidergic cells is not affected by deficits in MeCP2

The analysis of AVP-if and OT-if somata revealed that there were no significant differences in cell density between *Mecp2*-het and WT female mice ($p > 0.05$ in all cases, Table 13). Likewise, no significant differences were found between *Mecp2*-null and WT males (Fig. 20 a, b, and table 14).

Table 13: Quantitative analysis of average AVP and OT-ergic somata/mm^2 and innervation in different brain nuclei of *Mecp2*-het females and their WT siblings. Values are presented as mean±SEM. We did not find statistically significant differences between genotypes in any of the brain nuclei analysed. Abbreviations: *AC/ADP*, nucleus of the anterior commissure/anterodorsal preoptic nucleus; *AcbC,* nucleus accumbens, core; *AcbSh,* nucleus accumbens, shell; *BSTMPI,* bed nucleus of the stria terminalis, medial division, posterointermediate part; *Ce,* central amygdaloid nucleus; *DMH,* dorsomedial hypothalamic nucleus; *DR,* dorsal raphe nucleus; *LHb,* lateral habenular nucleus; *LS,* lateral septum; *MeA,* medial amygdaloid nucleus, anterior part; *MePD,* medial amygdaloid nucleus, posterodorsal part; *Pa,* paraventricular hypothalamic nucleus; *SCh,* suprachiasmatic nucleus; *SON,* supraoptic nucleus; *vlPAG,* ventrolateral periaqueductal grey; *vmStP,* ventromedial striatopallidum.

	AVP-ir cells/mm^2		AVP-ir crossing fibres		OT-ir cells/mm^2		OT-ir crossing fibres	
	WT	*Mecp2*-het	WT	*Mecp2*-het	WT	*Mecp2*-het	WT	*Mecp2*-het
AC/ADP	25.33 ± 13.89	8 ± 3.89	-	-	146.67 ± 49.98	162 ± 50.86	-	-
AcbC	-	-	-	-	-	-	18.10 ± 2.4	13.1 ± 0.93
AcbSh	-	-	-	-	-	-	5.6 ± 1.39	11.7 ± 2.93
BSTMPI	0 ± 0	0.56 ± 0.61	2.3 ± 0.97	2.42 ± 1.36	9.33 ± 3.06	9.44 ± 1.74	57.9 ± 4.94	54.67 ± 10.18
Ce	-	-	-	-	-	-	19.4 ± 4.3	9.83 ± 3.18
DMH	-	-	44.2 ± 13.68	29 ± 11.67	-	-	101.5 ± 24.84	74 ± 17.94
DR	-	-	-	-	-	-	28.8 ± 3.09	34.67 ± 7.82
LHb	-	-	0 ± 0	0 ± 0	-	-	1.1 ± 0.58	0.08 ± 0.08
LS	-	-	0.25 ± 0.11	0.25 ± 0.11	-	-	9.65 ± 1.73	11.8 ± 1.65
MeA	5 ± 2.85	5.33 ± 3.74	8.38 ± 4.54	11.6 ± 2.59	7.5 ± 1.43	3.33 ± 1.05	17.13 ± 2.81	18 ± 4.62
MePD	0 ± 0	2.5 ± 2.5	1 ± 0.73	1.85 ± 1.44	5 ± 5	2.08 ± 2.28	11 ± 2.5	10.22 ± 1.58
Pa	13.99 ± 4.54	12.26 ± 2.53	-	-	59.74 ± 6.01	70.95 ± 4.15	-	-
SCh	38.67 ±22.45	10.67 ± 4.52	-	-	-	-	-	-
SON	52 ± 10.73	50.56 ± 10.35	-	-	138 ± 19.54	130.56 ± 21.67	-	-

Values are presented as mean±SEM

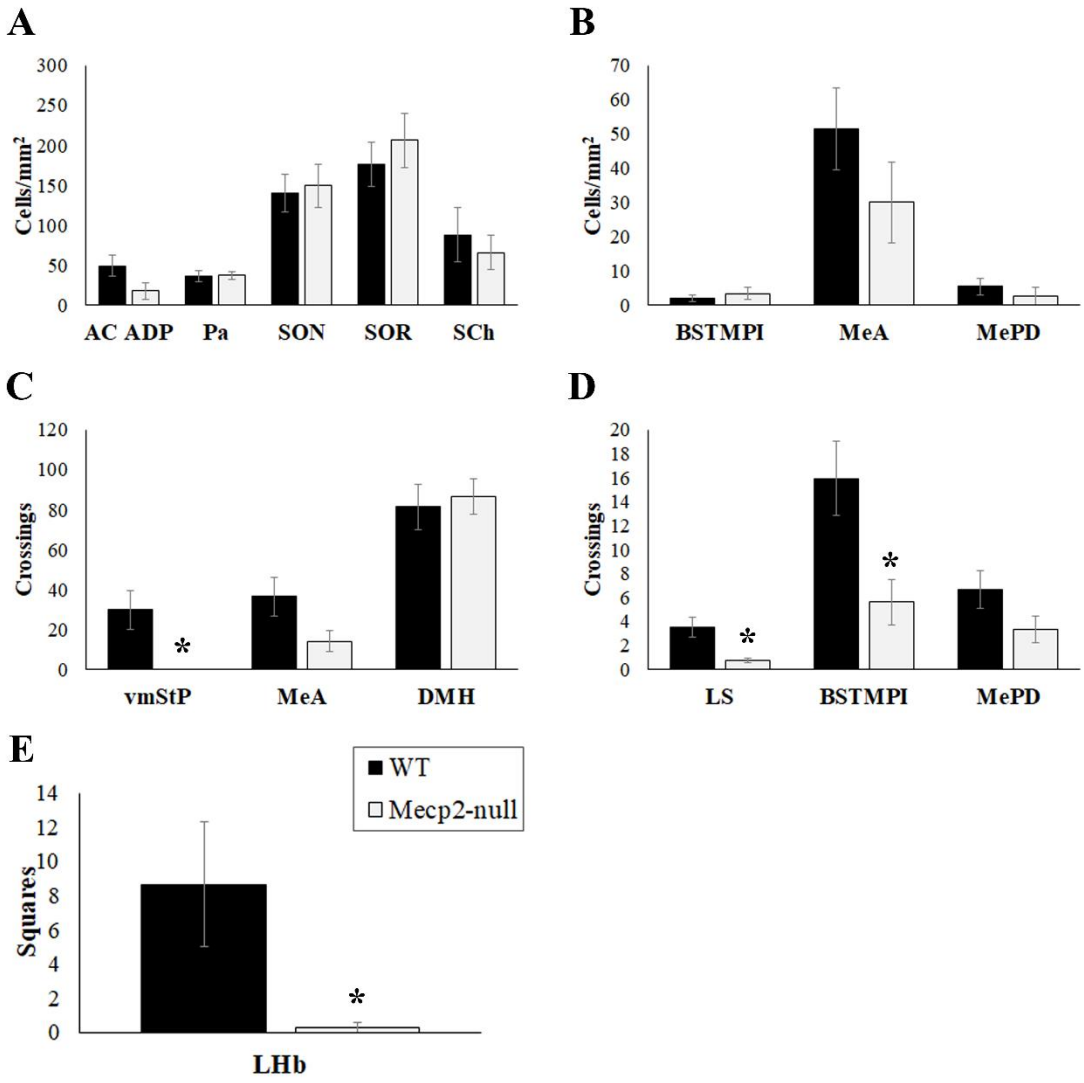


Figure 20: Bar chart showing the density of **a**, **b)** AVP-immunofluorescent (cells/mm^2) and **c**, **d)** fibres crossings in different brain nuclei of WT (black bars) and *Mecp2*-null males (grey bars). Due to the intricate mark of AVP found in the **e)** LHb, we represent the number of squares instead of crosses of fibres for this nucleus. Statistical analyses (Student's T-test or Mann–Whitney U test) revealed **a**, **b)** no effect of genotype for AVP-ir cell density, but **c–e)** a significant reduction of testosterone-dependent AVP-ergic innervation in *Mecp2*-null males as compared WT siblings. Abbreviations: *AC/ADP*, nucleus of the anterior commissure/anterodorsal preoptic nucleus; *BSTMPI,* bed nucleus of the stria terminalis, medial division, posterointermediate part; *DMH,* dorsomedial hypothalamic nucleus; *LHb,* lateral habenular nucleus; *LS,* lateral septum; *MeA,* medial amygdaloid nucleus, anterior part; *MePD,* medial amygdaloid nucleus, posterodorsal part; *Pa,* paraventricular hypothalamic nucleus; *SCh,* suprachiasmatic nucleus; *SON,* supraoptic nucleus; *SOR,* retrochiasmatic part of the supraoptic nucleus; *vmStP,* ventromedial striatopallidum. Values are presented as mean±SEM. *p<0.05

Table 14: **Quantitative analysis of average OT-ergic somata/mm² and innervation in different nuclei of *Mecp2*-null males and their WT controls.** We did not find statistically significant differences between genotypes in any of the brain nuclei analysed except for lateral habenula. Abbreviations: *AcbC,* nucleus accumbens, core; *AcbSh,* nucleus accumbens, shell; *BSTMPI,* bed nucleus of the stria terminalis, medial division, posterointermediate part; *Ce,* central amygdaloid nucleus; *DMH,* dorsomedial hypothalamic nucleus; *DR,* dorsal raphe nucleus; *LHb,* lateral habenular nucleus; *LS,* lateral septum; *MeA,* medial amygdaloid nucleus, anterior part; *MePD,* medial amygdaloid nucleus, posterodorsal part; *Pa,* paraventricular hypothalamic nucleus; *SON,* supraoptic nucleus; *vlPAG,* ventrolateral periaqueductal grey; *vmStP,* ventromedial striatopallidum ** p<0.01

	OT-ir cells/mm²		**OT-ir crossing fibres**	
	WT	***Mecp2*-null**	**WT**	***Mecp2*-null**
AC/ADP	125.24 ± 16.98	123.33 ± 26.92	**-**	**-**
AcbC	-	-	24.21 ± 6.33	21 ± 5.68
AcbSh	-	-	9.64 ± 2.06	4.4± 1.66
BSTMPI	8.57 ± 1.91	7.33 ± 2.67	67.29 ± 5.29	47.3 ± 11.98
Ce	-	-	33.79 ± 6.74	20.3 ± 3.60
DMH	-	-	121.71 ± 13.11	96.4 ± 14.16
DR	-	-	42 ± 12.6	41 ± 8.2
LHb	-	-	1.29 ± 0.32	**0 ± 0****
LS	-	-	12.96 ± 1.12	11.45 ± 2.13
MeA	12.86 ± 2.76	7.33 ± 3.4	39.64 ± 7.39	20.9 ± 6.53
MePD	3.57 ± 2.31	0 ± 0	14.86 ± 1.20	15.55 ± 2.22
Pa	77.24 ± 11.64	80.95 ± 9.12	-	-
SON	103.33 ± 20.09	79.33 ± 24.64	-	-
vlPAG	-	-	50.43 ± 15.12	39.50 ± 8.95
vmStP	-	-	13.79 ± 1.78	12.60 ± 2.65

Values are presented as mean±SEM ** p<0.01

Nonapeptidergic innervation is not affected by genotype in females

We did not find significant differences between *Mecp2*-het and WT females in any of the analysed areas, both in AVP-ergic and in OT-ergic innervation ($p > 0.05$ in all cases, Table 13). Thus, the partial deficiency of MeCP2 that occurs in heterozygous individuals fails to affect both the pattern of distribution and the density of nonapeptidergic innervation in young adult female mice.

Testosterone-dependent AVP-ergic innervation is reduced in the brain of Mecp2*-null males*

The quantitative analysis of AVP-ergic innervation showed significant reduction in absence of AVP-ergic fibres in several sexually dimorphic nuclei in the brain of *Mecp2*-null males (Fig. 20 c-e). Specifically, AVP-if innervation was significantly reduced in *Mecp2*-null males in the vmStP (t = 3.043, p=0.023), LS (t = 2.794, p=0.019), BSTMPI (t = 2.569, p=0.028), and LHb (Mann–Whitney, p=0.018). By contrast, immunofluorescent fibres in MeA, MePD and DMH were not significantly different between genotypes (all p > 0.05, Fig. 20c, d). Additionally, the analysis of AVP-ir fibres from DAB immunostaining (Figure 21) revealed a significant reduction in dorsal endopiriform cortex (dEn; t = 5.009, p=0.002) and ventral hippocampus (vHip; Mann–Whitney test, p=0.02) of *Mecp2*-null males as compared to WT siblings, but not in dorsolateral PAG (dlPAG), vlPAG or DR (all p > 0.05, Fig. 21). In the MePD, we found a significant effect of genotype in DAB samples (t = 2.415, p=0.036) that did not reach statistical significance in the immunofluorescent samples, albeit there was a trend in the same direction.

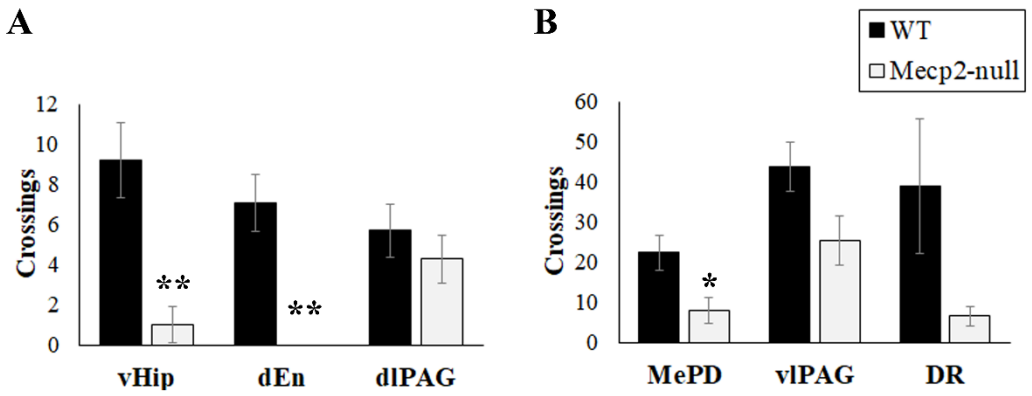


Figure 21: Bar chart showing AVP-ir innervation in samples immunostained with DAB, in WT (black bars) and *Mecp2*-null males (grey bars). Similar to AVP-if assayed by immunofluorescence, results from statistical analyses (Student's T-test or Mann–Whitney U test) showed a significant decrease of testosterone-dependent AVP-ergic innervation in *Mecp2*-null males as compared to WT. Abbreviations: *dEn*, dorsal endopiriform cortex; *dlPAG,* dorsolateral periaqueductal grey; *DR,* dorsal raphe nucleus; *MePD*, medial

amygdaloid nucleus, posterodorsal part; *vHip*, ventral hippocampus; *vlPAG,* ventrolateral periaqueductal grey. Values are presented as mean±SEM. *p<0.05; **p<0.01

OT-ir innervation is decreased in the lateral habenula of Mecp2*-null mice*

In general, we did not find differences between genotypes in the density of OT-if innervation (Table 14, all p > 0.05) in males, except in the case of the LHb, where we found a significant decrease of OT-if fibres in *Mecp2*-null males as compared to WT (p=0.02, Table 14). Interestingly, in females we found a trend towards reduction of OT-ir innervation in *Mecp2*-het females (p=0.09), with 3 out of 5 WT females showing scarce innervation but only 1 out of 6 females showing a little immunoreactivity.

NADPH-diaphorase stained cells are increased in the dorsal striatum and accumbens core of Mecp2*-null males.*

In DAB-immunostained samples, we also performed histochemical detection of NADPH-diaphorase activity, as it helps delimitate the nuclei of interest (Otero-Garcia et al., 2014). Interestingly, NADPH-diaphorase (NADPHd) activity has been shown to increase with castration, so we hypothesised that $NADPHd^{+}$ activity would be higher in the brain of *Mecp2*-null males.

In agreement with our hypothesis, we found a significant increase of NADPH-diaphorase stained cells in the dSt and AcbC of *Mecp2*-null mice compared to WT males (Figure 22 a, b). By contrast, we did not find significant differences in the other analysed nuclei (p>0.05 in all cases; Figure 22 c, d), although we observed that diaphorase staining was less intense in preparations from WT males as compared to *Mecp2*-null males. This suggests that NOS activity is higher in a region-specific manner in *Mecp2*-null males.

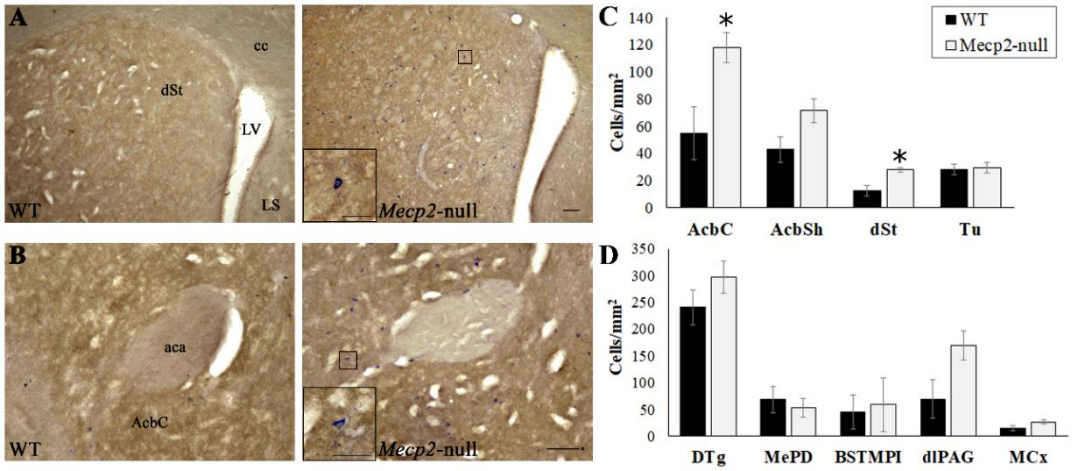


Figure 22: Example of NADPH-diaphorase staining in the a) dSt and b) AcbC of WT and *Mecp2*-null males. Nitrergic cells appear dark blue (insets). **c**, **d)** Bar charts showing the density of nitrergic cells in WT (black bars) and *Mecp2*-null (grey bars) males. The density of nitrergic cells is significantly increased in the AcbC and dSt, but not in other areas studied. Data are presented as mean±SEM. Scale bars, 100 µm; inset scale bar 50 µm. Abbreviations: *aca*, anterior commissure; *AcbC*, nucleus accumbens core; *AcbSh*, nucleus accumbens shell; *BSTMPI*, posterointermediate part of the medial division of the bed nucleus of the stria terminalis; *cc*, corpus callosum; *dlPAG*, dorsolateral periaqueductal grey; *dSt*, dorsal striatum; *DTg*, dorsal tegmental nucleus; *LS*, lateral septum; *LV*, lateral ventricle; *MCx*, motor cortex; *MePD*, posterodorsal part of the medial amygdala; *Tu*, olfactory tubercle.

Experiment 2.2 Effect of lack of MeCP2 in aggressive behaviour

Mecp2-*null males do not attack and display reduced chemoinvestigation of intruders and increased self-grooming*

Aggressive behaviour is known to be influenced by testosterone (Brain & Haug, 1992). Thus, we tested WT and *Mecp2*-null mice in a resident-intruder paradigm to evaluate aggressive behaviour against a WT intruder. Since the aggressiveness of our WT males was low, we combined attack and chase parameters and analysed them as one. We found that while 31% of WT resident mice attacked or chased the WT intruder, none of the *Mecp2*-null mice attacked or chased the WT intruder (Chi square test = 4.97; p=0.026). However, since the WT resident animals were scarcely aggressive, we did not find significant differences in time spent attacking/chasing the intruder (WT resident: 7.30 ± 2.73 s, *Mecp2*-null resident: 0 ± 0 s, Mann–Whitney

U test, p=0.083, Fig. 23 a). Additionally, we analysed the time spent by the resident in chemoinvestigation of the intruder, a socially-directed behaviour. Here, *Mecp2*-null male residents spent significantly less time chemoinvestigating the WT intruders, as compared to the time WT residents took to investigate WT intruders (Mann–Whitney U test, $p < 0.018$; Fig. 23 b). All these results point to a lack of interest of *Mecp2*-null males towards their conspecific intruders, at least in the RI test (but see discussion for other possible interpretations). Interestingly, we found that social and agonistic behaviours were apparently substituted in the *Mecp2*-null resident males by self-grooming, as they spent significantly more time grooming than WT residents (Mann–Whitney U test, p=0.005; Fig. 23 c). We cannot exclude that this behaviour is reflecting a stereotypic behaviour, similarly to what was reported previously in mouse models of ASD as compared to WT (A. D. Chang et al., 2016; Wu et al., 2019).

We finally included a third group of agonistic pairs in our analysis, formed by WT residents and *Mecp2*-null intruders. Since all our measures point towards a decrease in testosterone levels, which also control masculine pheromonal production, we hypothesised that *Mecp2*-null males would have lower levels of the "male pheromone" Darcin (Roberts et al., 2010, 2012), and therefore might elicit a decreased aggression or a different pattern of investigation from WT residents. Indeed, analysis of the urine of *Mecp2*-null males confirmed lack of Darcin (Martinez-Rodríguez et al, 2020). Data from this RI experiment revealed that WT residents investigated significantly more *Mecp2*-null intruders than WT intruders (Student's t-test, $t = -3.882$, $p < 0.001$; Fig. 23 b). However, WT residents did not show significant differences in time spent attacking/chasing WT or *Mecp2*-null intruders (Mann–Whitney U test, $p > 0.05$, Fig. 23a), probably due to a floor effect in the levels of aggression. This suggests that WT residents "see" *Mecp2*-null animals as "novelty" that calls for their attention/investigation.

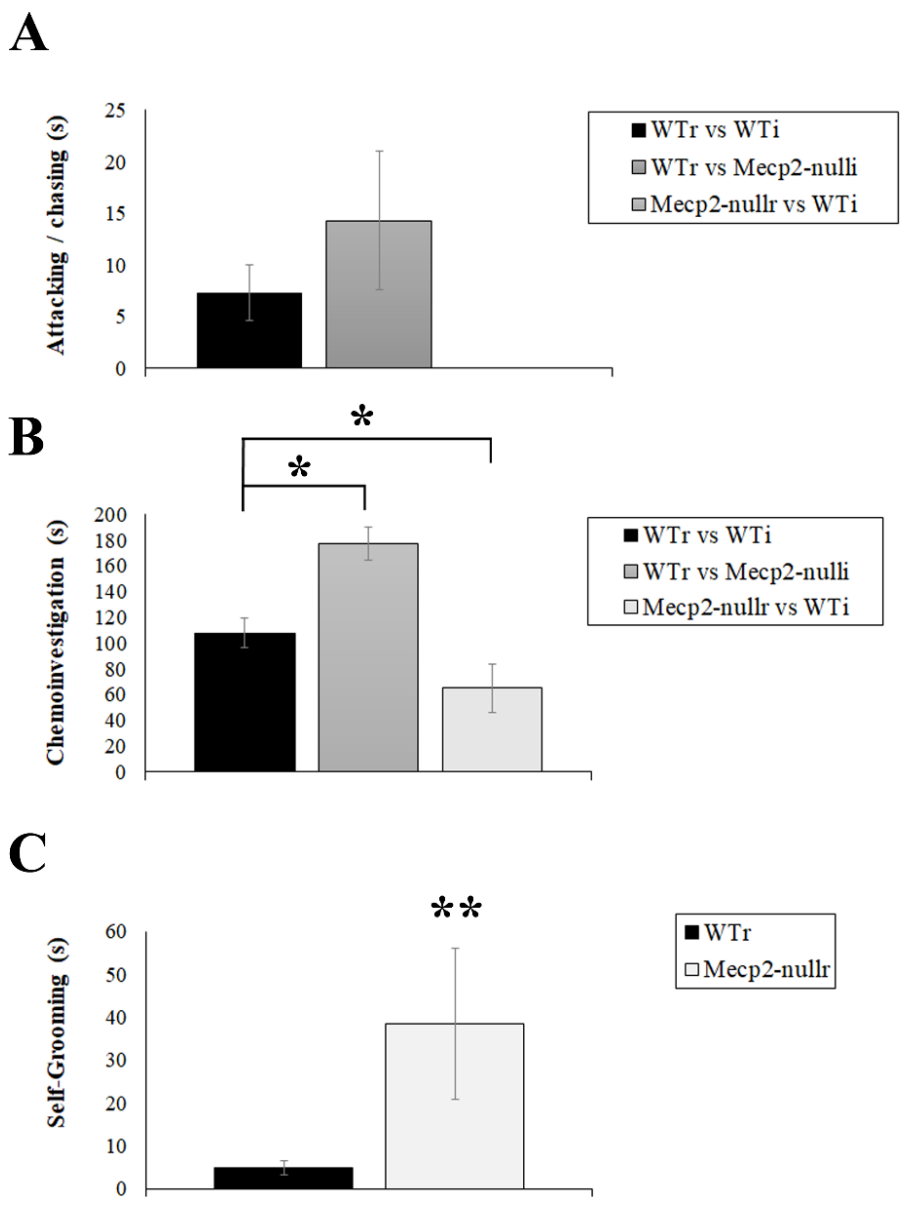


Figure 23: Behavioural parameters analysed in the resident animals during the RI-test. First, we analysed **a)** aggressive behaviours, **b)**chemoinvestigation, and **c)** self-grooming in the residents of the Wa_{ter} vs WT_i (black bars) and *Mecp2*-$null_r$ vs WT_i (light grey bars) conditions, and then we analysed the same parameters in the residents of WT_r vs WT_i (black bars) and WT_r vs *Mecp2*-$null_i$ (dark grey bars).

DISCUSSION

In this study, we investigated the distribution and density of nonapeptidergic somata and innervation in young adult *Mecp2*-null males and *Mecp2*-het females, as compared to their WT siblings. Overall, females do not show significant differences between genotypes in either AVP-ergic or OT-ergic distribution. By contrast, we found a significant reduction in AVP innervation in *Mecp2*-null males, specifically in the sexually dimorphic nuclei of the social brain network. Additionally, we found a significant reduction of OT innervation in *Mecp2*-null males specifically in the LHb, which is not sexually dimorphic. Since the main neuroanatomical changes found in the nonapeptidergic system were specific to the testosterone dependent AVP-ergic innervation, we further analysed features that are dependent on the gonadal status of males. Specifically, we analysed the density of nitrergic cells and aggressive behaviour, and found significant deficits in both of them that we discuss below.

The distribution of AVP and OT cells is not affected by lack or deficit of MeCP2

AVP and OT are both synthesised in the Pa and SON hypothalamic nuclei. Besides, AVP-if somata are also present in the SCh and AVP-if and scarce OT-if somata in Me, BST and accessory nuclei. Distribution of AVP and OT in our sample of WT and *Mecp2*-mutant mice matched previous studies in mice by Otero-Garcia et al. (2014, 2016) in CD1 strain and Rood et al. (2013) in C57BL/6N strain. In general, neurons were either AVP-if or OT-if, although we found colocalization of both neuropeptides in AC/ADP and Pa, as previously described (Otero-Garcia et al., 2016). These results suggest that deletion of MeCP2, previously described as key in the regulation of AVP in the Pa (Murgatroyd et al., 2009), is not sufficient to produce a major deficit in AVP production. To our knowledge, a regulation of the OT gene by MeCP2 has not been described; however, the AVP and OT genes are located in adjacent regions of the

same chromosome, separated by only 12 Kb (Summar et al., 1990), and both peptides are co-expressed in some neuronal populations (Otero-Garcia et al., 2016), suggesting a close transcriptional regulation of both genes. As with AVP, no qualitative differences were observed in OT distribution between genotypes. However, a caveat in this study is that immunohistochemistry is not directly quantitative of the level of nonapeptides. Thus, future experiments should address the possibility that nonapeptidergic mRNA or protein levels are affected in *Mecp2*-mutant mice, using more sensitive techniques such as RNAScope, or CLARITY analysis.

Mecp2-heterozygous females show no discernible deficits in the nonapeptidergic systems

We did not find significant differences in nonapeptidergic distribution or innervation between *Mecp2*-het females and their WT littermates. Lack of differences between genotypes in females could be attributed to (i) the presence of one *Mecp2* allele in females that could be sufficient to prevent alterations in AVP or to (ii) a lack of testosterone-dependent AVP innervation in females, assuming that absence of AVP in sexually dimorphic nuclei in *Mecp2*-null males is mainly testosterone-dependent. In addition, we must also consider possible age effects in our mice, analysed at 8 weeks old. As explained in Chapter 1, at this age, male *Mecp2*-null mice are already manifesting the overt phenotype of the syndrome, whereas female *Mecp2*-het mice do not show the complete symptomatology until, at least, 3 months old (Guy et al., 2001; our own data, see Chapter 3). In this sense, further investigations of AVP/OT-ergic systems in older females are required to elucidate possible impairments in both nonapeptidergic systems, similarly to what studies from the lab discovered in the case of DCX-ir cells (see Chapter 1).

Mecp2-null males exhibit significant deficits in testosterone-dependent AVP-ergic innervation and OT-if in LHb

As described in previous reports (Otero-Garcia et al., 2014; Rood et al., 2013), AVP-ergic innervation is sexually dimorphic in some brain nuclei. Specifically, Rood et al. (2013) described that AVP-ergic innervation of LS, Me, BST, LHb, PAG, vHip, and DR nuclei was dependent on gonadal steroids and more abundant in males than in females, whereas AVP fibres in hypothalamic areas, such as DMH, were not dependent on gonadal steroids and did not show sex differences.

Our data in *Mecp2*-null mice show that there is a specific decrease in AVP-ergic innervation in all the testosterone-dependent nuclei, but not in the DMH. Thus, our data are consistent with an effect of lack MeCP2 in AVP production through indirect testosterone-dependent mechanisms. In this sense, Auger et al. (2011) showed that circulating gonadal steroid hormones modify the methylation status of some steroid responsive gene promoters and, consequently their expression levels. In accordance, methylation of AVP promoter is regulated by testosterone signalling in the BST of adult male rats (Auger et al., 2011). Moreover, sexually dimorphic AVP-ergic innervation in the LS is dependent on the action of estradiol (presumably aromatised from testosterone) via ERα receptor (Scordalakes & Rissman, 2004), which is also regulated by MeCP2 in the brain (Westberry et al., 2010). *Mecp2*-null males display internal testicles (Guy et al., 2011), a feature consistent with lower levels of testosterone production and possible deficits in signalling through ERα (Cederroth et al., 2007). Although future studies ought to directly measure androgen levels in *Mecp2*-null mice to prove a reduction in testosterone levels, our data obtained from previous studies from our lab, showed that *Mecp2*-null males display a reduction of MUPs and lack the masculine pheromone darcin, supporting this assumption (Martínez-Rodríguez et al., 2020).

We acknowledge that other deficits displayed by *Mecp2*-null males, such as broad or specific deficits in pheromonal production or impaired kidney function, could

account for this physiological effect, but we think that the most likely explanation is that of reduced testosterone levels. Indeed, darcin production is absent in females and castrated males but present in “sick” males (see supplementary information from Martínez-Rodríguez et al., 2020).

In addition to an indirect action through hormonal deficits, deficits in MeCP2 have been shown to directly regulate the sexually-dimorphic AVP-ergic innervation in rats. Thus, a transient reduction of MeCP2 during the first three postnatal days via focal injections of siRNA in the Me leads to a transient decrease of both androgen receptor (AR) and AVP mRNA in the Me of 14-day-old rat males (Forbes-Lorman et al., 2012). Interestingly, rats subjected to this treatment at early postnatal days showed no lasting effects on the levels of AR at 7.5 months old, but a long-lasting deficit in the density of AVP-ergic somata in centromedial amygdala and BST, and a reduction of LS innervation. In fact, the expression of *Mecp2* gene is sexually dimorphic in the brain of rats during the steroid-sensitive period (Kurian et al., 2007), suggesting a key role of this gene in the development of sexually dimorphic systems. In summary, the mechanism by which MeCP2 regulates AVP production may be an indirect effect over gonadal hormone production, a direct gene regulation, or both.

Surprisingly, we also found a significant decrease in the scarce OT innervation in LHb, a feature that, to our knowledge, has not been previously described as sexually dimorphic, in our *Mecp2*-null males compared to their WT siblings. Low levels of testosterone could also contribute to this deficit, since the metabolite of dihydrotestosterone, 5α-androstane-3β,17β-diol, is able to regulate OT expression through ER-β activation (Hiroi et al., 2013), a type of estrogenic receptor found in the LHb (Shughrue et al., 1997). Further, we found a trend towards reduction on the OT innervation of the LHb in *Mecp2*-het females. We cannot exclude that lack of statistical significance in our sample is due to lack of sensitivity of the technique, since in this region OT-ir is scarce, or due to reduced number of subjects. Taking all

these aspects into consideration, OT innervation in the LHb in this model should be re-analysed in the future.

Consequences of nonapeptidergic deficits for behaviour in *Mecp2*-null mice

Alterations in AVP-ergic and OT-ergic signalling may impair the proper functioning of the social brain network. For instance, deficits in AVP-ergic innervation in BST/Me and nuclei to which they project could be affecting social and aggressive behaviours in *Mecp2*-null males (Modi & Sahin, 2018) which is consistent with our results obtained in the RI test (see below). The MePD is interconnected with BSTPM to control socio-sexual behaviours mediated by pheromones, as well as defensive behaviours against predators (Pardo-Bellver et al., 2012; Tong et al., 2019). Both MePD and BST are intimately modulated by circulating hormones, such as progestogens, androgens, and oestrogens (de Lorme et al., 2012; Pardo-Bellver et al., 2012; Zancan et al., 2017) due to the large number of cells that expresses steroid receptors.

Despite the extensive innervation of AVP in MePD, the density of V1aR is sparse, and how the AVP could modulate social behaviour in adult males remains poorly understood (C. J. W. Smith et al., 2019). Finally, LS and vHip are connected to each other, allowing LS to integrate socio-sexual information from the amygdala with spatial and contextual information from the vHip (Campbell et al., 2009; Pardo-Bellver et al., 2012). This pathway provides adequate responses to each social situation and, therefore, it has been proposed that its connectivity could be impaired in ASD patients.

It was recently found that LHb is involved in the regulation of social preference in rats (Benekareddy et al., 2018) and aggression in mice (Golden et al., 2016). Therefore, deficits of both AVP- and OT-ergic innervation in this structure could contribute to the social abnormalities displayed by *Mecp2*-null mice. LHb integrates

information from the hypothalamus. In this sense, the LHb regulates the serotoninergic system between the DR and other nuclei, such as the amygdala, BST, LS, hippocampus, and preoptic area (Rood et al., 2013). Serotonin, together with NO and AVP, modulate social and aggressive behaviours in rodents (Agustín-Pavón et al., 2009; Angoa-Pérez & Kuhn, 2015; Ferris et al., 1997; Hashikawa et al., 2017), whereas serotonin blocks aggression and territorial behaviour, and AVP promotes aggression in the anterior hypothalamus in males. It has been described that serotonin agonists promote AVP and OT synthesis. Likewise, AVP administration stimulates synthesis and release of serotonin in some brain nuclei (Auerbach & Lipton, 1982; Jørgensen et al., 2003). In support of this hypothesis, deficit in serotonin levels has previously been reported in *Mecp2*-null males (Santos et al., 2010; Vogelgesang et al., 2017). Likewise, treatments based on stimulation of the serotonin transmission are able to improve the phenotype of *Mecp2*-null males (Ricceri et al., 2013) and *Mecp2*-het females (Filippis et al., 2015). Consequently, it is likely that lack of MeCP2, causing misbalances in the metabolism of NO and serotonin and production of gonadal hormones, could be affecting AVP- and OT-ergic innervation in a region-specific manner and, therefore, impairing social and aggressive behaviours in *Mecp2*-null mice.

Abnormal aggression and social behaviour in *Mecp2*-null males

In the RI test, *Mecp2*-null residents do not display aggressive behaviours against the intruder, which is consistent with a decreased AVP innervation and increased NADPHd+ (indicative of increased production of NO) found in those males. Both, AVP and NO, have been long related with the modulation of aggressive behaviours in male mice (Marie-Luce et al., 2013; S. Robinson et al., 2012; Trainor et al., 2007). Thus, decreased AVP innervation [in particular in the LS, (Veenema et al., 2010)], and possibly an increase in NO production in the brain of our *Mecp2*-null males, could contribute to the alterations found in *Mecp2*-null behaviours in the RI test, such as reduced aggression of the intruder and territoriality.

Interestingly, it has been shown that overexpression of MeCP2 can increase aggressive behaviour in both mice and humans (Tantra et al., 2014), mirroring the lack of aggression that we found in *Mecp2*-null mice. Of note, we cannot exclude the possibility that, being *Mecp2*-null mice smaller than the WT intruders, this can constitute a confounding factor in the results obtained, as the size of WT animals can be "intimidatory" towards the smaller *Mecp2*-null animals. In addition, we found a reduction in chemoinvestigation of the intruder by *Mecp2*-null males. Additionally, previous results from our lab demonstrated that *Mecp2*-null males do not suffer from anosmia, since a habituation–dishabituation test showed that both WT and *Mecp2*-null males are able to detect both urine and rose odours (Martínez-Rodríguez et al., 2020). Thus, the reduced chemoinvestigation points towards a general lack of interest in the intruder by *Mecp2*-null residents. Another possible explanation could be that *Mecp2*-null animals exhibit behaviours, such as self-grooming, an activity to which they devoted about a 13% of the time of the test, whereas WT residents devoted a mere 1.6% of the time to this behaviour. We did not directly measure locomotion in these tasks, so potential locomotor deficits of *Mecp2*-null mice could influence the observed results. Finally, an increased somatosensory sensitivity of *Mecp2*-null mice, which could render unpleasant direct contact with conspecifics, cannot be discarded (see Chapter 3 and Flores Gutiérrez et al., 2020). In this sense, 67% of *Mecp2*-null males escaped abruptly from the resident WT during the interaction, in front of only 38% of the WT residents confronted to a WT intruder, and none of the WT residents confronted to a *Mecp2*-null intruder.

Curiously, *Mecp2*-null intruders were significantly more investigated by WT residents. Provided that, male sexual pheromones give information about strain, sex and fertility of rodents (Brennan & Kendrick, 2006), and that *Mecp2*-null males show low levels of MUPs and darcin in the urine (Martínez-Rodríguez et al., 2020), we suggest that increased investigation of *Mecp2*-null intruders by WT residents could

be due to lack of information given by male pheromones. In this line, it would be interesting in future studies to further explore the social dimension of *Mecp2*-null mice in social interaction tests, including the use of *Mecp2*-null mice as stimulus animals and exploring the behavioural features of and towards *Mecp2*-het females.

Limitations, conclusions and future directions

Taken together, our results in *Mecp2*-null male mice reveal several abnormalities in sexually-dimorphic, testosterone dependent, neuroanatomical (nonapeptidergic innervation), and behavioural features (aggression and social investigation). These deficits could be due both to a direct involvement of MeCP2 in the regulation of expression of several genes (AVP, ERα) or to indirect effects due to the impact of lack MeCP2 in gonadal development, and consequently in the hormonal status of the mice.

Thus, when studying neurodevelopmental disorders, such as RTT and other *MECP2*-related conditions, it is important to consider possible effects in hormonal signalling that could account for some of the deficits observed. In this sense, different levels of gonadal hormones have been implicated in sexual differences in the incidence and severity of some diseases, such as autism, and psychiatric and cognitive disorders (Akinola & Gabriel, 2018; Romano et al., 2016). Future works should study in depth the mechanism leading to the loss of sexually-dimorphic features in *Mecp2*-null males, and check the possible effect of pharmacological manipulations of nonapeptidergic systems in the amelioration of behavioural symptoms in this mouse model.

CHAPTER 3. EFFECT OF *MECP2* DEFICIENCY IN NOCICEPTION AND PAIN PROCESSING

INTRODUCTION

The concept of pain

The experience of pain has been described as a complex combination of sensory-discriminatory information of a noxious stimulation, with affective-emotional and cognitive-evaluative dimensions, and autonomic reactions (Price, 1999; Thienhaus et al., 2002.) In 2020 the International Association for the Study of Pain (IASP) came up with a new revised definition of pain as "An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage", which is expanded upon by the addition of six key Notes and the etymology of the word pain for further valuable context.

- Pain is always a personal experience that is influenced to varying degrees by biological, psychological, and social factors.
- Pain and nociception are different phenomena. Pain cannot be inferred solely from activity in sensory neurons.
- Through their life experiences, individuals learn the concept of pain.
- A person's report of an experience as pain should be respected.
- Although pain usually serves an adaptive role, it may have adverse effects on function and social and psychological well-being.
- Verbal description is only one of several behaviours to express pain; inability to communicate does not negate the possibility that a human or a nonhuman animal experiences pain.

Pain has an emotional impact leading to different responses on each patient (Giannoni-Pastor et al., 2016). It can also be classified in different types according to its specific characteristics. On one side, pain can be classified according to its utility or aim in:

- **Adaptative pain**, whose main objective would be to guarantee survival and/or healing of the individual.
- **Maladaptive or dysfunctional pain**, when presented as a pathological condition per se. This kind of pain tends to be persistent or recurrent, without any protective of healing purpose (Costigan et al., 2009; Woolf, 2004).

In the case of human patients, we can also consider the source of pain and distinguish between:

- **Stimulus-evoked pain**, which can be described as either hyperalgesia (increased or exaggerated pain response to a normal stimulus) or allodynia (painful response to a non-noxious or innocuous stimulus). In terms of sensory loss, the term hypoalgesia can be used when there is a decreased sensitivity to a nociceptive stimulus. This type of pain can also be further subdivided on the basis of the evoked stimulus modality (e.g., mechanical, heat, cold, chemical; Woolf & Mannion, 1999). This kind of pain can be quantitatively measured by using sensory testing, which allows experts to classify the pain of patients based on the mechanisms and to choose the best treatment to target that mechanism (Baron et al., 2010; Cruz-Almeida & Fillingim, 2014).
- **Stimulus-independent or spontaneous pain**. This pain appears to be spontaneous, instead of triggered by an identifiable stimulus. It can also be subclassified as paroxysmal (sudden and severe) or continuous, and can be

described as aching, cramping, crushing, shooting, and burning (Jensen et al., 2001).

In terms of proximity of the damaged tissue to the surface, two different kinds of pain can be described:

- **Internal pain**, also known as visceral pain.
- **External pain**, caused by cutaneous injuries, extreme thermal conditions, etc.

Despite these classifications, some aspects are shared by different pain categories. Once sensitised, an individual can perceive pain evoked by a stimulation that was previously experienced as non-painful, in a process known as allodynia. Further, an exacerbated and prolonged pain sensation can be generated in response to a noxious stimulation in a condition called hyperalgesia (Costigan et al., 2009; Woolf, 2004).

By comparison, the term of nociception describes a sensory processing of a noxious stimulus, from the periphery to the brain via the pain/nociceptive pathway (Coutaux et al., 2005; Steeds, 2016; Woolf, 2004, 2011), generating a peripheral neuronal response to noxious stimuli that have the potential to damage the tissue (Dubin & Patapoutian, 2010). A nociceptive stimulus capable of activating this pathway is termed allogenic stimulus (Coutaux et al., 2005).

The nociceptive pathways and principal modulators: the neuromatrix

From this definition, pain is inferred as a personal and subjective ordeal, the result of multiple interactions between the signalling pathway and its modulation by higher cerebral centres (Bie et al., 2011; Marchand, 2008; Steeds, 2016; Todd, 2010; Woolf, 2004). Thus, noxious information is processed by a widely-distributed, hierarchically interconnected neural network in the brain, referred as neuromatrix [reviewed in (J. Chen, 2009; M.-G. Liu & Chen, 2009)]. The nociceptive pathway

starts at the peripheric nociceptors, which will transmit the information to the second (spinal cord) and third order neurons. This information will reach the cortical regions in the brain, where it elicits the perception of pain. Signalling is then transmitted through different areas (including the spinal cord, thalamus and brainstem) through separately parallel pathways for the processing of the noxious information. Each parallel pathway will contribute differently to the final noxious experience. By this statement, IASP considers that pain can only be experienced if the noxious stimulation is interpreted by the cortex to produce a multidimensional experience, which separates the concepts of pain and nociception.

Furthermore, each pain category is also characterised by a unique combination of neurochemical changes (neurotransmitters such as glutamate and substance P; and neuromodulators like BDNF) at the peripheral and central levels along the nociceptive pathway (S. P. Hunt & Mantyh, 2001).

BDNF is a neurotrophin necessary for neuronal development and peripheral pain mechanisms (Merighi et al., 2008), with a well stablished role in both inflammatory and neuropathic pain (Ha et al., 2001; Y. T. Lin et al., 2011; Obata & Noguchi, 2006; Uchida et al., 2013). BDNF modulates sensory neurotransmission and nociceptive pathways at both the spinal and supraspinal levels, with an important role in the development of central sensitization that underlies many forms of hyperalgesia (Merighi et al., 2008).

Likewise, injured tissues are responsible for the local release and production of different molecules (bradykinin, histamine, serotonin, ATP, prostaglandins, cytokines, neuropeptides, etc.). All these transducers will affect to different parts of the cell, as well as directly or indirectly lead to changes in gene expression, which result in prolonged changes in the nervous system. In response to noxious stimulation and the subsequent inflammation, some genes like cFos will get their expression increased (Gao & Ji, 2009; S. P. Hunt et al., 1987; Todd et al., 2002;

Williams et al., 1990). It is of special relevance to consider that cFos expression only takes place in the nuclei, and its basal levels are very low, thus it is commonly used as a marker of cell activation.

Epigenetics and pain perception

Some studies suggest an interplay between epigenetics and pain. An influence of epigenetic factors has been reported to regulate transcriptional alterations during different pain states (Crow et al., 2015; Denk & McMahon, 2012; Géranton, 2012; Géranton & Tochiki, 2015b; Mmed et al., 2012; Z. Zhang et al., 2011). Likewise, there is an association between epigenetic changes and pain in humans (Rahn et al., 2013; Tajerian et al., 2011). Furthermore, epigenetic regulation may participate in the transition of acute to chronic pain by supporting maladaptive molecular changes (Géranton, 2012; Géranton et al., 2007; Géranton & Tochiki, 2015a; Rahn et al., 2013; Z. Zhang et al., 2011).

Although there are no studies correlating abnormal pain sensitivity and severity of mutation, a potential relationship between hyposensitivity and specific mutations in gene *MECP2* has been reported (Downs et al., 2010). Additionally, odds ratios for decreased pain sensitivity seem to be highest for p.R168X, a mutation often regarded as severe (Bebbington et al., 2008; Downs et al., 2008; Neul et al., 2008), and two rather milder mutations, p.R306C (Neul et al., 2008; Schanen et al., 2004) and C terminal deletions (Smeets et al., 2005).

Although MeCP2 is involved in multiple sensory and nociceptive processes, it remains unclear how alterations in the expression of this protein affect pain experience or perception in RTT (Martin, 2017). As a possible explanation, it has been proposed that MeCP2 is important for mu opioid receptor (MOR) regulation. MeCP2 may bind to the methylated regions of the *Oprm1* gene promoter, repressing its expression and leading to lower MOR levels in the dorsal root ganglion (DRG). Decreased MOR expression in the peripheral nervous system contribute to the

development of neuropathic pain, whilst MeCP2 knockdown enhances the analgesic effect of morphine in these cases (Sun et al., 2021). However, unpublished results from our laboratory showed that MOR levels are not affected in symptomatic *Mecp2*-het females (6 months old). Considering all this information, MeCP2 plays a relevant role in the development and function of highly relevant components of the nociceptive circuitry.

Additionally, MeCP2 contributes to an early cascade of molecular events through the release of serotonin, which are necessary for the initiation of pain states (Géranton et al., 2007, 2008). On this basis, post-translational modifications of MeCP2 may contribute to the initiation and possibly the maintenance of pain states. Thus, alterations in *MECP2* could therefore interfere in the response to noxious stimulation, and decreased levels of functional MeCP2 protein may directly or indirectly reduce pain sensitivity. Manners et al also suggested that hyposensitivity in RTT patients could be due to an increase in miRNAs that binds and repress translation of *MECP2*, thus decreasing MeCP2 levels (Manners et al., 2015).

Furthermore, MeCP2 seems to be important in the regulation of BDNF expression (Wang et al., 2006). Decreased levels of BDNF have been found in *Mecp2*-null mice, which may contribute to hyposensitivity in those males. By contrast, BDNF release in the dorsal horn of neuropathic pain models has been associated with thermal hyperalgesia (Miletic & Miletic, 2002; Pezet & McMahon, 2006). However, other modes of action could be mediating mechanical sensitivity (Manners et al., 2015). Thus, the reduction in endogenous BDNF could be one of the contributing factors leading to altered pain sensitivity in RTT.

Pain and nociception in Rett syndrome

There is some evidence suggesting that pain perception is altered in patients with RTT (Downs et al., 2010; Hagberg, 1995, 2002; Neul et al., 2010; Peters et al., 2013). Indeed, decreased pain sensitivity has been diagnosed in some girls with RTT (Downs

et al., 2010; Hagberg, 2002; Neul et al., 2010; Peters et al., 2013). As an example, Hagberg described in 1995 how a girl suffering from RTT laughed when placing her hand through a candle flame (Hagberg, 1995). Alterations in pain sensitivity in RTT differ between visceral and external pain. Indeed, carers and close relatives seem to report that perception of internal pain is less impaired or even increased when compared to external pain (Downs et al., 2010). This could be due to both vagal and spinal afferents contributing to the sensory experience in visceral pain where emotional and autonomic components are particularly strong (Bielefeldt et al., 2005; R. H. Hunt & Tougas, 2002). Although preclinical investigations suggest that reflexive pain circuitry is intact (Samaco et al., 2008), high-order discriminatory behaviour may be impaired in those girls. Downs reviewed that, in a population-based Australian study, 75.2% of cases of RTT reported an abnormal pain response, with decreased pain sensitivity in 65% of them. In addition, 70.6% of patients with mutations in the amino acid T158, located at the C terminus of the methyl-CpG binding domain of MeCP2 showed decreased pain sensitivity (Downs et al., 2010).

Downs et al also appreciated how that proportion with abnormal responses was different depending on the age. Lower proportion of patients with abnormal response to pain was observed in the youngest age-group (up to 6 years of age). However, the group of 6 to 11 years of age presented the highest rates of abnormal responses (Downs et al., 2010). Thus, altered pain perception seems to increase by the age of 6 to 11 years old in humans. This period of age of 6-10 years corresponds to the third stage of RTT syndrome (Hagberg & Witt-Engerstrom, 1986). A stage of stabilization in which social interaction seems to come back and, therefore, could be considered as the stage in which RTT girls are most cognitively alert. On the same line, Coleman performed a parent survey revealing that 81% of subjects with RTT seem to be insensitive to pain (Coleman et al., 1988). However, we must not forget that parent surveys are subjective and based on personal perception of pain responses.

Although the literature suggests that pain perception diminishes in old age in healthy humans, most of these results are equivocal and inconsistent. As an example, pressure pain threshold is usually lower in old adults compared with younger adults, but there seem to be no differences in contact heat pain thresholds between old and younger adults (El Tumi et al., 2017). However, some evidence revealed that younger children (6 to 8 years) are more sensitive to noxious stimuli than older children (reviewed in El Tumi et al., 2017). There is tentative evidence that old adults may be more sensitive to mechanically evoked pain, but not heat evoked pain, than young adults. However, there is a need for further studies on age-related changes in pain perception.

In opposition to basal pain perception, delayed responses to noxious stimuli (injections, burns, falls, trauma, etc.) must also be considered as observed in some cases of RTT (Downs et al., 2010). This is consistent with results obtained from some animal models exposed to injury, in which increased sensitivity was delayed compared to WT animals (Géranton et al., 2007).

The problem of measuring pain in patients of Rett syndrome

One of the main problems in measuring pain lies in its multidimensional domains, such as pain severity, sensory qualities of pain, pain beliefs, the affective state, subjectivity to pain, and the way each person communicates about their pain, among others. Thus, it is important to consider all these various dimensions that contribute to the construct of pain, in order to choose valuable methods that accurately provide for assessment of each dimension. On this basis, multiple qualitative and quantitative techniques have been commonly used to test sensory nerves and pain in both research and clinical settings. In the specific case of humans, verbal and non-verbal communication of pain have commonly been used to measure pain in patients. As an example, facial expression constitutes a powerful way to communicate pain (Arif-Rahu & Grap, 2010; Kappesser, 2019), especially

when working with people suffering from dementia (Lautenbacher & Kunz, 2017). However, difficulties analysing facial expression stem from its qualitative dimension. In addition, depending on the age or even condition of each patient, facial responses may vary or even be absent, even in the presence of painful states. That is the case of RTT patients, and one of the main considerations we should address when analysing pain in girls affected by this condition. Something similar happens with RTT patients when using techniques based on analysing variations in heart rate, since lower heart rate variations has been reported in this disorder (Acampa & Guideri, 2006; Guideri et al., 2001). By contrast, some studies have proposed quantitative sensory testing protocols as a most accurate way to measure nociception and pain perception in both patients and animal models (Barrot, 2012; Deuis et al., 2017; Kostek et al., 2016; Larson et al., 2019). These tests are based on quantitative techniques that measure tactile sensation and pain threshold for pressure and heat (Kostek et al., 2016).

Due to the significant motor and communicative impairments, it remains difficult to determine what pain "looks like" in patients of RTT. In fact, some studies have provided subjective and objective evidence that individuals with RTT experience recurring and chronic pain for which facial expression appears intact. Communication deficit, motor impairment and high frequency of abnormal autonomic responses (Julu et al., 2001) contribute to the challenge for such assessment in RTT. Additionally, a large proportion of RTT patients described less sensitivity to potentially painful stimuli due to the lack of manifestation of usual characteristic behaviours associated with responses to pain, including crying, wincing, tachycardia, and muscle tension, among others (Stallard et al., 2002). For this reason, it is of relevance to estimate whether results pointing to hypoalgesia are influenced by a real decreased sensitivity of pain or due to a reduced ability to express this pain. Elucidating this causal relationship would potentially lead to improved life quality in those girls. For girls suffering from Rett Syndrome, it

becomes a fundamental goal to build the capacity for movement and communication in everyday life, with a deeper understanding of motor deficits, the potential role of the environment, and technological advances in assisted communication systems. Thus, in this chapter, we explored mechanical and thermal sensitivity in a group of young-adult (2 months old) and older-adult (6 months old) of *Mecp2*-het and WT females. We also analysed our results in terms of age, weight, severity of the syndrome, and motor ability of those mice.

MATERIALS AND METHODS

Animals

For these experiments we used a first group of 24 female mice (WT females, n=11; *Mecp2*-het females, n =13) purchased from The Jackson Laboratory (B6.129(C)-*Mecp2*$^{tm1.1Bird}$). Females from this group were tested at different ages for a longitudinal analysis (see Table 15) which included Von Frey test (VF), clasping test, and weight and gait analyses. At the age of 6 months, and one week after finishing the last test from the longitudinal analysis, animals were tested in the Hot Plate test. Twelve of these females (WT females, n=5; *Mecp2*-het females, n =7) were sacrificed 90' after the Hot Plate test to analyse neuronal activation patterns by means of immunohistochemical detection of the product of the early-immediate gene cFos.

An additional group of 12 female mice (WT females, n=6; *Mecp2*-het females, n=6) from our established colony (*Mecp2*-het females from Jackson Laboratory; stock #003890, B6.129P2(C)-*Mecp2*$^{tm1.1Bird/J}$ crossed with C57Bl/6J WT males) were only tested for the hot plate test (HP) at the age of 2 months, and sacrificed 90' after finishing the test for the analysis of cFos expression.

Table 14: Number of animals used for each experiment classified by their age of sacrifice and genotype, and age at which each experiment was performed. Abbreviations: *HP,* hot plate; *mo,* months old; *VF*, Von Frey.

Age of sacrifice	Total	Female	Experiment	Age for each experiment				
				2mo	3mo	4mo	5mo	6mo
2 months	12	WT: 6 *Mecp2*-het: 6	HP test, cFos	X				
6 months	24	WT: 11 *Mecp2*-het: 13	VF test	X	X	X	X	X
			Clasping test	X	X	X	X	X
			Weight analysis	X	X	X	X	X
			Gait analysis			X	X	X
			HP test (a subset for cFos)					X

Mice were housed in groups of 3–6 animals in standard laboratory cages with controlled humidity and temperature (22 °C), a 12:12-h light/dark cycle, and water

and food available ad libitum. All the procedures were carried out in strict accordance with the EU directive 2010/63/EU. The protocols were approved by the Ethics in Animal Experimentation Committee of the University of Valencia.

Genotyping and histology

Genotyping and histology procedures were carried out as described in Chapter 1.

Immunohistochemical techniques

cFos permanent immunostaining and Nissl staining

We obtained c-Fos permanent immunostained preparations in one out of the five parallel series obtained from 12 (WT females, n=5; *Mecp2*-het females, n =7) of the 24 animals sacrificed at the age of 6 months.

We followed the same procedure as described in chapter 1 for the AVP permanent immunodetection (see Table 16). We combined cFos immunodetection with Nissl staining to reveal the cytoarchitecture of the brain slices. After DAB, slices were (i) incubated for 12 seconds with a solution of 0.25% Cresyl Violet Acetate, to stain Nissl substance in the cytoplasm; (ii) washed briefly in tap water to remove any residual salts; (iii) dehydrated in ethanol; (iv) cleared with xylene and (v) cover-slipped with Entellan.

Table 16: Primary and secondary antibodies selected for cFos immunohistochemistry.

Experiment		Primary Antibody		Secondary Antibody	
cFos	DAB	Goat anti-cFos	1:1000 Santa Cruz Biotech. Inc sc-52-G	Biotinylated Horse anti-Goat IgG	1:250 Vector Labs BA9500

Immunohistochemical analysis and quantification

Analysis and quantification of cFos positive cells

For cFos quantification, an experimenter blind to genotype and sex of mice obtained photographs with the conventional light (Leica Leitz DMRB) at previously selected

bregma levels of different brain regions such as the AcbC, Pa, paraventricular thalamic nucleus (PV), Ce, and PAG (see Table 17).

To analyse the number of cFos-ir nuclei, we first split the pictures by RGB channels and selected the blue one to avoid Nissl-staining to interfere with the counting. Then, an experimenter blind to genotype manually counted the cFos-ir nuclei with the multipoint plugin of Image-J. For the Pa, we normalized the number of cFos-ir cells dividing the counting of cells per picture by the area of Pa in each picture (μm^2).

cFos-ir somata

Nucleus	Approximate mm to Bregma
AcbC	+1.10, +0.86
Pa	-0.70, -1.06
PV	-1.34, -1.58
Ce	-1.34, -1.46
PAG	-4.24, -4.48

Table 17: Nuclei and Bregma levels selected for cFos quantification in *Mecp2*-het and WT female mice. Abbreviations: *AcbC*, nucleus accumbens core; *Ce*, central amygdaloid nucleus; *Pa*, paraventricular hypothalamic nucleus; *PAG*, dorsolateral periaqueductal grey; *PV*, paraventricular thalamic nucleus.

Behavioural procedures

Clasping and weight analysis

Since severe obesity correlates with hyposensitivity in humans (Torensma et al., 2017), we measured weight monthly and behaviour displayed by the animals when they were suspended and held by the tail for a maximum of 15 seconds (from 2 to 6 months old). A WT mouse would separate and move both hind and fore paws trying to liberate when held by the tail. By contrast, symptomatic *Mecp2*-mutant mice will shrink their hind and/or fore paws (clasping), a specific and typical behaviour described in this mouse model as well as in other mouse models of neurological disease (Garriga-Canut et al., 2012; Guy et al., 2001; see Figure 24a).

Similar to what happens in patients suffering from Rett syndrome, onset of symptomatology does not happen at the same age in all of the *Mecp2*-mutant mice

(Guy et al., 2001). Thus, for pain perception experiments we also analysed their clasping behaviour to classify the *Mecp2*-mutant females as either symptomatic or pre-symptomatic. Study of clasping behaviour also allowed us to classify the animals as either early symptomatic (those who showed clasping by the age of 4 months, using 50% of the animals as cut-off) or late symptomatic females (onset of clasping starts after at the age of 5 months; see figure 24b) for upcoming analysis.

Von Frey test (VF)

Following the SUDO methodology described in (Bonin et al., 2014), we tested mechanosensitivity using 8 von Frey filaments (from filament 2 to 9; Aesthesio, San José, CA). Von Frey test was performed monthly between the ages of 2 to 6 months. Animals were placed in separated chambers suspended above a mesh grid. For habituation, animals were allowed to walk freely through the chamber for 30 minutes the day before the test, as well as for 5 minutes prior to the experiment. Von Frey test consisted of 5 consecutive trials separated by 2 minutes of break. In each trial, the filament was pressed against the plantar surface of the right hind paw of the animal until the filament buckled, and held for a maximum of 3 seconds. The mouse withdrawing the paw was considered a positive response, whilst negative responses were considered when the animal did not move. Flinching immediately upon removal of the filament was also noted as a positive response. For the first trial, we started with filament 5 and progressed according to the up-down method described in Bonin et al., 2014 (in case of a positive response, a thinner filament was selected for the subsequent trial, whereas negative responses lead to the selection of a thicker filament; see Figure 25b).

For the analysis, we considered the number of the filament used for the last trial, and added 0.5 in the cases in which reaction of the animal was negative, and subtracted 0.5 in the cases in which its reaction was considered as positive. Using the validated formula described in Bonin et al., 2014, we calculated the paw

withdrawal threshold (PWT) from the number of this last filament (+0.5/-0.5) converted to force and expressed in grams, following the equation:

$$PWT_{force} = 10^{(x*F+B)}$$

Where F corresponds to the filament number (+0.5/-0.5), and B is determined from the next equation:

$$Log(bending\ force) = x * Filament\ number + B$$

For the mouse filament set, x=0.240 and B=-2.00.

Hot Plate test (HP)

Both groups of animals were tested for thermosensitivity in a hot plate test 90 minutes before sacrifice. For this experiment, mice were placed over a hot plate (set at 52ºC) for 30 seconds (Fig. 26a), and their behaviour recorded. We analysed the latency of the animals to lick their paws, the standard measure in this test. In addition, we noticed that most of the *Mecp2*-het performed an odd walking backwards movement when placed in the HP, a movement never seen in the WT females (see Fig. 26a'). We interpret that, since most of the animals were significantly motor impaired at this age, according to clasping and gait analysis data, this walking backwards represents an avoidance movement when trying to withdraw the paws. Thus, for each animal we recorded both latency to lick paws and latency to walk backwards, and analysed them separately as well as a single measure as latency to first avoidance movement (either lick or walk).

Paw print test

To determine possible motor impairments, we analysed the normal gait of WT and *Mecp2*-mutant females once a month from 4 to 6 months of age. To do so, we applied non-toxic washable tempera to the plantar surface of the hind paws (black paint) and fore paws (red paint). Then, animals were allowed to run through a delimited path over a filter paper (see Fig. 27a).

For the analysis, we took different measures from 4 consecutive steps of each animal: the distance (mm) between the two hindlimbs, distance between the two forelimbs, and distance between same side forelimbs as well as for the same side hindlimbs (see Fig. 29a; similar to what described in Vogel Ciernia et al., 2018).

Statistical analysis

For the immunohistochemical analysis and hot plate test, data were analysed using the software IBM SPSS Statistics 22.0. We first checked the data for normality (Shapiro–Wilk test) and homoscedasticity (Levene's test). Next, we evaluated the differences between genotypes using Student's t test or Mann–Whitney U test when appropriate in the case of cFos and latency to first avoidance. For the latency to lick paws and walk backwards, we used Kaplan-Meier analysis since not all the females showed these two behaviours. Weight, and results from Von Frey test and paw print tests were analysed with an ANOVA for repeated measures, considering genotype as inter-subject factor, and the different measures as intra-subject factor. Significance was set at $p < 0.05$.

RESULTS

Mecp2-het females suffer from more severe overweight and display clasping behaviour as the symptomatology of the syndrome progresses

We measured clasping behaviour monthly and weight of *Mecp2*-het and their wild type siblings females from 2 to 6 months of age.

We could appreciate that, by the age of 2 months, less than 10% of the *Mecp2*-mutant animals displayed clasping. This percentage of symptomatic animals progressively increased with the age, with about a 50% of the animals showing clasping at the age of 4 months. At 6 months of age, almost all *Mecp2*-het females could be considered as symptomatic. As expected, none of the WT animals showed clasping behaviour (see Fig. 24b). Thus, we classified *Mecp2* females as early symptomatic (n = 7) or late (n = 6).

We performed an ANOVA for repeated measures with weight at different ages/measurements as intra-subject variable and genotype as inter-subject variable. As expected, data showed an effect between age and increase of weight in both genotypes (WT, $F_{(1,19)}$=6.148, p=0.002; *Mecp2*-het, $F_{(1,19)}$=43.275, p<0.001; Fig. 24c). Furthermore, there is also an effect of genotype, with *Mecp2*-het females being heavier than WT females ($F_{(1,22)}$=24.648, p<0.001; Fig. 24c). Moreover, this increase of weight remains significant at every age of measurement (2m.o., $F_{(1,22)}$=13.712, p=0.001; 3m.o., $F_{(1,22)}$=17.291, p<0.001; 4m.o., $F_{(1,22)}$=26.578, p<0.001; 5m.o., $F_{(1,22)}$=25.604, p<0.001; 6m.o., $F_{(1,22)}$=27.816, p<0.001).

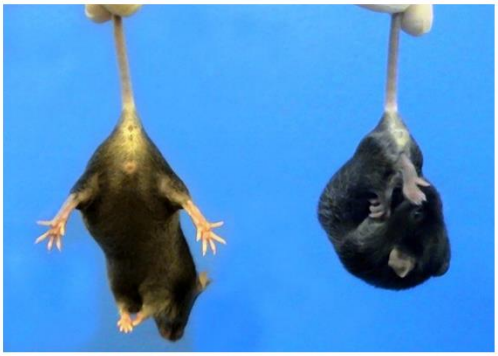
We also separately compared the weight of early and late symptomatic females with the group of WT females. Results revealed a significant increase in weight that was present in both early ($F_{(1,16)}$=68.746, p<0.001; Fig. 24c) and late ($F_{(1,15)}$=9.925, p=0.007; Fig. 24c) symptomatic *Mecp2*-het females compared to the WT. Similar to the previous analysis, significance was present at each point of age.

In order to compare early vs. late symptomatic females, we calculated the fold change for both groups, dividing each measure by the mean of WT at each point of age. The ANOVA revealed a significant increase in the weight of early symptomatic females compared to the late symptomatic ones at the age of 2 to 4 months old (2m.o., $F_{(1,11)}$=5.866, p=0.034; 3m.o., $F_{(1,11)}$=9.032, p=0.092; 4m.o., $F_{(1,11)}$=6.404, p=0.028) but not at the age of 5 or 6 months. These results suggest that excessive gain of weight in our *Mecp2*-het females is correlated with the progression and, therefore, severity of the symptomatology. Although both, early and late symptomatic mice present higher weight compared to the WT, early symptomatic females suffer from more overweight at earlier stages, since they start manifesting the syndrome at this age. On the other side, severe overweight in late symptomatic mice starts by the age of 5 to 6 months (matching with the onset of symptomatology) and reaching the weight of their mutant siblings.

We also measured the weight in the group of females sacrificed at the age of 2 months, to compare the results obtained between genotypes within this group and the group of 6 months old females. Student's t test revealed no significant differences between genotypes in the weight of these females (t=-0.257, p=0.82). The lack of difference in this group might be due to the different rearing conditions in the animal facility.

Thus, weight of *Mecp2*-het females is significantly higher compared to the WT after the age of 2 months and onward. Notably, the weight of *Mecp2*-het females correlates with severity of symptomatology of the syndrome, suffering from more severe overweight and obesity as the disease progresses.

A



B

Code	2 mo	3 mo	4 mo	5 mo	6 mo
5024					
5025					X
5026				X	X
5027			X	X	X
5028			X	X	X
5029			X	X	X
5030					X
5031	X	X	X	X	X
5032				X	X
5033		X	X	X	X
5034			X	X	X
5036				X	X
5037		X	X	X	X
% of symptomatic females	7.7%	23.1%	53.8%	76.9%	92.3%

C

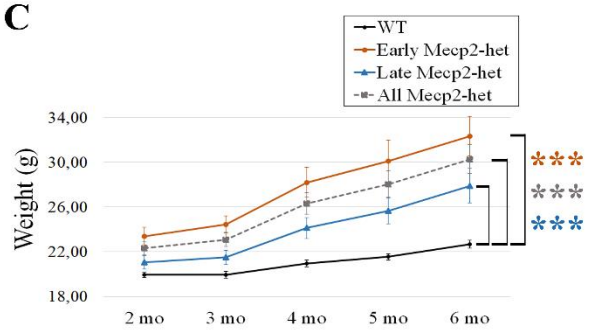


Figure 24: clasping and weight analysis in WT and *Mecp2*-het females. a) WT mouse (left) and a symptomatic *Mecp2*-mutant mouse (right) when hold by the tail. While a WT mouse would separate and move both hind and fore paws trying to liberate, symptomatic *Mecp2*-mutant mice will shrink their hind and/or fore paws (clasping). Picture courtesy of Carmen Agustín Pavón. **b)** Table summarizing the onset and percentage of *Mecp2*-het females, subdivided into early symptomatic (orange) and late (blue), showing clasping across experimental timepoints. **c)** Measures of weight in WT (black) and all *Mecp2*-het females together (grey) and subdivided into early symptomatic (orange) and late (blue) across experimental timepoints. *Mecp2*-het females are significantly heavier at every age compared to the WT females. Likewise, both early and late symptomatic *Mecp2*-het females were significantly heavier at every measurement compared to the WT females. Values are presented as mean±SEM. ***p<0.001

Early symptomatic *Mecp2*-heterozygous females are hyposensitive, while late symptomatic females are hypersensitive in the Von Frey test

For the Von Frey test, we analysed the data with an ANOVA of repeated measures. We first compared the WT females with the whole group of *Mecp2*-het females. As

a result, we found an effect of repeated measures in the VF that was only present in *Mecp2*-het females ($F_{1,19}$=4.866, p=0.007; Fig. 25c), meaning that WT females showed similar responses to VF test at each stage, whilst *Mecp2*-het females varied their response to the VF test depending on the stage and, therefore, with severity of the symptoms. However, when considering the whole group of mutant females, we saw no significant effect of genotype in any of the VF tests.

Next, we decided to compare the group of WT females with the early and late symptomatic heterozygous females separately. In the case of early *Mecp2*-het females, the ANOVA still revealed this effect of VF ($F_{1,13}$=3.011, p=0.058, Fig. 25c), meaning that VF results vary throughout the consecutive expositions, but there is no significant effect of genotype. However, we observed a slight difference in the last VF test (6 m.o.), in which early symptomatic *Mecp2*-het females showed a trend of hyposensitivity (more pressure needs to be applied to trigger the paw withdrawal) compared to WT ($F_{1,16}$=3.575, p=0.077). Conversely, we could see a significant hypersensitivity of late symptomatic *Mecp2*-het females compared to the WT, but only in the second ($F_{1,15}$=6.320, p=0.024) and third ($F_{1,15}$=,11.484 p=0.004) VF test (age of 3 and 4 months, respectively, see Fig. 25c).

Finally, we calculated the fold change of both groups of mutant females, normalizing the data with respect the mean from the WT group for each test, and compared data from the early to the late symptomatic females. In this case, a significant difference could be appreciated in the VF2 and 3 (same ones as when comparing the late symptomatic to the WT). In both cases, late symptomatic females showed a significant hypersensitivity (VF2, $F_{1,11}$=16.751, p=0.002; VF3, $F_{1,11}$=5.299, p=0.042) compared to the early symptomatic ones.

In general, the response of *Mecp2*-het females to the VF test significatively differs from the WT. For the first VF (at the age of 2 months when almost none of the animals manifest clasping behaviour), both early and late symptomatic females start

at the same point in the graph and display hypersensitivity compared to the WT females. For the subsequent VF tests, the responses of both groups differs among themselves, as well as with the group of WT females. On the one hand, early symptomatic females get progressively close to the WT response in the graph, although remaining slightly hypersensitive with regard to the WT. On the other hand, late symptomatic females remain significantly hypersensitive compared to both early symptomatic and WT females. By the age of 5 months, when almost 77% of the animals showed clasping, this situation in *Mecp2*-het mice started to revert and early symptomatic females started showing hyposensitivity compared to WT (although it is not significant), whilst late symptomatic females remained hypersensitive but get closer to the WT in the graph. From this point on, early symptomatic females got more hyposensitive and would continue to distance themselves from the WT. Similarly, at the age of 6 months, late symptomatic females started showing hyposensitivity compared to WT, following the trend of the early symptomatic mice.

Thus, our results suggest that the more severe the symptomatology of this syndrome is, the more hyposensitive the animals seem to be. However, it is likely that overweight and motor impairments developed by the progression of the syndrome could be interfering with the results. This hypothesis is supported by the fact that pre-symptomatic females show a clear hypersensitivity compared to WT, which remains significant in the case of females with later onset of symptomatology (late symptomatic *Mecp2*-het females).

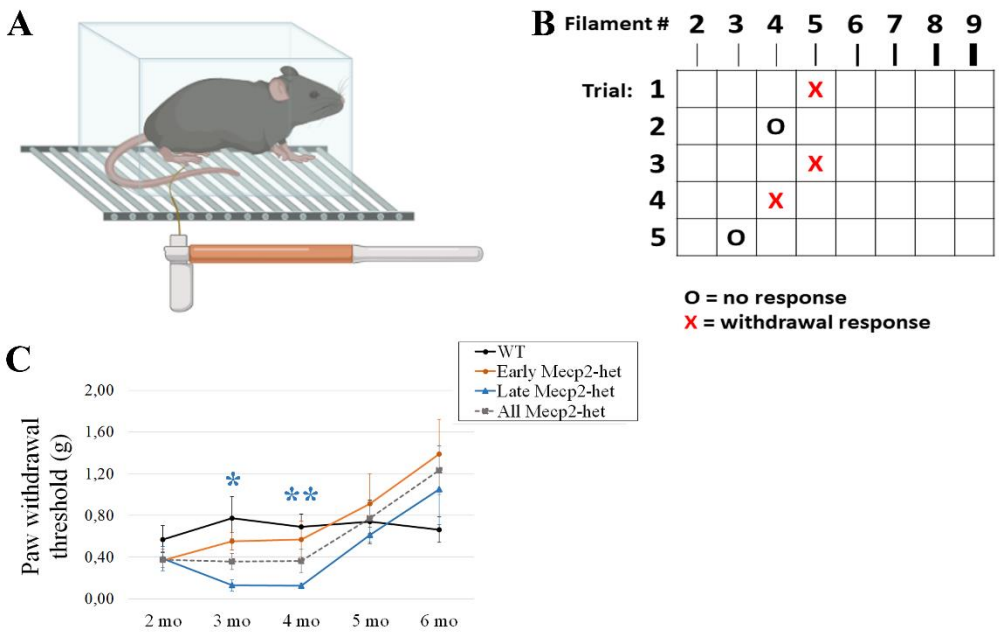


Figure 25: Von Frey analysis in our WT and *Mecp2*-het female mice. a) Illustration of VF test in which each animal was placed in a separated chamber suspended above a grid. The filament was then pressed against the plantar surface of the right hind paw until the filament buckled, and held for a maximum of 3 seconds. Picture created with Biorender.com **b)** Representation of the up-down Von Frey paradigms described by SUDO method a simplification of the VF paradigms described in (Chaplan et al., 1994). The mouse withdrawing the paw was considered a positive response (X), whilst negative responses were considered when the animal did not move (O). Flinching immediately upon removal of the filament was also considered as a positive response. We started with filament 5 and progressed according an up-down method (for positive responses a minor filament was selected for the subsequent trial, negative responses lead to the selection of a higher filament. **c)** Paw withdrawal response (PWD) of WT (black) and all *Mecp2*-het females together (grey) and separated into early symptomatic (orange) and late (blue) in the VF test across the consecutive measures. No significant differences between genotypes were found when comparing the whole group of *Mecp2*-het females with the WT. However, at the age of 3 and 4 months, late symptomatic *Mecp2*-het females were significantly hypersensitive compared to the WT females. We also appreciate a significant difference in the PWT between early and late symptomatic females at the age of 3 and 4 months old, being the late symptomatic females more hypersensitive in the VF test. Values are presented as mean±SEM. *p<0.05

Mecp2-heterozygous symptomatic females show hypersensitivity in the hot plate

We studied the groups of *Mecp2*-het and WT females of 2 and 6 months of age separately, since the tests were performed in different moment. First, we analysed the latency of to lick the paws. However, due to motor impairments caused by the progression of the disease, half of *Mecp2*-het females did not withdraw the paws, but performed and odd walking backwards movement when placed in the HP (see video in Fig. 26a'). Thus, we also measured the latency to first avoidance for each animal, considering either licking the paws or walking backwards.

For the group of females of 6 months old, we first compared the WT and the whole group of *Mecp2*-het females. We analysed the latency to lick paws and walk backwards by means of a Kaplan–Meier log-rank test, since some of the animals did not display these behaviours, and therefore, we had some censored values. We observed that, although all of the WT females licked their paws in the HP, only 71.4% of *Mecp2*-het females showed this behaviour. Conversely, 64.6% of *Mecp2*-het females displayed a walking backwards, whilst none of the WT females did. The Kaplan-Meier analysis revealed no significant differences between genotypes in the latency to lick paws ($\chi^2(1) = 0{,}097$, $p = 0.756$; Fig. 26b), but revealed a significant effect of genotype in walking backwards ($\chi^2(1) = 10{,}414$, $p = 0.001$; Fig. 26b'). For the latency to first avoidance, Student's t test revealed a significant reduction in the *Mecp2*-het females compared to the WT ($t=4.101$, $p<0.001$; Fig. 26b''), suggesting a hypersensitivity of *Mecp2*-het females in the HP. This hypersensitivity was also present when comparing the early and late symptomatic females with their WT controls, since we found a significant decrease in the latency to first avoidance in both early (Student's t test, $t=3.805$, $p=0.002$) and late (Student's t test, $t=3.389$, $p=0.004$) *Mecp2*-het females (data not shown).

In the group of 2 months old females, all WT and *Mecp2*-het females showed the licking paw behaviour, and 50% of the *Mecp2*-het females and none of the WT

showed the walking backwards. However, the Kaplan-meier analysis revealed no significant differences between genotypes in the latency to lick paws ($\chi^2(1) = 0{,}669$, $p = 0.414$; Fig. 26c), and a trend in walking backwards ($\chi^2(1) = 3.669$, $p = 0.055$; Fig. 26c'). Additionally, in the case of latency to first avoidance, Student's t test revealed no significant differences between genotypes ($t = 1.438$, $p = 0.181$; Fig. 26c'').

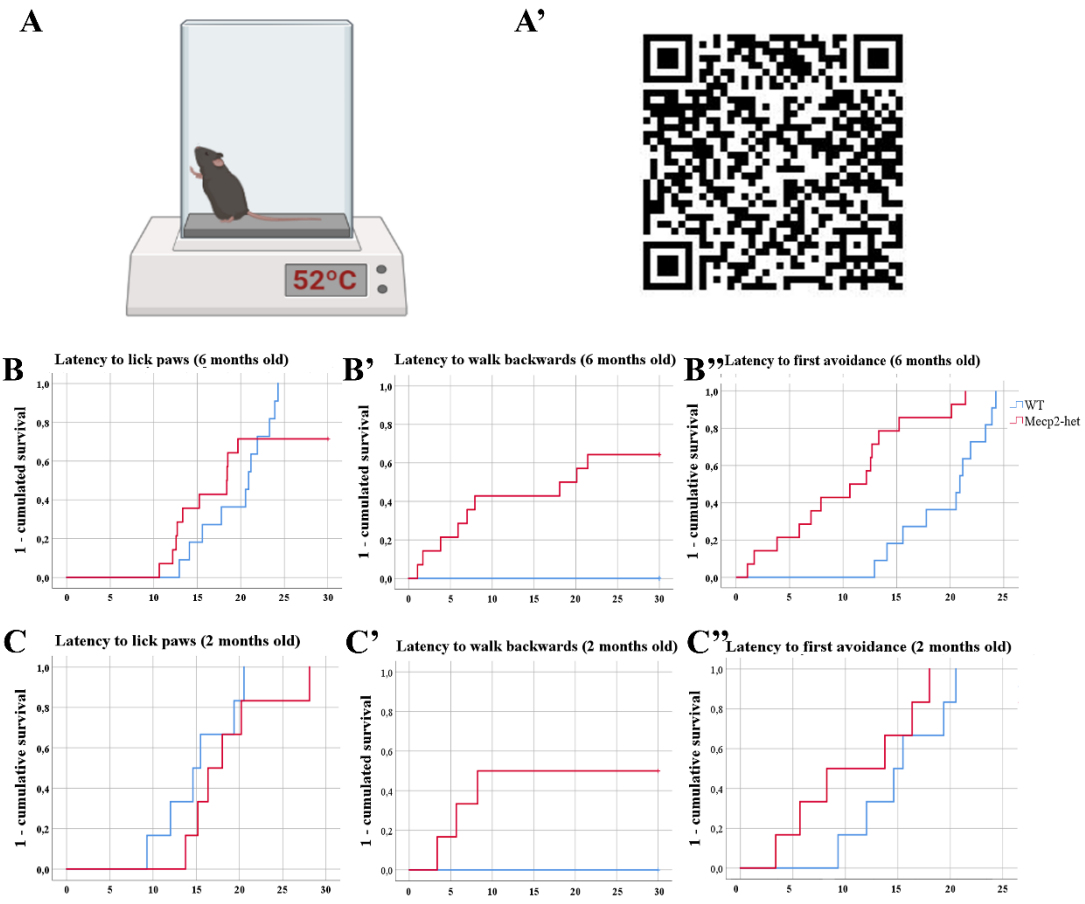


Figure 26: Hot plate test analysis in WT and *Mecp2*-het females. a) Illustration of Hot Plate test in which mice were placed over a hot plate (set at 52°C) for 30 seconds. Image created with Biorender.com **a')** Representative video illustrating the walking backwards displayed by half of *Mecp2*-het females, possibly due to their motor impairments. **b)** Latency to lick paws, **b')** walk backwards and to **b'')** first avoidance in the group of WT (blue) and *Mecp2*-het (red) females of 6 months of age. **c)** Latency to lick paws, **c')** walk backwards and to **c'')** first avoidance in the group of WT (blue) and *Mecp2*-het (red) females of 2 months of age. Although licking paws was present in all females from the group of 2 months old, not all the *Mecp2*-het females of 6 months old showed this behaviour (around 70%). By contrast, we observed that the walking backwards was present in more than 60% of *Mecp2*-het females

of 6 months old, as well as in half of the 3 months old *Mecp2*-het females, but none of the WT females showed this response at any age. In addition, latency to walking backwards in *Mecp2*-het females was lower compared to the latency to lick paws, suggesting that at least half of the *Mecp2*-het females displayed this walking backwards as a predominant response to heat stimulus. Only the group of 6 months old, the latency to first avoidance was significantly lower in the *Mecp2*-het females compared to WT, suggesting that symptomatic *Mecp2*-het females are hypersensitive in the hot plate test.

In general, our results show that symptomatic (either early or late symptomatic according to the onset of symptomatology) *Mecp2*-het females present lower latency to first avoidance in the HP test. Thus, *Mecp2*-het females show hypersensitivity in the HP test compared to the WT. However, due to the motor demands of the test and the motor impairments described for these animals, it is important to consider not only the latency to lick, but also other avoidance behaviours, like the odd walking backwards displayed by the heterozygous females.

Gait analysis shows that hind base print is increased in *Mecp2*-heterozygous females compared to the wild type

To determine possible motor impairments, we analysed the normal gait of WT and *Mecp2*-mutant females once a month from 4 to 6 months of age. To do so, we took different measures from 4 consecutive steps of each animal: the distance (mm) between the two hindlimbs, distance between the two forelimbs, and distance between same side forelimbs as well as for the same side hindlimbs (see Fig. 27a). For the analysis, we combined distance between same side forelimbs (right and left-limb strides) as a unique measure (forelimb stride), and did the same for the same side hindlimb (hindlimb stride).

The ANOVA revealed no significant differences in the case of hindlimb stride, but showed a significant effect of genotype in the case of the forelimb stride only in the last measure (at the age of 6 months; $F_{(1,22)}=11.345$, $p=0.003$; Fig. 27b) with a decreased length of stride in the case of *Mecp2*-het females compared to the WT controls. This decrease at the age of 6 months, was also maintained when comparing

separately the group early ($F_{(1,16)}$=10.189, p=0.006; Fig. 27b) and late ($F_{(1,15)}$=4.741, p=0.046; Fig. 27b) symptomatic females with the WT. Thus, discrepancies in the forelimb stride seems to start at the age of 6 months with the progression of the severity of the symptomatology.

We also noticed a significant increase in the length of the hindlimb base of *Mecp2*-het females compared to the WT ($F_{(1,22)}$=8.791, p=0.007; Fig. 27c), present only at the age of 4 ($F_{(1,22)}$=5.940, p=0.023) and 5 ($F_{(1,22)}$=6.130, p=0.021) months, but not at the age of 6. Conversely to what we saw with the forelimb stride, differences in the hindlimb base were only maintained when comparing the WT females to the early symptomatic females ($F_{(1,16)}$=16.382, p=0.001; Fig. 27c), but not to the late symptomatic ones. In the case of forelimb base, the ANOVA showed no effect of genotype.

Overall, our data suggest that some motor impairments could be interfering in the normal walking of our *Mecp2*-het females. Moreover, severity of these motor impairments becomes more evident with the progression of the disease and, thus, the symptomatology. Differences found in the length of steps could indeed interfere in the behaviour displayed by *Mecp2*-het females in the HP and VF tests, creating a confounding factor. Therefore, motor demands need to be considered when choosing behavioural tests for *Mecp2*-mutant mice.

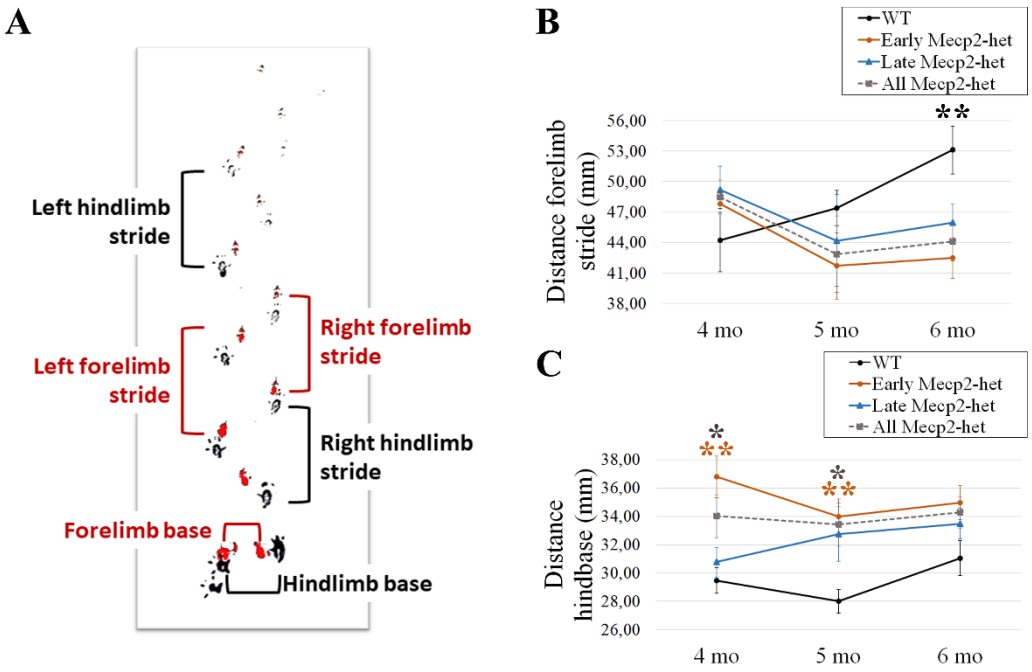


Figure 27: Paw print test in WT and *Mecp2*-het females by the ages of 4 to 6 months old. **a)** For the paw print test, mice were applied with non-toxic washable tempera at the plantar surface of the hind paws (black) and fore paws (red), and allowed to run through a delimited way over a filter paper. For the analysis, we took different measures from 4 consecutive steps of each animal: the distance (mm) between the two hindlimbs, distance between the two forelimbs, and distance between same side forelimbs as well as for the same side hindlimbs. **b)** Distance forelimb stride (includes measures from right and left) in our WT (black) and *Mecp2*-het females (grey) by the age of 4 to 6 months old. *Mecp2*-het females showed a significant shorter distance compared to the WT at the age of 6 months. The group of *Mecp2*-het females was then separated in early (orange) and late (blue) symptomatic, and compared to the WT females (black). Both early and late symptomatic females showed shorter distance in the forelimb stride compared to the WT females. **c)** The distance of hindbase was significantly higher in the case of *Mecp2*-het females compared to the WT females by the age of 4 and 5 months; and in early symptomatic *Mecp2*-het females of this same age, but not in the late symptomatic ones, compared to the WT females. Values are presented as mean±SEM. *p<0.05; **p<0.01

cFos levels are significantly reduced in the PAG of symptomatic *Mecp2*-het females

Both groups of animals (2 and 6 months old) were sacrificed 90 minutes after the HP test to examine neuronal activation patterns with an immunohistochemical detection of cFos. In the case of 6 months old mice, we observed a significant

decrease of cFos-ir cells in the PAG of *Mecp2*-het females compared to the WT females (Mann-Whitney U test, p=0.004; Fig. 28a). This difference was maintained when separately analysing the groups of early (Mann-Whitney U test, p=0.04) and late (Mann-Whitney U test, p=0.04) *Mecp2*-het symptomatic females. Conversely, we found no significant differences between genotypes in the AcbC, Pa, PV or Ce (Fig. 28a, a'), nor in any of the regions analysed in the group of 2 months old (all p>0.05, Fig. 28 b, b').

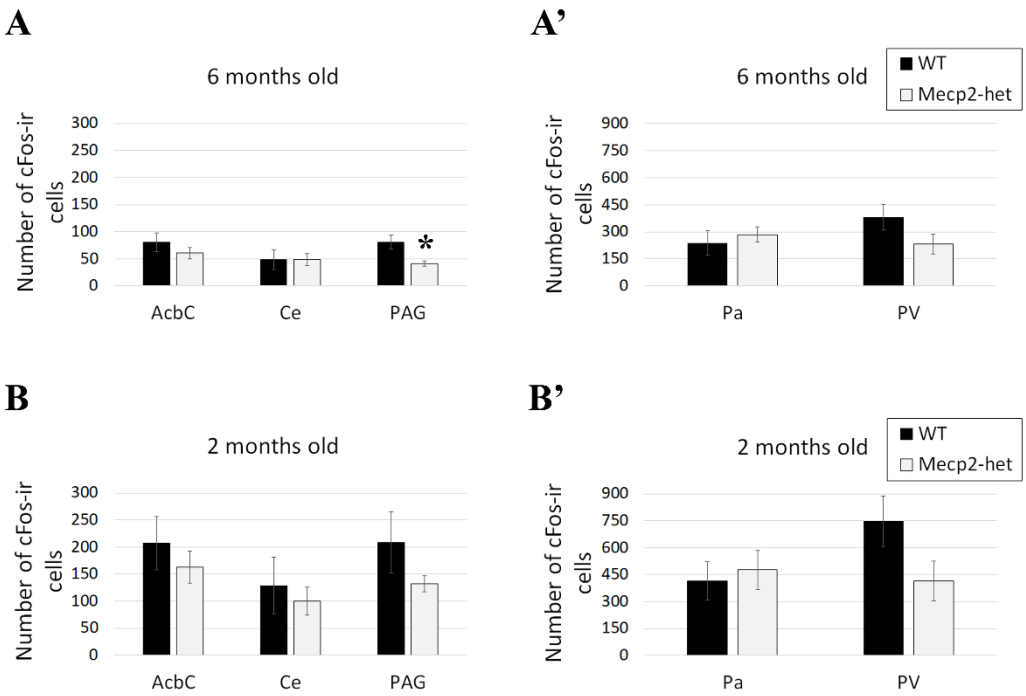


Figure 28: Number of cFos positive cells in the WT (black bars) and *Mecp2*-het females (grey bars) of a, a') 2 and b, b') 6 months old. Only in the group of old females (6 months of age), we found a significant decreased of cFos-ir cells in the PAG of *Mecp2*-het females compared to the WT. No significant differences were found in any of the other regions analysed, nor in the group of young females (2 months of age). Values are presented as mean±SEM. *p<0.05

DISCUSSION

In this chapter, we explored weight and pain sensitivity in *Mecp2*-heterozygous females and compared it to their wild type siblings. Given that onset of symptomatology does not happen at the same age in all mutant females, we monthly analysed clasping behaviour to classify them into symptomatic (which display clasping behaviour, shrinking their hind and/or fore paws when held by the tail) and pre-symptomatic females (which separate and move their hind and fore paws trying to liberate). We saw that, in terms of clasping behaviour, almost all mutant females remained in a pre-symptomatic state at the age of 2 months (did not show clasping behaviour). Percentage of symptomatic females progressively increased, with almost all heterozygous females displaying clasping behaviour by the age of 6 months. Analysing clasping behaviour also allowed us to subdivide the group of *Mecp2*-het females into early symptomatic (have already showed clasping by the age of 4 months, when almost 50% of the mutant females are symptomatic) or late symptomatic (display clasping after the age of 4 months old) females. We also evaluated weight of the animals. This measure seems to correlate with severity of symptomatology, with *Mecp2*-mutant females also suffering from more severe overweight and obesity compared to the WT animals as the syndrome progresses. Furthermore, pre-symptomatic *Mecp2*-het females display increased mechanical and thermal sensitivity in the Von Frey and Hot plate tests. However, it is important to consider that motor symptoms, suggested by abnormal gait observed in the paw print test, together with overweight, could be interfering in results obtained from the analysis of pain sensitivity in symptomatic *Mecp2*-het females. Finally, cFos activation levels were decreased in the PAG of *Mecp2*-het females, a main region involved in pain perception.

Overweight and clasping correlate with severity of symptomatology in *Mecp2*-heterozygous female mice

Since there are some pieces of evidence that correlate severe obesity with hyposensitivity in humans (Torensma et al., 2017), we decided to analyse body weight in our females. Adipose tissue secretes proinflammatory polypeptides that may affect multiple organ systems, leading to the hyposensitivity described. A possible connexion between different mutations in gene *Mecp2* and different levels of severity of symptomatology has also been proposed.

The mouse model we employed for our experiments was first designed by Adrian Bird and Jacky Guy in 2001 (Guy et al., 2001). *Mecp2*-null males from this strain suffer from severe neurological symptoms by the age of 6 weeks, while *Mecp2*-het females do not show overt behavioural symptoms until several months of age. Furthermore, Guy and Adrian described a correlation between exhibition of clasping behaviour and progression of the syndrome with age. On this basis, severity of the symptomatology in *Mecp2*-null males worsens by the age of 7-weeks (lifespan of *Mecp2*-null males is about 8 weeks of age), when these mice start to manifest clasping behaviour (Guy et al., 2001). Thus, we decided to use this behaviour to determine onset of severe symptomatology for each animal, and to classify them into pre-symptomatic and symptomatic.

Symptomatology in this mouse model includes motor impairment and differences in body weight. Guy et al. also specified that body weight of those animals was variable depending on the genetic background. While *Mecp2*-null males with C57BL/6 background usually suffered from underweight by the age of 4 weeks, F1 animals obtained after crossing with a 129 strain showed the opposite effect (Guy et al., 2001). In this second strain, *Mecp2*-null males exhibited the same weight as their wild type siblings by the age of 8 weeks. Moreover, those who survived this point of age, also become heavier than WT males, showing an obvious increase in deposited fat (Guy et al., 2001). Discrepancies between the two strains could be

explained by the presence of one or more modifier genes mediating the effects of MeCP2 on body weight, as Guy et al speculated.

However, weight of *Mecp2*-het females was not analysed or described in their publication. As with *Mecp2*-null males with 129-strain background, *Mecp2*-het female mice significatively exceed the weight of WT females. On this basis, as the syndrome progresses and the symptomatology exacerbates, *Mecp2*-het females significantly increased their weight above normal, suffering from severe overweight. Thus, lack of MeCP2 may directly or indirectly interfere with weight, promoting overweight and obesity in those mice. This is consistent with metabolic disturbances observed in patients of RTT, and with the suggested role of MeCP2 in co-ordinating liver lipid metabolism with the NCoR1/HDAC3 corepressor complex (Kyle et al., 2016). Furthermore, restoration of lipid metabolism through administration of statin drugs alleviates motor symptoms in *Mepc2*-mutant mice and increases their lifespan (Buchovecky et al., 2013), suggesting a possible correlation between progression of the syndrome, overweight, and severity of symptomatology.

Mechanical sensitivity in the Von Frey test seems to be dependent on severity of symptomatology in *Mecp2*-het females

Different behavioural methods have been developed to quantify "pain-like" behaviours or nociception. These methods can be subdivided into: stimulus-evoked (based on evoking withdrawal behaviours, including modalities like mechanical, heat or cold) and non-stimulus evoked methods (grimace scales, burrowing, weight bearing and gait analysis; Deuis et al., 2017). The manual Von Frey test, developed by the physiologist Maximilian von Frey, is a stimulus-evoked method commonly used to determine mechanical thresholds of nociception in rodents (reviewed in Deuis et al., 2017).

Although a majority of the literature suggests that nociception is decreased in RTT patients, we cannot discard the possibility of an undetected hyperalgesia. As an

example, reduced pain sensitivity has been considered as a typical feature of autism [American Psychiatric Association 1987; American Psychiatric Association 2000]. However, increased heart rate has been recorded in children and adolescents with autism during venepuncture (Tordjman et al., 2009). Since heart variability has been related to self-reported pain experiences, heart rate has commonly been used to quantify stress or pain in humans and mouse models. In this particular case, although parental report suggested decreased reactions during venepuncture, as well as to other accidental noxious stimuli, increased heart rate suggests that pain sensitivity in autism may not be decreased, but subject to different expression. Additionally, two other small studies have also not provided support for the concept that pain insensitivity is a feature associated with either mild (Bromley et al., 1998) or more severe (LaChapelle et al., 1999) intellectual disability. Thus, we cannot discard that the same situation could be happening in RTT. In fact, some recent reports evidence nonverbal pain expression in RTT (Barney et al., 2015; Symons et al., 2013).

However, in the specific situation of RTT patients, heart rate cannot be carelessly used as an accurate measure of nociception. This is due to the decreased variability in heart rate manifested by these girls, which seems to correlate with age and clinical severity of the syndrome (Acampa & Guideri, 2006; Guideri et al., 2001). This is consistent with differences between the groups of early (manifesting a more severe symptomatology) and late symptomatic female mice. When we compared differences in the VF test between the whole group of *Mecp2*-het and the WT females, we saw no significant differences. This lack of significance may suggest that mechanical sensitivity does not seem to be altered in *Mecp2*-het females, at least in the range of age of 2 to 6 months. However, when subdividing the group of mutant females in early and late symptomatic, we could appreciate how severity of the syndrome affects mechanical sensitivity in those mice. As a result, we could observe a slight trend towards hyposensitivity in early symptomatic *Mecp2*-het females

compared to WT that was present only by the age of 6 months. This trend may suggest that, with the progression of the syndrome and the increase of severity of symptomatology, *Mecp2*-het females may display hyposensitivity in the VF test. However, we cannot discard a possible effect of weight interfering in the paw withdrawal, since early symptomatic females are significatively heavier than late symptomatic females by the age of 2 to 4 months.

Surprisingly, and conversely to what is described in literature (Downs et al., 2010; Samaco et al., 2008), we found that late symptomatic females were hypersensitive to VF compared to WT, but only by the age of 3 to 4 months. In addition, by this age, we found a significant hyposensitivity in early symptomatic females when comparing to the late symptomatic ones. By the age of 3/4 months, late symptomatic females have not manifested clasping behaviour yet; suggesting that onset of syndrome has not yet occured. This is something important to consider, since the majority of behavioural tests analysing nociception are performed with already-diagnosed patients. Diagnosis of RTT is mainly based on symptomatology. Thus, analysis of nociception does not encompass possible alterations in pain perception occurring before the onset of symptomatology and/or prior to the clinical diagnosis. We also must consider that, since the methodology we employed for our experiments does not differ from literature, differences in background mutations between different strains of mice could alter the results obtained.

Based on this progression of symptoms, and considering that the VF test forces the need to elicit paw withdrawal in mice, we cannot discard an effect of motor disability interfering in our results. In addition, weight of the animal may hinder the paw withdrawal in early symptomatic mutant females. Additionally, we must consider a possible effect of delayed response in the VF by heterozygous mice. Application of stimuli in the VF test only lasts 3 seconds. In addition, results from this test are only measured as positive or negative responses in terms of paw-withdrawal, and it does not analyse the latency to the avoidance. Considering this, we cannot discard that

longer duration of application could be more suitable for RTT models. This possibility was first suggested by Merbler et al, when analysing different exposition to noxious stimuli in patients with RTT. They only found significant evidence for the VF test, and not for other noxious stimuli with lower exposition. They suggested that these discrepancies could be due to differences in duration of exposure, since VF stimuli in clinical application with humans have longer application time than other stimuli (Merbler et al., 2020).

Overall, our data suggest that hypersensitivity is present in pre-symptomatic states of the syndrome. Moreover, the more severe the symptomatology of this syndrome is, the more hyposensitive the animals seem to be. However, these results should be interpreted with caution, since some other variables (such as overweight, motor impairment or delayed responses) could be hiding a possible non-manifested hyperalgesia in RTT patients.

Symptomatic *Mecp2*-het females are hypersensitive to the hot plate test, displaying an unusual expression of pain compared to WT animals

The exposure of peripheral sensory nerve endings to elevated temperatures can evoke sensations of warmth, heat, or even pain. Typically, the sensation of warmth is elicited at temperatures of 34–37◦C, while the sensation of pain is elicited at temperatures of 42–48◦C (Defrin et al., 2006; Pertovaara et al., 1996; Rolke et al., 2006). On this basis, the hot plate test constitutes another stimulus-evoked measure for nociception, typically used with rodents.

In standard conditions, the hot plate test allows analysis of the latency displayed by the animal to lick its paws. However, we observed that half of *Mecp2*-het females do not withdraw or lick their paws, but performed and odd walking backwards movement when placed in the HP. This is something that the group of Samaco also observed (Samaco et al., 2008). They described that their *Mecp2*-mutant females did not lick their paws, but displayed paw withdrawal instead. However, they did not

find hyperalgesia, but hypoalgesia. As discussed for the VF test, we cannot discard an effect of overweight and/or motor impairments interfering in this latency. Thus, we decided to analyse the latency to first avoidance, considering either licking the paws or walking backwards.

Although we found no significant differences between genotypes in the group of 2 months old, we obtained some interesting conclusions from the group of 6 months of age. We saw a significant reduction in the latency to first avoidance and to lick paws in the case of *Mecp2*-het females compared to WT. Those results suggest a hypersensitivity caused by mutations in *Mecp2*, which is consistent with results obtained with 8 months old MeCP2-308 male mice (Vigli et al., 2021), another mouse model for RTT. This hypersensitivity was also present when separately comparing the group of early and late symptomatic females with the WT group. These results are inconsistent with experiments from the group of Samaco et al, showing that female *Mecp2*(+/-) mice on two different genetic backgrounds display robust hyposensitivity in the HP (Samaco et al., 2013). Previous reports from this group working with males, showed a dissociation between the tail flick and hot plate results in their *Mecp2*-mutant males compared to the WT (Samaco et al., 2008). Thus, Samaco et al suggested a possible impairment in primary deficit in pain recognition rather than in the peripheral sensation of pain. It is important to consider that, since these last experiments were made with males instead of females, possible differences between sexes could also be interfering.

Results obtained in the HP test support our hypothesis of a masked hypersensitivity in *Mecp2*-null males. In fact, when analysing only the latency to lick paws in the group of late and early symptomatic females, we saw no significant differences compared to WT. Thus, hypersensitivity was only present when considering the odd walking displayed by these mutant females. In accordance with that, although in Samaco et al. 2013 it is not described whether they observed this odd walking on their mutant females, they observed that their mutant males only displayed

hindlimb paw withdrawal in the HP, and never licked their paws (Samaco et al., 2008), favouring the hypothesis of a different behavioural manifestation of pain-like responses, instead of a real reduced nociception.

Gait analysis of *Mecp2*-het females is slightly affected after onset of symptomatology and could be affected by their weight

Gait abnormalities, together with motor dysfunction, correspond to a common criterion for diagnosis in Rett syndrome (Neul et al., 2010). Gait impairment has been described in 99% of patients with classical RTT, which is also present in at least 57% of patients suffering from syndromic or non-syndromic intellectual disability (reviewed in Kaur & Christodoulou, 2019). In some other alterations, like Angelman syndrome, gait ataxia is first noted by the age of 6 months in humans. However, in the case of RTT, it is not overtly evident in the first six months of age. This delay corresponds to the fact that most RTT patients only develop locomotor disruption after the regression phase of the syndrome. Thus, gait abnormalities seem to not be manifested during the pre-symptomatic stages of the syndrome in humans.

Since many of the features of RTT have successfully been modelled in *Mecp2* mutant animals, motor deficits are the most common and consistent phenotype in both male and female mouse (R. Z. Chen et al., 2001; Guy et al., 2001; L. Robinson et al., 2012; M. Shahbazian et al., 2002; Vogel Ciernia et al., 2018), and rat (Vogel Ciernia et al., 2018) models of RTT. Mice carrying *Mecp2* mutations develop uncoordinated gait and reduced spontaneous movement between the age of three and eight weeks of age (R. Z. Chen et al., 2001; Guy et al., 2001; L. Robinson et al., 2012; M. Shahbazian et al., 2002). As an example, MeCP2-e1 mutant male and female mice exhibit early motor deficits by the age of 2 months, with significant differences in stride length, hind base and paw separation (Vogel Ciernia et al., 2018). By the age of 3 to 5 months, motor impairment became evident in the gait of mutant females. These females also develop beam-walking as well as rotarod disability. However, by the age of 4-5 months, only the differences in stride length remained significant.

Although we did not analyse gait before the age of 4 months, their results are consistent with our findings, with significant differences in the hindlimb base at the age of 4 and 5 months. However, these differences were lost by the age of 6 months. Thus, abnormalities in hind base seem to be present for only a short period of time between the ages of 3 and 5 months in *Mecp2*-het females. Additionally, our results revealed that those discrepancies were only present when comparing the group of early symptomatic females with the WT, suggesting a possible effect of severity of the syndrome over gait abilities. In addition, differences in forelimb stride were only present at the age of 6 months in our *Mecp2*-het females. Thus, abnormalities in limb stride may appear with the progression of the symptomatology, and be maintained after the age of 6 months.

In the case of male mice, Gadalla et al appreciated subtle changes in gait parameters in a colony of *Mecp2*-stop/y hemizygous male mice (Gadalla et al., 2014). Conversely, to what they found in females; abnormal gait preceded the onset of symptomatology of RTT in those males. In addition, previous studies reported significant abnormalities in several gait parameters in symptomatic animals (Kerr et al., 2010; L. Robinson et al., 2012; Santos et al., 2010).

Vogel et al also hypothesized that increased body weight, at least in mutant females, significantly influenced their results obtained from motor tests, suggesting an exacerbation of motor disruption by metabolic phenotype in this model. This is consistent with our results from the weight analysis, suggesting a correlation between weight and progression of the syndrome. On this basis, hind base distance was significantly more sensitive to weight changes in *Mecp2*-e1$^{-/+}$ heterozygous females (Vogel Ciernia et al., 2018), something that could be happening in *Mecp2*-het females. Moreover, we cannot discard other factors, like age or even nociceptive sensitivity, which could be interfering in abnormal gait.

Pattern of brain activity

Nociception starts with stimulation of nociceptors, which will transmit information to the second order neurons placed in the spinal cord. Then, information is transmitted to third order neurons, travelling through different areas including the spinal cord, thalamus, and brainstem. Finally, it terminates at different cortical areas in the brain, eliciting the perception of pain. In addition, different parallel pathways are implicated in the processing, thus contributing to the final noxious experiences [reviewed in (J. Chen, 2009; M.-G. Liu & Chen, 2009)].

The protein cFos is widely used as a marker of cell activation after noxious stimuli, to analyse brain regions that may be involved in nociception. This is due to basal levels of cFos, which are very low. Furthermore, increased levels of cFos protein have been reported in neurons from the superficial DHSC after noxious inputs such as inflammation, and electrical or chemical stimulation (Dubner & Ruda, 1992; Todd et al., 2002; White et al., 2011). On this basis, sacrificing the animals 90 minutes after hot plate exposure, allowed us to explore different brain regions that could be over or under activated after heat in *Mecp2*-het females. We decided to analyse cFos levels after excessive heat exposure in different brain regions, which are involved in nociception, namely the nucleus accumbens, the paraventricular thalamic and hypothalamic nuclei, central amygdala, and the periaqueductal grey.

Different pathways between PFC and other regions such as the amygdala, thalamus, hypothalamus, and PAG have been described. All these connections affect pain perception to a greater or lesser extent. Thus, considering our data from the longitudinal analysis, we hypothesized possible alterations in cell activation within these nuclei after hot plate exposition in our *Mecp2*-het females. As an example, some publications suggest that pathological conditions trend to increase thalamocortical inputs to the somatosensory cortex and PFC, contributing to chronification of pain through an increase in the activity of the insula (Henderson et

al., 2013). In addition, increased functional connectivity between the amygdala and multiple cortical, subcortical, and cerebellar regions is found in pain patients (Simons et al., 2014). Also, increased functional connectivity between the nucleus accumbens and PFC, as well as between mPFC-amygdala-accumbens, has been reported in patients with chronic back pain, linking this pathway with pain persistence (X. Huang et al., 2001). Likewise, abnormal activity in the PFC and Acb after peripheral nerve injury has been related to tactile allodynia (P. C. Chang et al., 2017). We found no significant differences in any of these analysed regions, suggesting that deficits in MeCP2 do not affect neuronal activation in any of these nuclei (neither at pre-symptomatic or symptomatic stages). Thus, and in accordance with the group of Samaco, pain perception does not seem to be altered by the absence of MeCP2.

PAG nucleus is considered as the primary control centre for descending pain modulation and relief. This nucleus receives its principal inputs from the cortical-PAG projections. In addition, vlPAG is functionally connected with other brain regions, such as the ACC, mediating descending pain modulation. On the other hand, dorsolateral regions of PAG are connected to PFC, striatum and hippocampus, all of them implicated in executive functions projections (Reviewed in Ong et al., 2019). Previous reports involved mPFC-PAG projections with modulation of autonomic responses to pain (Mai, Juergen K; Paxinos, 2011). Also, the PFC-amygdala-dorsal PAG pathway has been implicated in fear-conditioned analgesia, which means a reduction in pain response after repeated expositions to a context of pain (Butler et al., 2011). We found a decreased activation of PAG in *Mecp2*-het females, but only in the symptomatic ones (6 months of age). Considering the role of PAG in executive functions, under-activation of this region may be leading to the abnormal gait we observed, as well as possible delayed responses to noxious stimuli.

Limitations, conclusions and future directions

Although research based on pain perception in patients of RTT is increasing, discerning between an actual impairment in pain perception and possible alterations and discrepancies in the displayed pain expression proves essential to guarantee an improvement in their life quality. Thus, exploring the underlying biological pathways of pain perception, processing and expression, constitutes the basis of this objective. Likewise, knowing the limitations of techniques and procedures employed is important as well. It becomes necessary to adapt the protocols to the most suitable options, always considering the possible delayed responses, communicative and motor impairments that may complicate typical manifestations those responses, differences in age, sex (possibly caused by hormonal disbalances), genetic background (when working with animal models), or basal low heart rate variability. As an example, results from the VF test could be further validated using a Hargreaves apparatus, where a single beam of light is pointed towards the hind leg and experimenters can easily assess withdrawal responses. Also, knowing how different stages of the syndrome affect pain sensitivity, would help to better adapt specific treatments for these girls, and consider the most suitable safety strategies as well.

GENERAL DISCUSSION

In the present dissertation, we focus in analysing the anatomical, behavioural and sensory deficits in the brain of the most widely used mouse model for Rett syndrome. Understanding how *Mecp2* deficiency affects the neuroanatomy and behaviour in our mice may help for the development of more accurate therapies and treatments, improving life quality of patients suffering this condition.

On this basis, we first investigated possible alterations in the population of immature neurons. Results obtained from BrdU experiment suggest that mutations in *Mecp2* do not compromise cell survival of SVZ-generated neurons. Moreover, increased population of immature DCX^+ cells in the olfactory system of young-adult *Mecp2*-null males, suggest that lack of MeCP2 may impair the process of neuronal maturation in a region-specific way. This increase is consistent with olfactory biopsies from RTT patients showing defective and immature OSNs in the olfactory epithelium (Ronnett et al., 2003). Moreover, some researchers have suggested that RTT physiopathology is caused by a lack of maintenance of brain, instead of a defective brain development or a rapid deterioration. Also, the process of neuronal maturation can vary depending of environmental stimuli. As an example, early life stress, in a paradigm of maternal separation, alters the levels of DCX^+. On one side, MS might promote neuronal maturation in the OBGr, and tangled neurons of Pir in WT and *Mecp2*-het females. On the other side, MS exacerbates the consequences caused by deficits of MeCP2 in the Tu of *Mecp2*-het females, dSt of WT and *Mecp2*-null males, and vSt and Pir (in both tangled and complex neurons) of *Mecp2*-null males. Thus, stressful environmental stimuli in early life may vary the effect of mutations in *Mecp2* in the levels of immature neurons in the olfactory system. This opens the possibility for the development of more specific treatments focused on the promotion of neuronal maturation in RTT patients.

On this basis, exploring how environmental enrichment in early life interferes in this process, could lay the foundations for new therapeutic approaches. However, how some "internal stimuli" also affects the levels of immature neurons should also be considered. As an example, our results revealed that negative consequences of MeCP2 deficiency are dependent on *Mecp2*-dosage and, therefore, severity of symptomatology. On this basis, *Mecp2*-null males, which do not express any MeCP2, develop symptoms earlier than females, who are heterozygous for the mutation. Consequently, symptomatology gets worse in males than females of the same age. Thus, the negative consequences of MeCP2 deficiency can be appreciated earlier in males (around the 6^{th}/7^{th} weeks of age), whilst in the case of females, some of them are only visible after the 6^{th} month of age. Therefore, age of subjects significantly modifies the results obtained when working with this strain of mice. This is consistent with our results obtained from *Mecp2*-het female from 6/8 weeks of age, not showing significant differences between genotypes. However, unpublished results from our lab revealed that *Mecp2*-het females have more DCX^+ cells in the Pir than WT females (Sevilla-Ferrer et al., 2021). Moreover, some reports have described that beneficious X-chromosome inactivation patterns in some patients of RTT leads to milder phenotypes. Thus, dosage of *Mecp2* and variations X-chromosome inactivation patterns need to be considered as well.

Due to its extensive symptomatology and the wide extension of its consequences, understanding how this condition works remains a challenge for the research community. Therefore, RTT has been misdiagnosed. Even part of its symptomatology remains unclear. All this slows the development of effective and specific therapies for those patients. As an example, due to the social deficits described in patients of RTT, this syndrome was first considered as an ASD. However, recent reports revealed that these girls usually seek social interaction more often than children with autism do. However, this higher social interest is masked by their

motor and communicative deficits. Considering this, we analysed the social brain and some of the social and aggressive normative behaviours in our mice.

We observed that although general distribution of AVP and OT-ergic somata and innervation was not affected in our mice, testosterone-dependent AVP innervation was significantly reduced specifically in the sexually dimorphic nuclei of the sociosexual brain network of young-adult *Mecp2*-null males. This is consistent with the internal testicles described in this mouse model (Guy et al., 2001) and the lower levels of MUPs and lack of masculine pheromone darcin (both of them testosterone-dependent) found in the urine of *Mecp2*-null males (Martínez-Rodríguez et al., 2020). All of this, together with the increased chemoinvestigation displayed by WT residents towards *Mecp2*-null intruders, supports the hypothesis of a lower testosterone and male pheromone production in *Mecp2*-null males. In addition, it is known that MeCP2 directly regulates the sexually-dimorphic AVP-ergic innervation in rats. Surprisingly, we also found a significant decrease in our *Mecp2*-null males in the scarce OT innervation within the lateral habenula, which is not sexually dimorphic, that could also be related to deficits in testosterone levels (Hiroi et al., 2013; Shughrue et al., 1997).Therefore, an interplay between lack of MeCP2 and deficits on testosterone may lead to these reductions in AVP and OT-innervation, suggesting a key role of this gene in the development of sexually dimorphic systems. In summary, the mechanism by which MeCP2 regulates AVP and OT production may be an indirect effect over gonadal hormone production, a direct gene regulation, or both.

Alterations in AVP-ergic and OT-ergic signalling may impair the proper functioning of the social brain network, leading to abnormal behaviours in male mice. This might explain abnormal aggression and social behaviour in our *Mecp2*-null males compared to the WT in the RI test: *Mecp2*-null residents do not display aggressive behaviours against the intruder. This is due to the decreased AVP innervation, as well as the increased $NADPHd^{+}$ (indicative of increased production of NO, also

implicated in modulation of aggressive behaviours) found in those males. Increased NO production in the brain of our *Mecp2*-null males, could contribute to the alterations found in *Mecp2*-null behaviours in the RI test, such as reduced aggression of the intruder and territoriality. Of note, we cannot exclude the possibility that, being *Mecp2*-null mice smaller than the WT intruders, this can constitute a confounding factor in the results obtained, as the size of WT animals can be "intimidatory" towards the smaller *Mecp2*-null animals. In fact, in the experiment of maternal separation, WT mice were heavier than *Mecp2*-null males in both MS and naïve groups, thus supporting this difference in size. In addition, we found a reduction in chemoinvestigation of the intruder by *Mecp2*-null males, which does not seem to be caused by a possible anosmia, since *Mecp2*-null animals are able to detect and discriminate between odours (Martínez-Rodríguez et al., 2020). Thus, this reduced chemoinvestigation points towards a general lack of interest in the intruder by *Mecp2*-null residents. However, we cannot discard the possibility that the increased self-grooming displayed or potential locomotor deficits in *Mecp2*-null males may influence in the chemoinvestigation. This would be consistent with the abnormal gait observed in chapter 3, and motor impairment observed in symptomatic *Mecp2*-het females interfering in in results obtained from the VF and HP tests.

An increased somatosensory sensitivity of *Mecp2*-null mice, which could render unpleasant direct contact with conspecifics, cannot be discarded (see Chapter 3 and Flores Gutiérrez et al., 2020). In fact, there are discrepancies in the current bibliography about pain sensitivity in both RTT patients and mouse models. While part of the literature suggests that patients of RTT are hyposensitivity, the other part defend that RTT patients show different expression of pain, possible leading to a misunderstanding in pain sensitivity in RTT. On this basis, we have explored pain sensitivity in a longitudinal studio with *Mecp2*-het and WT females from 3 to 6 months of age.

First, we must remember that onset of syndrome does not happen at the same age in patients or mouse modes. In addition, this onset correlates with severity of symptomatology in RTT. Since Guy and Adrian described a correlation between exhibition of clasping with the progression of the syndrome with age (Guy et al., 2001), we analysed clasping in our *Mecp2*-het female mice from 3 to 6 months of age. This way, we classified them into early (showing clasping by the age of 4 months) and late symptomatic females (display clasping after the 4 months of age) for later analysis. Moreover, some evidences have correlated severe obesity with hyposensitivity in humans (Torensma et al., 2017). We also found that weight of our *Mecp2*-mutant female correlates with severity of symptomatology, with *Mecp2*-het females suffering from more severe overweight and obesity as the syndrome progresses. This is also consistent with results from the weight analysis in our experiment of maternal separation, where our six-weeks-old *Mecp2*-het females were significantly lighter than the WT females. By this age, most of *Mecp2*-het females remains in a pre-symptomatic state, thus supporting an interplay between deficits in MeCP2 and overweight (which is dependent on the age of onset of the syndrome), and severity of symptomatology.

When we explored pain sensitivity, we observed that late symptomatic females (with slighter symptomatology) are hypersensitive in the VF test compared to the WT, but only by the age of 3 and 4 months. Conversely, early symptomatic females (with more severe symptoms) showed a trend towards hyposensitivity in this same test. However, we cannot discard an effect of motor impairment and overweight (both of them exacerbated by the progression of the disease) hindering the paw withdrawal in the VF test. This is consistent with the abnormal gait of *Mecp2*-het females observed in the paw print test. Therefore, we must consider all possible variables that could be hiding a possible non-manifested hyperalgesia in mice with severe symptomatology. In agreement with differences in pain responses, we observed that majority of *Mecp2*-het females do not withdraw or lick the paws in

the HP test. Instead, they performed and odd walking backwards movement. On this basis, motor impairments and overweight could be the cause of abnormal gait and difficulties in licking their paws, thus leading to this odd walking instead. When we analysed the latency to first avoidance (considering either licking the paws or walking backwards), we found that the group of 6-months-old *Mecp2*-het females showed hypersensitivity in the HP test, compared to the WT females. This hypersensitivity was also present when comparing separately the group of early and late symptomatic females with the WT group. By contrast, we found no significant differences between genotypes in the group of 2 months old, suggesting that higher thermosensitivity in *Mecp2*-het females also correlates with progression of the syndrome. However, significant hypersensitivity was only present when considering the odd walking displayed by these mutant females, and not when only considering licking their paws. Thus, abnormal responses must be taken into account when analysing pain perception in RTT. In addition, decreased cFos activation levels in the PAG of *Mecp2*-het females may lead to lower analgesic effects controlled by this region, supporting the hypothesis of a hidden hypersensitivity in our symptomatic *Mecp2*-het females.

Differences between early and late symptomatic females are of special relevance since majority of behavioural test analysing pain and nociception are performed with already diagnosed patients. In addition, age of animals should also be considered when working with animal models. Considering that diagnosis of RTT is mainly based on symptomatology, analysis of nociception does not encompass possible alterations in pain perception occurring before the onset of symptomatology. Thus, pain sensitivity protocols should be adapted and consider the motor impairments, overweight, and delayed and different responses to noxious stimuli displayed by RTT patients and animal models.

Although Rett is a condition mostly affecting females, traditionally most research has been done in the male murine model. In the present dissertation we

demonstrate the relevance of using females in different stages of age and, therefore, of the disease progression. Likewise, we analysed some secondary symptoms, such as testosterone levels, weight, gait, or abnormal responses to certain stimuli, which have not been studied in depth, but may interfere in the conclusions obtained from other analysis.

CONCLUSIONS

1. Lack of MeCP2 does not seem to affect cell survival of newly generated neurons in the subventricular zone that migrate to the dSt and OB.

2. Lack of MeCP2 increases the number of immature DCX^+ cells in the olfactory system in a region-specific manner, mainly affecting the piriform cortex and the olfactory tubercle but not the OB, and thus suggesting a possible impairment in the process of neuronal maturation.

3. Early life stress, in a paradigm of maternal separation, affects this population of DCX^+ cells in a region, MeCP2-dossage, and sex-specific manner. We hypothesized that maternal separation promotes neuronal maturation in the OBGr and Pir of WT and *Mecp2*-het females; whilst it exacerbates the consequences of MeCP2 deficiency impairing the neuronal maturation of cells in the Tu of *Mecp2*-het females, dSt of WT and *Mecp2*-null males, as well as vSt and Pir of *Mecp2*-null males.

4. Lack of MeCP2 leads to a decreased testosterone-dependent AVP-innervation in the social brain of *Mecp2*-null males, affecting specifically the vmStP, LS, BSTMPI, LHb, vHip, and dEn.

5. Absence of MeCP2 also decreases OT-ergic innervation in the LHb of *Mecp2*-null males, which is also testosterone-dependent.

6. An interplay between lack of MeCP2 and a testosterone deficit may lead to reductions described in AVP and OT-innervation, suggesting a key role of this gene in the development of sexually dimorphic systems.

7. Mutations in *Mecp2* also lead to increased $NADPHd^{+}$ activity, which is indicative of increased production of nitric oxide.

8. Alterations within the nonapeptidergic system suggest lower levels of testosterone in *Mecp2*-null males and, together with the increased levels of nitric oxide, may lead to alterations in aggressive and social male behaviours in mice.

9. *Mecp2*-null males do not display aggressive behaviours against the intruder in the resident-intruder test, suggesting reduced aggressiveness. However, an intimidatory effect due to the higher size of WT males compared to the *Mecp2*-null should be considered.

10. *Mecp2*-null males spend less time chemoinvestigating the intruder, and more time in self-grooming. This could be due to different factors such as a loss of interest towards the intruder, a higher somatosensory perception that could render unpleasant direct contact with conspecifics, or motor deficits that may complicate the approach.

11. *Mecp2*-mutations in females lead to severe overweight and obesity as the syndrome progresses, suggesting an interplay between deficits in MeCP2 and overweight, which is dependent on the severity of symptomatology.

12. Late symptomatic females are hypersensitive in the Von Frey test by the age of 3 and 4 months, while early symptomatic females show a trend towards hyposensitivity, suggesting that different responses to mechanosensitivity correlate with onset and severity of symptomatology. We must consider all possible variables that could be hiding a possible non-manifested hyperalgesia, such as motor impairment and overweight.

13. Half of the symptomatic *Mecp2*-het females do not withdraw or lick their paws in the hot plate test, but perform and odd walking backwards movement, probably due to their motor impairment and/or overweight. Thus, abnormal responses to noxious stimuli must be considered when analysing pain perception in RTT.

14. MeCP2 deficiency leads to hypersensitivity in the hot plate test, but only in symptomatic females, suggesting that higher thermosensitivity in *Mecp2*-het females also correlates with progression and severity of the syndrome.

15. Mutations in *Mecp2* lead to a decreased cFos activation within the PAG of *Mecp2*-het females after thermal stimulation, probably leading to lower analgesic effects controlled by this region.

16. Different results and responses obtained in early and late symptomatic females are of special relevance since a majority of behavioural tests analysing pain and nociception are performed with already diagnosed

patients, and do not consider possible alterations in pain perception occurring before the onset of symptomatology.

17. Although RTT is a syndrome mostly affecting females, traditionally most research has been done in the male murine model. Our results highlight the importance of using females in different stages of syndrome progression to fully understand the onset and interaction of the hallmark symptoms of the RTT phenotype.

RESUMEN EN CASTELLANO

INTRODUCCIÓN GENERAL

El síndrome de Rett

El síndrome de Rett (OMIM #312750) es una enfermedad que afecta al correcto neurodesarrollo de 1 de cada 10-15.000 niñas de todo el mundo. Aunque está considerada como una enfermedad rara, sigue siendo la segunda causa de discapacidad intelectual de origen genético en mujeres, justo por detrás del síndrome de Down (Christodoulou, 2001). Las niñas afectadas se desarrollan con aparente normalidad hasta los 6-18 meses de edad, momento en el que sufren un estancamiento en el desarrollo (Christodoulou, 2001) y empiezan a manifestar la sintomatología característica de este síndrome: regresión motora y cognitiva, convulsiones, dificultades respiratorias, movimientos repetitivos de manos, bajo peso (Schultz et al., 1993), alteraciones en la percepción del dolor (Downs et al., 2010) y alteraciones del sistema circulatorio (Acampa & Guideri, 2006; Hagberg, 2002), entre otras.

A día de hoy sigue sin existir una cura específica para el síndrome de Rett, siendo la mayoría de los tratamientos paliativos. La esperanza de vida varía desde los 2 años hasta los 40-50 años (Christodoulou, 2001). Debido a la gran cantidad de sistemas que se ven alterados, las niñas afectadas necesitan ayuda en su día a día. Por ello, entender los procesos biológicos subyacentes de este síndrome supone un punto clave en la mejora de su calidad y esperanza de vida.

MECP2 como principal causa del síndrome de Rett

Al menos un 95% de los casos del síndrome de Rett están causados por mutaciones en el gen *MECP2 (Methyl-CpG-binding protein 2)*, mientras que el 5% restante se deben a mutaciones en las regiones reguladoras de la transcripción del mismo (Neul et al., 2008; Shin et al., 2013; Zoghbi, 2016; Zoghbi et al., 1999). Este gen se localiza

en el cromosoma X_{q28}, configurándose el síndrome de Rett como una alteración dominante ligada al cromosoma X que afecta a mujeres heterocigotas, mientras los niños hemicigotos no llegan a término o mueren durante su primer año de vida de encefalopatía neonatal (Hagberg et al., 1983; Santos et al., 2009).

El gen *MECP2* codifica la proteína MeCP2, un regulador de la transcripción que se encuentra sobre todo en el sistema olfativo y corteza cerebral (Akbarian et al., 2001; Coy et al., 1999; Lee et al., 2014; Lombardi et al., 2015; M. D. Shahbazian et al., 2002). La expresión de este gen es dependiente de región, género y momento del desarrollo, existiendo un mecanismo muy preciso de control de niveles de MeCP2 (Chao et al., 2007; Kishi & Macklis, 2004; Mullaney et al., 2004; M. D. Shahbazian et al., 2002). Además, está implicado en procesos como la maduración neuronal, complejidad neurítica, sinaptogénesis, organización de la cromatina, potenciación a largo plazo y plasticidad sináptica (S. Cohen et al., 2011; Degano et al., 2014; Jones et al., 1998; Kishi & Macklis, 2004; Krishnan et al., 2017; H. Li et al., 2011; Mullaney et al., 2004; Nan et al., 1998).

Modelo animal empleado para el estudio del síndrome de Rett

Para la presente tesis hemos utilizado el modelo de ratón diseñado por Guy y sus colaboradores en 2001 (*Mecp2*$^{tm.1Bird}$). Este modelo es el más empleado en Rett, modelizando los problemas motores y respiratorios del síndrome, así como algunos de los fenotipos comportamentales y la menor esperanza de vida (Guy et al., 2001). Además, y de forma similar a lo descrito en pacientes de Rett, estos ratones también presentan alteraciones morfológicas a nivel cerebral, con neuronas de pequeño soma y reducida complejidad dendrítica.

Fisiopatología del síndrome de Rett

Aunque el síndrome de Rett es considerado una enfermedad del neurodesarrollo, existe la posibilidad de que se deba a defectos en el mantenimiento neuronal (Akbarian et al., 2001; R. Z. Chen et al., 2001; Guy et al., 2001). Ello explicaría el

retraso en la manifestación de la sintomatología. Sin embargo, no existen evidencias que sugieran que la proliferación, supervivencia y/o apoptosis celular estén alterados en Rett (Agustín-Pavón et al., 2016; Smrt et al., 2007; Tsujimura et al., 2009), ni de que exista una pérdida neuronal significante. Por ello, se ha planteado la hipótesis de una deficiente maduración neuronal, provocando que los neuroblastos no consigan diferenciarse en neuronas plenamente funcionales (D. Armstrong et al., 1995; W. G. Chen, 2003; D. R. S. Cohen et al., 2003; Duncan Armstrong, 2005; Smrt et al., 2007).

A lo largo del primer capítulo decidimos investigar la distribución de doblecortina, un marcador de neuronas inmaduras. Nuestra hipótesis sugiere que déficits en los niveles de MeCP2 podrían incrementar la DCX en los ratones *Mecp2*-mutantes. Además, se ha demostrado que el estrés a edades tempranas es capaz de promover la maduración neuronal en ratones, especialmente en experimentos de separación materna (Bath et al., 2016). Sabiendo que MeCP2 es un regulador transcripcional epigenético que actúa dependiendo del ambiente (Sharifi & Yasui, 2021), especulamos que la separación materna a edades tempranas podría interferir en dicho proceso de maduración neuronal.

Por otro lado, muchas niñas con Rett desarrollan problemas sociales, algunos de ellos relacionados con el aislamiento social. Sin embargo, se ha visto que, tras la adolescencia, muchas son capaces de recobrar parcialmente su habilidad para socializar, e incluso mantienen un mayor contacto visual y buscan iniciar interacciones sociales (Olsson & Rett, 1985). Por este motivo, se ha sugerido una relación entre los déficits sociales con otros problemas motores o de comunicación.

En lo referente a comportamientos sociales en mamíferos, el sistema nonapeptidérgico desempeña un papel importante. Por este motivo, en el capítulo 2 exploramos el cerebro social de ratones jóvenes salvajes y mutantes para el gen *Mecp2*. Posibles alteraciones en dicho sistema podrían estar interfiriendo en las

alteraciones sociales encontradas en ratones *Mecp2*. Por ello, decidimos analizar también algunos de los comportamientos sociales típicos en ratones machos, mediante la realización de un test de residente-intruso. Nuestra hipótesis se basa en que los animales mutantes presentan alteraciones en los sistemas nonapeptidérgicos, así como en su agresividad.

De igual modo, se han asociado comportamientos menos ansiosos a ratones modelo de Rett, basándose en que éstos pasan más tiempo en los brazos abiertos del test de laberinto en cruz elevado. Sin embargo, algunos investigadores, han apuntado que este comportamiento puede deberse al hecho de que éstos pudiesen experimentar sensaciones más dolorosas de lo normal, al rozar sus vibrisas con las paredes de los brazos cerrados. De esta forma, no se trataría de una preferencia por los brazos abiertos, sino una evitación de los cerrados por incomodidad. Por este motivo, debemos considerar siempre otros aspectos de la sintomatología de Rett que puedan estar interfiriendo en los resultados obtenidos. Algo similar ocurre con el análisis de percepción de dolor. A día de hoy resulta difícil esclarecer si realmente experimentan una menor percepción del dolor, o si los problemas de comunicación y movilidad podrían estar impidiendo una adecuada expresión del mismo. Por este motivo, en el capítulo 3 decidimos realizar un estudio longitudinal para explorar la nocicepción, en términos de mecano y termo-sensibilidad en un grupo de hembras. Además, analizamos algunas de sus características motoras y la evolución de su peso, con el objetivo de dilucidar posibles factores que puedan influir en la expresión del dolor.

OBJETIVOS

El objetivo general de la presente tesis doctoral es analizar algunos aspectos neuroanatómicos y funcionales que han sido poco explorados en el modelo murino de síndrome de Rett. Este objetivo general puede detallarse en una serie de objetivos que enumeramos a continuación:

1. Explorar el número de células proliferativas en los bulbos olfativos y estriado de ratones macho *Mecp2*-nulos para analizar la supervivencia de los progenitores celulares localizados en la zona subventricular.

2. Describir la distribución y densidad de células marcadas con doblecortina, un marcador de neuronas inmaduras, en el cerebro de ratones jóvenes.

3. Investigar el efecto del estrés temprano, utilizando el paradigma de separación materna, en la densidad de neuronas que expresan doblecortina.

4. Analizar la distribución de vasopresina y oxitocina, dos nonapéptidos relacionados con la regulación de comportamientos sociales en ratón, en el cerebro social de ratones jóvenes.

5. Analizar la expresión de NADPH, implicado en la modulación indirecta de comportamientos agresivos en ratones machos.

6. Estudiar los comportamientos sociales y agresivos en ratones macho jóvenes.

7. Examinar la nocicepción, en términos de mecanosensibilidad, en un estudio longitudinal en hembras jóvenes y adultas.

8. Explorar otros síntomas tales como el peso, los déficits motores o "clasping" que podrían interferir en la expresión de dolor en ratones.

9. Explorar la sensibilidad térmica en hembras jóvenes presintomáticas y hembras adultas sintomáticas heterocigotas.

10. Explorar los patrones de activación neural tras la exposición a la prueba de placa caliente en hembras mutantes para *Mecp2*.

CAPÍTULO 1

El sistema olfativo supone un buen modelo de estudio para mutaciones en el gen *MECP2*, debido a los altos niveles de proteína MeCP2 encontrados en el mismo. Además, junto con el giro dentado del hipocampo (DG), constituye un reservorio de neurogénesis adulta en roedores, configurándose como un buen modelo de estudio de generación y maduración neuronal en el cerebro adulto (Matarazzo & Ronnett, 2004; Ronnett et al., 2003). La función del sistema olfativo se inicia en las neuronas sensoriales olfativas que detectan olores y envían la información química a través de las fibras nerviosas. Dicha información llega hasta los glomérulos de los bulbos olfativos, y será posteriormente enviada a distintas regiones de la corteza olfatoria, entre las que destacan la corteza piriforme y el tubérculo olfativo.

En el sistema olfativo existe un grupo de células progenitoras en la zona subventricular (SVZ) que proliferan y viajan a través de la corriente migratoria rostral hasta los bulbos olfativos, donde se diferenciarán e integrarán como neuronas maduras granulares y periglomerulares (Shipley et al., 2004; Yang et al., 2004). Aunque se sabe que la proliferación y apoptosis no se encuentra alterada en el cerebro de estos ratones, decidimos estudiar la supervivencia de las neuronas generadas postnatalmente en la SVZ analizando la expresión de BrdU (un análogo de la timidina que se integra en el DNA de las células en proliferación).

Para estudiar el proceso de maduración neuronal, analizamos la distribución y cantidad de células que expresan doblecortina (DCX). Ésta es una proteína asociada a microtúbulos que se expresa en neuroblastos en migración y neuronas en diferenciación (F. Francis et al., 1999; Gleeson et al., 1998), es decir, en neuronas inmaduras. En el cerebro de ratón adulto, existe una población de neuronas unipolares que expresan DCX en la capa II de la corteza piriforme y que pueden diferenciarse en función de su morfología en complejas y enmarañadas (“tangled”, según Benedetti et al., 2020; M. Á. Gómez-Climent et al., 2008; Rubio et al., 2016),

siendo las primeras más grandes, con mayores ramificaciones y, por tanto, situándose en un punto de maduración más avanzado que las segundas, las cuales tienden a ser de menor tamaño, con menores procesos dendríticos y más redondeadas. Estas neuronas son generadas durante la etapa embrionaria y permanecen en estado inmaduro en el ratón adulto, desapareciendo progresivamente con la edad (M. Á. Gómez-Climent et al., 2008; Rubio et al., 2016) al ir madurando y diferenciándose en neuronas glutamatérgicas que se integrarán en la circuitería del piriforme (Rotheneichner et al., 2018). A medida que desciende la expresión de DCX en las células inmaduras, éstas empiezan a expresar NeuN, utilizada normalmente como marcador de neuronas maduras. Cuando la expresión de ambas proteínas coincide, se considera que la neurona se encuentra en transición desde un estado inmaduro hacia uno maduro.

MeCP2 está implicado en el control de periodos críticos del desarrollo postnatal, en los que existe una alta plasticidad y sensibilidad a los cambios del entorno (Picard & Fagiolini, 2019). Cabría esperar, por tanto, que la exposición a estrés en edades tempranas, algo que parece ser común en pacientes con Rett, pudiese influir y modificar el epigenoma de estas niñas. Además, la exposición a estrés temprano en forma de separación materna es capaz de promover la maduración neuronal en el hipocampo de ratones machos (Bath et al., 2016). De esta forma, analizar las interacciones entre la separación materna y déficits de MeCP2 podría ayudar a entender mejor cómo influyen los estímulos ambientales en Rett.

Realizamos, por tanto, tres análisis distintos: supervivencia celular (Experimento 1), densidad de neuronas inmaduras (Experimento 2), y su interacción con el estrés a edad temprana (Experimento 3).

Para el **Experimento 1**, administramos BrdU vía intraperitoneal a un grupo de machos para posteriormente realizar una inmunohistoquímica para detectar células $BrdU^{+}$. encontramos células marcadas con BrdU en el estriado ventral y dorsal, en

las zonas cercanas a los ventrículos laterales y zona subventricular, así como en las capas glomerular y granular de los bulbos. En el caso del Piriforme, sólo encontramos un par de células esparcidas. Sin embargo, no encontramos diferencias significativas en el análisis de BrdU en ninguna de las áreas analizadas, sugiriendo que la ausencia de MeCP2 no afecta a la supervivencia de los neuroblastos generados postnatalmente en la zona subventricular.

Para el análisis de neuronas DCX^+ del **Experimento 2**, realizamos una inmunohistoquímica frente a DCX y una doble inmunofluorescencia para detectar DCX y NeuN, analizando el número de células DCX^+ y que co-expresasen DCX/NeuN en distintas regiones. Encontramos un aumento significativo en el número de neuronas DCX^+ en el piriforme de los machos nulos para *Mecp2*, así como en una población de neuronas doblecortina localizada en la capa I del tubérculo olfativo que no había sido descrita en profundidad en la bibliografía hasta el momento. Por otro lado, las poblaciones de células DCX en hipocampo y estriado no parecen estar alteradas, lo que sugiere que la falta de MeCP2 sólo afecta a dichas poblaciones neuronales en determinadas regiones. En el caso de las neuronas en transición ($DCX^+/NeuN^+$), tampoco encontramos diferencias significativas. Todo ello apuntaría a un posible retraso en el proceso de maduración de las neuronas DCX^+ específico de regiones del sistema olfativo. Además, la falta de diferencias significativas entre genotipos tanto en la densidad como tamaño celular de la capa II del piriforme, descarta que este incremento de células DCX^+ pueda deberse a una mayor densidad celular y apunta hacia una maduración neuronal postnatal defectuosa. Aunque desconocemos si las células DCX^+ del tubérculo son de origen embrionario o si se generan en el adulto, vemos que aparecen en dos estados distintos: formando agrupaciones de células DCX^+ (menor tamaño, más empaquetadas y situadas en el extremo más externo de la capa I del tubérculo) o de forma individual (mayor tamaño, núcleo redondeado y proyecciones más largas, comúnmente adyacentes a vasos sanguíneos y más cercanas a la capa II del tubérculo). Asimismo, estas

agrupaciones celulares tienden a estar rodeadas de células que expresan Ki67 (un marcador de proliferación celular), con algunas de ellas co-expresando ambos marcadores (Ki67 y DCX; Esteve-Pérez et al., 2022), por lo que podría existir algún tipo de proliferación adulta en esta región, o bien tratarse de células de origen embrionario que van madurando con el paso de los años, contribuyendo a procesos de plasticidad neuronal.

En el caso de las hembras, no encontramos diferencias significativas en ninguna de las áreas analizadas. Esto puede deberse a las diferencias en la edad de aparición de los síntomas que existen entre machos y hembras, lo cual apoyaría a su vez la hipótesis de que la fisiopatología de Rett se deba más a un defecto de mantenimiento del sistema nervioso que de desarrollo.

Para el **experimento 3**, trabajamos con un grupo de ratones machos y hembras separados de sus madres a edades tempranas, junto con un grupo naïve que se mantuvo en sus cajas con sus madres, y realizamos una inmunodetección frente a DCX. Descubrimos que la separación materna afecta al peso y proceso de maduración neuronal de forma diferente a machos y hembras, promoviéndola o disminuyéndola en función de la región. Estos resultados sugieren que la separación materna afecta a la población de neuronas inmaduras de forma diferencial en función del núcleo, sexo y genotipo. Por un lado, podría acelerar la maduración en la capa granular del bulbo y corteza piriforme en hembras jóvenes asintomáticas, mientras que en el tubérculo podría: (i) empeorar las consecuencias producidas por los déficits de MeCP2, ralentizando el proceso de maduración y aumentando el número de células DCX^+, o bien (ii) promover la proliferación celular en el tubérculo de las hembras heterocigotas asintomáticas. Se necesitaría una investigación más a fondo, aunque la primera hipótesis parece ser la más probable.

En el caso de los machos, la separación materna podría estar empeorando la patología de los machos nulos, incrementando su susceptibilidad a estresores

durante las primeras etapas de vida que afectan a la maduración neuronal, especialmente en la corteza piriforme. Esta hipótesis se ve apoyada una menor expresión de reelina (implicada en maduración y migración neuronal; Carceller et al., 2016; Tissir & Goffinet, 2003), en piriforme, tubérculo y DG en animales expuestos a separación materna. Dado que su expresión está regulada por MeCP2 (Sánchez-Lafuente et al., 2022), apuntaría hacia una menor migración y maduración neuronal causada la ausencia de MeCP2, y exacerbada por la separación materna. Futuros estudios son necesarios para esclarecer tanto el efecto de distintos tipos de estresores, como los mecanismos mediante los cuales el estrés puede afectar a las poblaciones de DCX^+ en ratones adultos.

La separación materna también es capaz de incrementar el peso, afectando sólo a ratones macho salvajes. El diferente efecto de la separación materna en función del sexo podría estar relacionado con las diferencias de niveles de MeCP2. De esta forma, diferentes niveles de MeCP2 conducen a diferencias tanto en la edad de inicio como en severidad de la sintomatología.

Podríamos decir que el presente estudio supone un interesante punto de inicio para investigar las consecuencias de déficits de MeCP2 en el desarrollo y funcionamiento del sistema olfativo, así como de la neurogénesis adulta no proliferativa (König et al., 2016). Dada la relevancia de algunos de los núcleos afectados en las capacidades cognitivas y desarrollo de comportamientos afectivos, una maduración neuronal deficiente en dichas áreas podría estar implicada en algunos de los déficits que caracterizan tanto Rett como los trastornos de espectro autista.

CAPÍTULO 2

Los comportamientos sociales están regulados por un conjunto de conexiones entre distintos núcleos que componen lo que se conoce como cerebro socio-sexual (Newman, 1999). En roedores, este cerebro social recibe una importante inervación del sistema olfativo, que se constituye como la modalidad predominante en la percepción de información social a través de feromonas (Crawley, 2012). La información en forma de olores tanto volátiles como no volátiles se procesa en los bulbos olfativos principal y accesorio, respectivamente. Posteriormente, la información viajará hasta la amígdala, en un proceso regulado por niveles de oxitocina (OT) y vasopresina (AVP). Estos dos neuropéptidos desempeñan un papel importante en la regulación de comportamientos sociales tanto en humanos como roedores (J. Goodson, 2008).

De esta forma, la decisión entre comportamientos de acercamiento o aversivos dependerán de la activación de diferentes núcleos, así como la neuromodulación a la que se vean sometidos por parte de la vasopresina y la oxitocina (J. Goodson, 2008). En algunas de las regiones diana de las neuronas que expresan AVP, las proyecciones vasopresinérgicas son sexualmente dimórficas y dependientes de los niveles de testosterona, por lo que existirá una mayor densidad de inervación en machos que en hembras (Otero-Garcia et al., 2014). Todo ello sugiere que el desarrollo y función del cerebro social, clave para el desarrollo y expresión de los comportamientos sociales, está regulado por componentes epigenéticos y hormonales.

En el capítulo 2, decidimos analizar las consecuencias del déficit de MeCP2 sobre la expresión de AVP y OT en el cerebro social de ratones jóvenes (Experimento 1). Sabiendo que gran parte de la inervación vasopresinérgica es dependiente de testosterona, y que el modelo de ratón empleado presenta testículos internos y es infértil (Guy et al., 2001), hipotetizamos que los niveles de ratones machos *Mecp2*-

nulos presentarán niveles menores de testosterona. Por ello, decidimos analizar otras características que se conocen están afectadas por los niveles de testosterona: (i) la densidad de células nitrérgicas (Experimento 1) y (ii) comportamientos agonistas en un test de residente-intruso (Experimento 2).

Para el **Experimento 1**, trabajamos con el tejido de ratones sacrificados a las 8 semanas de edad con los que realizamos dos inmunohistoquímicas: (i) una doble para marcar AVP y OT, (ii) y otra para marcar AVP junto con una tinción diaforasa para las células que expresan NADPH. Decidimos centrarnos en analizar aquellas áreas de mayor relevancia para el cerebro socio-sexual (ver la tabla 11).

En hembras no encontramos diferencias significativas. Esto puede deberse a: (i) la presencia del alelo *Mecp2* salvaje protector, o (ii) a la ausencia de inervación dependiente de testosterona en hembras. Tampoco podemos descartar un efecto similar al observado en el capítulo 1, en el que la edad implica que una ausencia sintomatología que nos impediría apreciar el efecto de la falta de *Mecp2*.

Por otro lado, encontramos una reducción significativa de las fibras vasopresinérgicas dependientes de testosterona en los machos *Mecp2*-nulos. Sabiendo que este modelo de ratón presenta testículos internos, y que presentan menores niveles de proteínas urinarias principales y darcina (una feromona masculina cuya síntesis es dependiente de testosterona; Martínez-Rodríguez et al., 2020), podríamos pensar que la falta de MeCP2 podría afectar a la producción de AVP de manera indirecta a través de mecanismos dependientes de testosterona.

En el caso de la oxitocina, solamente encontramos una reducción significativa en la habénula lateral de los machos *Mecp2*-nulos. El metabolito de la dihidrotestosterona (el 5α-androstane-3β,17β-diol) es capaz de regular la expresión de OT por medio de la activación de un tipo de receptor estrogénico que puede encontrarse en la habénula (Shughrue et al., 1997). Sin embargo, se necesita un análisis más específico para la inervación oxitocinérgica de este núcleo.

Por otro lado, en análisis de NADPH reveló un incremento de células $NADPH^+$ en el estriado dorsal y núcleo accumbens de los mismos. La actividad de NADPH se ve incrementada tras la castración en ratones machos, por lo que no es de extrañar que apreciemos un incremento de estas células en nuestros ratones nulos. Esto sugiere que la actividad de la sintasa de óxido nítrico (NOS) se encuentra incrementada en regiones específicas de los machos nulos.

Todo ello se traduce en un malfuncionamiento del circuito del cerebro social de estos ratones, afectando tanto a los comportamientos sociales como agresivos. Por otro lado, se han descrito déficits de serotonina en ratones *Mecp2*-nulos (Santos et al., 2010; Vogelgesang et al., 2017). La serotonina junto con la vasopresina y el óxido nítrico, modulan los comportamientos sociales y agresivos en roedores (Agustín-Pavón et al., 2009; Angoa-Pérez & Kuhn, 2015; Ferris et al., 1997; Hashikawa et al., 2017). Mientras la serotonina inhibe la agresión y comportamientos territoriales, la AVP en el hipotálamo anterior promueve la agresión en machos. Además, algunos agonistas de la serotonina promueven la síntesis de AVP y OT, así como la administración de AVP estimula la síntesis y liberación de serotonina en ciertos núcleos (Auerbach & Lipton, 1982; Jørgensen et al., 2003). Por tanto, probablemente la falta de MeCP2 esté causando desbalances en el metabolismo de serotonina y óxido nítrico, así como en la producción de hormonas gonadales, afectando así a la inervación de AVP y OT en determinadas regiones. Todo ello, causaría a su vez alteraciones en los comportamientos sociales y agresivos normativos en los ratones *Mecp2*-nulos.

Para el **Experimento 2** realizamos un test de residente-intruso. Encontramos que, mientras el 31% de los residentes salvajes atacaban al intruso, ninguno de los residentes *Mecp2*-nulos atacó al intruso. La ausencia de comportamientos agresivos en los ratones nulos concuerda con los déficits de AVP e incremento de $NADPHd^+$ del experimento 1, ambos relacionados con la regulación de comportamientos agresivos en ratones machos (Marie-Luce et al., 2013; S. Robinson et al., 2012;

Trainor et al., 2007). Por supuesto, no podemos excluir la posibilidad de que pueda deberse a un factor intimidante (siendo los salvajes bastante más grandes que los nulos).

Por otro lado, observamos que los residentes *Mecp2*-nulos empleaban mucho menos tiempo investigando al intruso que los residentes salvajes. Esto puede deberse a una falta de interés, o bien a que los residentes nulos empleaban significativamente más tiempo en acicalarse (un 13% del tiempo del test) que los salvajes (1.6% del tiempo). Sin embargo, no podemos descartar que este comportamiento de constante acicalamiento podría reflejar algunos de los comportamientos estereotípicos observados en modelos del espectro autista. Además, aunque no medimos de forma directa la locomoción de los ratones durante este test, puede existir algún déficit motor en los *Mecp2*-nulos que pueda estar influyendo. Otra hipótesis podría implicar una mayor sensibilidad somatosensorial por parte de los ratones nulos que generase una sensación desagradable ante el contacto directo con conespecíficos (ver capítulo 3 y Flores Gutiérrez et al., 2020). En este sentido, el 62% de los machos nulos escapaban de forma abrupta de los residentes salvajes durante la interacción, mientras que sólo el 38% de los residentes salvajes manifestaban este comportamiento.

Dado que nuestros datos apuntan a menores niveles de testosterona en sangre, decidimos incluir un tercer grupo de estudio formado por residentes salvajes expuestos a intrusos *Mecp2*-nulos. Gracias a este tercer grupo, pudimos observar que los residentes salvajes empleaban más tiempo investigando a los intrusos *Mecp2*-nulos que a los salvajes. Esto puede ser debido a una menor producción de feromonas por parte de los machos mutantes, siendo considerados estos como una "novedad" que aumentaba su atención y, por tanto, promovían una mayor investigación. Sin embargo, no presentaban diferencias en el tiempo empleado atacando o persiguiendo a los intrusos de ambos genotipos. En este sentido, sería

interesante explorar más a fondo la dimensión social de los machos nulos en test de interacción social, incluyendo a dichos ratones mutantes como estímulo animal.

Como conclusión, nuestros resultados revelan que la falta de MeCP2 produce importantes alteraciones neuroanatómicas y comportamentales dependientes de testosterona y, por tanto, sexualmente dimórficas. Estos déficits pueden deberse a un efecto directo de MeCP2 en la regulación de la expresión de genes como AVP o receptores de estrógenos, o bien a efectos indirectos debido a su impacto sobre el desarrollo gonadal y, consecuentemente, el estado hormonal. Por ello, al estudiar trastornos del neurodesarrollo, es importante tener en cuenta los posibles efectos en señalización hormonal que pueden derivar en los déficits observados. De hecho, variaciones en los niveles de hormonas gonadales han sido implicados en las diferencias sexuales que existen en la incidencia y severidad de algunas enfermedades tales como el autismo, o los desórdenes psiquiátricos y cognitivos (Akinola & Gabriel, 2018; Romano et al., 2016). Una investigación más exhaustiva ayudaría a entender mejor el mecanismo que deriva en la pérdida de características sexualmente dimórficas en machos nulos para *Mecp2*.

CAPÍTULO 3

El dolor se ha descrito como una sensación y experiencia emocional desagradable asociada con daño tisular tanto real como potencial. Es importante destacar que la descripción verbal es sólo uno de los muchos comportamientos que permiten expresar dolor y, por tanto, la incapacidad para comunicar una experiencia dolorosa no elimina la posibilidad de que dicha persona o animal no humano pueda experimentar dolor. El dolor no deja de ser el resultado de múltiples interacciones entre las vías de señalización y su modulación por parte de los centros cerebrales superiores (Bie et al., 2011; Marchand, 2008; Steeds, 2016; Todd, 2010; Woolf, 2004). Para poder hablar de dolor como tal, esta estimulación dolorosa debe ser interpretada por la corteza sensorial para producir una experiencia multidimensional que separa el concepto de dolor de la nocicepción.

Por su parte, el concepto de nocicepción describe un procesamiento sensorial de un estímulo doloroso, desde la periferia hasta el cerebro siguiendo las vías de dolor/nocicepción (Coutaux et al., 2005; Steeds, 2016; Woolf, 2004, 2011), y generando una respuesta neuronal periférica ante el estímulo doloroso (Dubin & Patapoutian, 2010).

En el caso de Rett, existen casos de niñas diagnosticadas con una menor sensibilidad al dolor (Downs et al., 2010; Hagberg, 2002; Neul et al., 2010). Sin embargo, algunos cuidadores han reportado que la percepción de dolor visceral parece estar menos alterada o incluso aumentada comparada con el dolor periférico (Downs et al., 2010). Aunque las investigaciones preclínicas sugieren que el circuito de dolor reflejo no se encuentra alterado en Rett (Samaco et al., 2008), los comportamientos asociados al dolor podrían estar alterados en estas niñas.

Medir o evaluar el dolor en niñas con Rett sigue suponiendo un reto a día de hoy debido a que, en humanos, la comunicación tanto verbal como no verbal (expresión facial, ritmo cardiaco, etc.) es la principal forma de medición del dolor en pacientes.

No obstante, en el caso de Rett, la expresión facial ante el dolor varía entre personas y edades, llegando incluso a estar ausente en algunos casos. Otro factor determinante a tener en cuenta son las alteraciones tanto motoras como comunicativas características de este síndrome. Por todo ello, a día de hoy sigue suponiendo un reto distinguir si las niñas con Rett realmente sienten menos dolor o si, por el contrario, son incapaces de manifestarlo de forma normativa.

En el presente capítulo decidimos realizar un estudio longitudinal para poder explorar la sensibilidad mecánica y térmica de un grupo de ratones hembra jóvenes y adultas (desde los 3 hasta los 6 meses de edad). También analizamos otros factores que pudiesen influir, tales como su peso, edad, severidad del síndrome o habilidad motriz.

Al igual que ocurre con las pacientes de Rett, el inicio de la sintomatología no ocurre a la misma edad en ratones mutantes para *Mecp2* (Guy et al., 2001). Por ello, decidimos analizar el "clasping" en el grupo de hembras (los animales mutantes sintomáticos encogerán sus patas traseras y/o delanteras en lugar de separarlas). Éste es un comportamiento típico y específico de este modelo animal, así como de otros modelos de enfermedades neurológicas (Garriga-Canut et al., 2012). El análisis del clasping nos permite además clasificar a las hembras heterocigotas en sintomáticas tempranas (manifiestan "clasping" a los 4 meses de edad) y tardías (lo manifiestan después). Observamos que, a la edad de 2 meses, menos de un 10% de las hembras heterocigotas manifestaban "clasping", porcentaje que se incrementa con la edad, llegando al 50% a los 5 meses (edad que elegimos como punto de corte), y al 92% a los 6 meses de edad. Tal y como era de esperar, ninguno de los animales salvajes mostró "clasping" a ninguna de las edades.

Puesto que existen estudios que relacionan la hiposensibilidad en humanos con la obesidad (Torensma et al., 2017), decidimos también analizar el peso de nuestras hembras. Observamos que el peso tanto de salvajes como heterocigotas aumentaba

con la edad, siendo las heterocigotas significativamente más obesas que las salvajes. Además, pudimos ver que las sintomáticas tempranas eran significativamente más obesas que las tardías. Esto sugiere una correlación entre la ganancia significativa de peso con la edad de aparición de los síntomas y, por tanto, severidad de los mismos. Por tanto, la falta de MeCP2 parece aumentar de forma directa o indirecta el peso. También se ha descrito un potencial papel de MeCP2 como coordinador del metabolismo lipídico en el hígado (Kyle et al., 2016). Además, la restauración del metabolismo lipídico por medio de fármacos de estatina es capaz de aliviar los síntomas motores en ratones mutantes para *Mecp2*, así como de incrementar su esperanza de vida (Buchovecky et al., 2013).

Utilizamos el test de Von Frey (VF) para analizar la mecano-sensibilidad de las mismas hembras. Sólo encontramos diferencias significativas al comparar de forma separada el grupo de sintomáticas tempranas y tardías con las salvajes. Sin embargo, se puede apreciar cómo las heterocigotas muestran una tendencia de hipersensibilidad comparada con las salvajes, hasta la edad de 5 meses, momento en que ambos grupos coinciden. A partir de este punto, las heterocigotas comienzan a separarse de las salvajes, mostrando una tendencia hacia la hiposensibilidad (ver figura 25). Por otro lado, observamos que las heterocigotas tardías mostraban una hipersensibilidad significativa en el VF a la edad de 3 y 4 meses, comparadas con las salvajes. Esto sugiere que, en estadíos presintomáticos, las hembras heterocigotas son hipersensibles, mientras que, a medida que el síndrome progresa y sus síntomas se agravan, parecen ir mostrando una mayor hiposensibilidad. Sin embargo, no podemos olvidar que muchos de estos síntomas incluyen alteraciones motoras y obesidad, los cuales podrían estar interfiriendo en la capacidad de estos ratones para retirar la pata durante el test. Las diferencias encontradas entre sintomáticas tempranas y tardías son un punto a tener en cuenta a la hora de trabajar con pacientes de Rett, puesto que muchos de los test que se realizan con estas niñas se

llevan a cabo cuando el síndrome ya ha sido diagnosticado y, por tanto, ya presentan sintomatología.

El test de placa caliente ("Hot plate test", HP) permite evaluar la sensibilidad a estímulos térmicos. Muchas de las hembras heterocigotas realizaban un movimiento extraño de retirada hacia atrás, algo que no ocurría en el caso de las hembras salvajes. Interpretamos que, dado que la mayoría de hembras heterocigotas son sintomáticas a la edad en que realizamos el HP (6 meses), muchas de ellas presentarían impedimentos motores severos, lo cual dificultaría su capacidad de lamerse las patas. Por eso, decidimos analizar tanto la latencia a lamerse las patas como a caminar hacia atrás, analizándolas de forma separada y combinándolas en una sola medida (latencia al primer comportamiento de evitación).

En esta medida, no apreciamos diferencias significativas entre genotipos en el grupo de hembras jóvenes (2 meses). En el caso de hembras viejas (6 meses), observamos diferencias significativas tanto en la latencia a lamer las patas, como en la latencia al primer comportamiento de evitación. En ambos casos, las hembras heterocigotas mostraban hipersensibilidad comparadas con las salvajes. Estos resultados sugieren que, en estados sintomáticos, las hembras heterocigotas siguen siendo hipersensibles a los estímulos térmicos. Además, nos recuerda que es importante tener en cuenta la demanda motriz que puedan requerir algunas de las pruebas realizadas cuando trabajamos con este tipo de modelos animales, que podrían estar enmascarando o interfiriendo en los resultados. Estos resultados apoyarían la hipótesis de una posible hipersensibilidad oculta o enmascarada por factores secundarios en ratones nulos para *Mecp2* (los cuales suelen ser completamente sintomáticos cuando se trabaja con ellos), algo que debe ser tenido en cuenta al trabajar con machos.

Para el análisis motor realizamos un análisis de la marcha. Pudimos observar un aumento significativo de la distancia de las patas traseras en heterocigotas a los 4 y 5 meses de edad. Esto podría deberse a una correlación directa entre la progresión del síndrome y la aparición de alteraciones motoras, o indirecta por medio de la obesidad que desarrollan las hembras heterocigotas, llevando a una mayor separación de las patas traseras. Apreciamos también una disminución significativa de la distancia de zancada de las patas delanteras derecha e izquierda en las heterocigotas de 6 meses, lo que concuerda con muchas de las alteraciones motoras observadas en el gateo de niñas con Rett (presentes en el 99% de los casos; Kaur & Christodoulou, 2019). Además, muchas de estas alteraciones no se manifiestan desde el primer momento en niñas, sino a medida que el síndrome progresa y empeora la sintomatología.

La proteína cFos es ampliamente utilizada como marcador de activación celular asociada al dolor, debido a que su expresión sólo se encuentra aumentada ante estímulos dolorosos (Dubner & Ruda, 1992; Todd et al., 2002; White et al., 2011). Encontramos una reducción significativa en el PAG de las hembras heterocigotas de 6 meses al compararlas con las salvajes. Esta región es considerada como el centro de control primario para la modulación y alivio del dolor. De hecho, la conexión entre el PAG y la corteza motora prefrontal parece estar implicada en la modulación de respuestas autonómicas al dolor (Mai, Juergen K; Paxinos, 2011), por lo que su menor activación podría alterar las respuestas motoras a estímulos dolorosos.

Como conclusión, es importante destacar la importancia de poder distinguir si los resultados obtenidos del análisis de dolor en pacientes de Rett se deben a alteraciones reales en la percepción de dolor reales o si, por el contrario, se debe a posibles discrepancias a la hora de expresar dicho dolor. También es importante tener en cuenta las limitaciones de muchas de las técnicas empleadas, así como las diferencias que pueden observarse en los resultados obtenidos en función de la progresión y severidad del síndrome.

DISCUSIÓN GENERAL

En la presente tesis, hemos centrado nuestra investigación en analizar déficits comportamentales y neuroanatómicos de un modelo de ratón de síndrome de Rett que habían recibido una atención menor que otras alteraciones más estudiadas (como la motora, intelectual o epilepsia). Ello podría ser de ayuda para el desarrollo de terapias y tratamientos más específicos y efectivos para este síndrome, mejorando así la calidad de vida de los pacientes de Rett.

Nuestros resultados del demostraron que las mutaciones en *Mecp2* no parecen comprometer la supervivencia de las células generadas en la zona subventricular. Además, la ausencia de MeCP2 parece incrementar los niveles de células que expresan doblecortina, un marcador de neuronas inmaduras, en áreas específicas del sistema olfativo tales como la corteza piriforme o el tubérculo olfativo. Esto apoya evidencias previas mostrando que mutaciones en *Mecp2* podrían derivar alteraciones en el proceso de maduración neuronal de forma dependiente de región.

Un hallazgo importante de esta tesis es que este proceso de maduración neuronal parece verse influido por estímulos ambientales como la separación materna. Este efecto es distinto en machos y hembras, y dependiente de región. Podría decirse, por tanto, que los estímulos estresores durante la edad temprana, varían el efecto de la mutación de *Mecp2*, aumentando o disminuyendo el número de neuronas inmaduras en determinadas regiones del sistema olfativo. Este efecto de la separación materna sugiere que las poblaciones de neuronas inmaduras no son estáticas, sino que pueden ser modificadas, abriendo las puertas a terapias que actúen sobre dichas poblaciones celulares y promuevan su maduración con el fin de aliviar los síntomas. En este sentido, explorar cómo otros factores como el enriquecimiento ambiental son capaces de influir en este proceso, podría suponer los cimientos para nuevos enfoques terapéuticos.

Por otro lado, descubrimos que la inervación de vasopresina dependiente de testosterona y, por tanto, sexualmente dimórfica, se encuentra, o incluso ausente, en determinadas áreas del cerebro social de los machos nulos para *Mecp2*. Esto, junto con el aumento en la investigación realizada por parte de los residentes salvajes hacia los intrusos nulos, sugieren menores niveles de testosterona en machos nulos. De hecho, se sabe que MeCP2 es capaz de regular de forma directa la inervación vasopresinérgica sexualmente dimórfica en ratas. Sorprendentemente, también encontramos menor inervación oxitocinérgica en la habénula lateral de los machos nulos. Aunque esta región no parece ser sexualmente dimórfica, sí que parece ser dependiente de niveles de testosterona (Hiroi et al., 2013; Shughrue et al., 1997). Todo ello sugiere la existencia de una interacción entre los déficits de MeCP2 y testosterona, promoviendo la reducción de esta inervación vasopresinérgica y oxitocinérgica.

Las alteraciones en el sistema de vasopresina y oxitocina estarían, a su vez, influyendo en los comportamientos típicos de estos machos. Algo parecido estaría ocurriendo con el sistema de óxido nítrico en estos machos. Nuestros resultados demostraron que la falta de MeCP2 incrementa la actividad de la $NADPHd^{+}$ (indicativo de una mayor producción de óxido nítrico). El óxido nítrico está, a su vez, implicado en la regulación de comportamientos agresivos en machos, de forma que, mayores niveles de óxido nítrico conducen a comportamientos menos agresivos. Todo ello explicaría la ausencia de agresión en el test de residente-intruso por parte de los ratones nulos para *Mecp2,* así como su falta de interés hacia el intruso. Sin embargo, no podemos obviar otros factores que podrían estar influyendo tales como un posible efecto intimidatorio, un mayor acicalamiento por parte de los nulos, problemas motores o incluso una mayor sensibilidad somatosensorial.

Existen también numerosas discrepancias en lo relativo a la sensibilidad al dolor en el caso de pacientes y modelos animales de Rett. Parte de la bibliografía defiende que los pacientes de Rett experimentan una menor sensibilidad al dolor, mientras

que otros estudios apuntan hacia lo contrario. Además, existe controversia a la hora de evaluar o medir la sensibilidad al dolor al trabajar con niñas con Rett, puesto que muchas de ellas carecen de las habilidades motoras o comunicativas necesarias como para expresar dicho dolor.

Observamos que, con la edad y progresión del síndrome y su severidad, aparecían algunos efectos adversos tales como un aumento desmesurado del peso, variaciones en la mecano-sensibilidad (hipersensibilidad en las sintomáticas tardías e hiposensibilidad en las tempranas), alteraciones motoras observables en la marcha de las mismas o hipersensibilidad a estímulos térmicos. Además, muchos de estos factores se influyen los unos a los otros: el sobrepeso o alteraciones motoras podrían dificultar la reacción a los estímulos dolorosos, modificando su respuesta a los mismos. Ello nos recuerda la importancia de valorar todos los posibles factores que puedan influir en las respuestas de estos ratones en las diferentes pruebas, y que podrían enmascarar una hiperalgesia no manifestada en animales con sintomatología severa. Además, estudiar a las hembras sintomáticas tempranas y tardías por separado, nos ofrece información valiosa a la hora de comprender mejor cómo evoluciona este síndrome, sobre todo considerando que la mayor parte de los test en pacientes de Rett se realizan cuando éstas ya son plenamente sintomáticas. En base a ello, muchos de los protocolos utilizados para analizar tanto la sensibilidad o dolor en pacientes y modelos de Rett como otros comportamientos y alteraciones, deben ser adaptados a las diferentes respuestas que puedan manifestar.

CONCLUSIONES

1. Los déficits de MeCP2 no parecen afectar a la supervivencia de las células generadas en la zona subventricular que pueblan los bulbos olfativos y estriado dorsal.

2. La falta de MeCP2 resulta en un aumento el número de neuronas inmaduras que expresan DCX en el sistema olfativo, de forma específica de región, afectando a la corteza piriforme y el tubérculo olfativo, pero no al bulbo olfativo. Ello sugiere que la falta de MeCP2 podría estar generando alteraciones en el proceso de maduración neuronal.

3. El estrés a edades tempranas, en forma de separación materna, afecta a esta población de células DCX^+ de manera específica de región, nivel de MeCP2 y sexo. Hipotetizamos que la separación materna promueve la maduración neuronal en el OBGr y Pir de hebras salvajes y heterocigotas; mientras que empeora las consecuencias de la falta de MeCP2, disminuyendo la maduración neuronal, en las células del Tu de las hembras heterocigotas, el dSt de machos salvajes y nulos, y el vSt y Pir de machos nulos.

4. La falta de MeCP2 produce una disminución de la inervación vasopresinérgica dependiente de testosterona en el cerebro social de ratones *Mecp2*-nulos, afectando específicamente al vmStP, LS, BSTMPI, LHb, vHip y dEn.

5. La ausencia de MeCP2 también disminuye la inervación oxitocinérgica en la habénula lateral, que también es dependiente de testosterona.

6. Una interacción entre la falta de MeCP2 y déficits de testosterona podrían llevar a las reducciones descritas en la inervación de AVP y OT descritas, sugiriendo un papel de este gen en el desarrollo de los sistemas sexualmente dimórficos.

7. Mutaciones en el gen *Mecp2* también producen un aumento de la actividad de $NADPHd^{+}$, lo cual es indicativo de una mayor producción de óxido nítrico.

8. Las alteraciones encontradas en el sistema nonapeptidérgico sugieren menores niveles de testosterona en los ratones *Mecp2*-nulos que, junto con los niveles aumentados de óxido nítrico, conducirían a alteraciones en los comportamientos agresivos y sociales de los ratones machos.

9. Los machos *Mecp2*-nulos no muestran comportamientos agresivos en el test de residente-intruso, sugiriendo una agresividad reducida. Sin embargo, debemos considerar la posibilidad de un efecto intimidatorio por parte de los machos salvajes, al ser éstos significativamente más grandes que los *Mecp2*-nulos.

10. Los ratones *Mecp2*-nulos dedican menos tiempo en investigar al intruso y más tiempo acicalándose. Esto puede deberse a diferentes factores como una pérdida de interés hacia el intruso, una mayor

somatosensibilidad que genere sensaciones desagradables al estar en contacto directo con los otros ratones, o a déficits motores que dificulten el acercamiento.

11. Las mutaciones en *Mecp2* en hembras producen un sobrepeso y obesidad más severos a medida que el síndrome progresa, sugiriendo una correlación entre los déficits de MeCP2 y el sobrepeso, que es dependiente de la severidad de la sintomatología.

12. Las hembras sintomáticas tardías son hipersensibles en el test de Von Frey a la edad de 3 y 4 meses, mientras que las sintomáticas tempranas muestran una tendencia hacia la hiposensibilidad, sugiriendo que las diferentes respuestas a la mecanosensibilidad se correlacionan con el momento de aparición y severidad de la sintomatología. Del mismo modo, debemos considerar todas las posibles variables que puedan estar ocultando una posible hiperalgesia no manifestada, como el sobrepeso o alteraciones motoras.

13. La mitad de las hembras heterocigotas sintomáticas no retiran o se lamen las patas en el test de placa caliente, sino que realizan un movimiento anormal de retirada hacia atrás, probablemente debido al sobrepeso que padecen y/o alteraciones motoras. Por lo tanto, se deben considerar todas las respuestas anormales ante estímulos dolorosos cuando se analiza la percepción del dolor en Rett.

14. Déficits en MeCP2 producen hipersensibilidad en el test de placa caliente, pero sólo en las hembras sintomáticas, sugiriendo que una

mayor termosensibilidad en las hembras heterocigotas se correlaciona con la progresión y severidad del síndrome.

15. Mutaciones en *Mecp2* producen una disminución de la activación cFos en el dlPAG de las hembras heterocigotas tras un estímulo térmico, probablemente produciendo un menor efecto analgésico modulado por esta región.

16. Los diferentes resultados y respuestas obtenidos en las hembras sintomáticas tempranas y tardías resultan de especial relevancia debido a que la mayoría de test para evaluar dolor y nocicepción se realizan en pacientes de Rett ya diagnosticadas, y no incluyen las posibles alteraciones que puedan ocurrir antes de la aparición de los síntomas.

17. Aunque el síndrome de Rett afecta principalmente a mujeres, gran parte de la investigación científica con modelos murinos se ha realizado en machos. Nuestros resultados resaltan la importancia de utilizar hembras en diferentes estadíos de la progresión del síndrome para así poder comprender completamente la aparición e interacción de los síntomas propios del fenotipo de Rett.

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