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The role of the methyl-CpG binding protein 2 (MECP2) in shaping vulnerability for psychopathology

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INDEX

INTRODUCTION

Chapter 1

1.1 Post-traumatic stress disorder

Exposure to traumas is defined in the 5th edition of the Diagnostic and Statistical Manual of Mental Disorder (DSM) as direct or witnessed exposure to threatened death, serious injury or sexual violence (American Psychiatric Association, 2013). According to the World Health Organization (WHO) this is an incredibly common experience worldwide, with 70% of people being exposed to traumatic events throughout their lives (Kessler et al., 2017). Percentages as well as trauma types variy across countries, depending on different sociodemographic and geographic predictors (Benjet et al., 2016).

Trauma exposure may lead to multiple adverse consequences for physical and mental health and is compulsory for the diagnosis of post-traumatic stress disorder (PTSD), a chronic mental disorder characterized by symptoms of persistent anxiety and hypervigilance (see paragraph 1.1.1) (American Psychiatric Association, 2013). It is worth noting that the onset of a chronic PTSD symptomatology is an uncommon eventuality in the aftermath of traumatic experiences, with only an average 5.6% of exposed individuals developing PTSD in the world, and resilience being the prevalent outcome (Koenen et al., 2017). Nevertheless, the high socio-economic costs and impressive burdens that PTSD symptomatology represents for the affected individuals (Koenen et al., 2017) urge the identification of vulnerability factors.

1.1.1 A clinical overview

Individuals who develop PTSD in the aftermath of a traumatic event experience a number of highly distressing and profoundly invalidating symptoms grouped in three main clusters: re-experiencing (intrusive memories or thoughts, in the form of nightmares and/or flashbacks), hyperarousal (continuous monitoring for potential threats, enhanced startle reactivity and sleep problems), and avoidance of trauma reminders (American Psychiatric Association, 2000). In the last update of the DSM a further category, comprising negative alterations in cognitions and mood, has been added (American Psychiatric Association, 2013). Also, PTSD has been moved from the "*Anxiety disorders*" to the newborn "*Trauma- and Stressor-Related disorders*" category (American Psychiatric Association, 2013) based on the fact that, unlike other nosologic groups, exposure to adverse events is the triggering factor, and is a fundamental criterion to be met for the diagnosis of PTSD (Pai et al., 2017; Shalev et al., 2017).

While trauma exposure is a prerequisite for the diagnosis, the clinical presentation of PTSD is highly variable and the relative predominance of different symptoms may change among individuals

or even over time in the same subject. Importantly, PTSD symptoms should last more than 1 month and should not be attributable to the physiological effect of a substance or other medical conditions. A proper assessment of trauma exposure and symptoms is necessary for PTSD diagnosis and is generally achieved through validated measures, often in the form of interviews or self-reports. Among them, the most used is the Clinician-Administered PTSD Scale (CAPS), a structured interview designated to be administered by clinicians or researchers with working knowledge of PTSD, conceived to make both a categorical PTSD diagnosis and to provide a measure of PTSD symptom severity (National Center for PTSD, 2017).

Symptom persistence varies across patients, with a mean duration of 6 years estimated by the World Health Organization (WHO, Kessler et al., 2017; Watson, 2019). The global burden represented by PTSD is further increased by its high comorbidity with numerous other medical conditions, ranging from depression, anxiety and substance abuse disorders to type 2 diabetes and cardiovascular diseases (Watson, 2019). Besides, PTSD diagnosis is associated with accelerated aging and premature death (Koenen et al., 2017; Watson, 2019).

Although PTSD represents a major health challenge, the currently available treatments are only partially effective. Trauma-focused cognitive behavioral therapies are considered the first-line treatments for PTSD. By revisiting the distressing elements of the traumatic event, these techniques help the patient to contextualize the traumatic memory, improving both avoidance and cognitive symptoms (Shalev et al., 2017). However, up to 50% of cases have been found non-responsive to psychological therapies (Kar, 2011; Richter-Levin et al., 2019). To increase therapeutic efficacy, it is widespread practice to combine psychotherapy with medication using selective serotonin or norepinephrine reuptake inhibitors (SSRIs or SNRIs), the first- line pharmacotherapies for PTSD (Hoskins et al., 2015; Marshall & Cloitre, 2000). Systematic data regarding the efficacy of such a combined approach, however, lack consistency, boosting the need for innovative or tailored interventions.

1.1.2 Psychobiological alterations in post-traumatic stress disorder

The complex clinical symptoms associated with PTSD psychopathology are attributable to alterations in a number of different systems or mechanisms regulating specific (patho)physiological and psycho(patho)logical processes. Numerous psychological and biological models have been proposed to explain PTSD symptomatology, which involve dysfunctions in emotion regulation, fear learning, contextual processing and salience detection and the associated brain circuits (Figure 1).

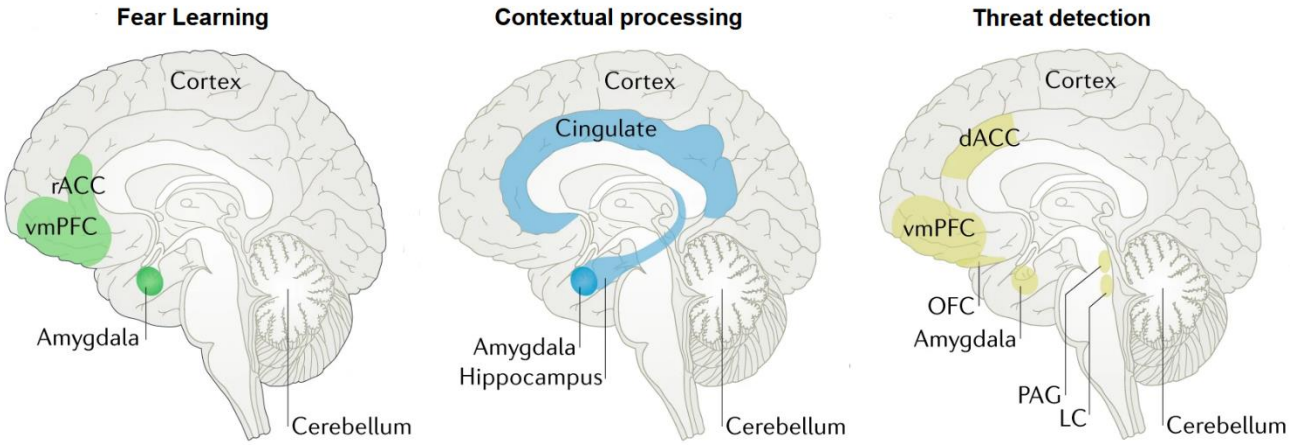

Figure 1 - Psychological mechanisms and brain networks implicated in post-traumatic stress disorder (PTSD) symptoms. The three mid.sagittal sections here illustrated highlight brain regions that have been linked to PTSD-related psychological processes: fear learning, contextual processing, and threat detection. dACC, dorsal anterior cingulate cortex; LC, locus coeruleus; OFC, orbitofrontal cortex; PAG, periaqueductal grey; rACC, rostral anterior cingulate cortex; vmPFC, ventromedial prefrontal cortex. Modified from Fenster et al., 2018.

Altered learning of fearful contents has been the prime candidate to explain the persistence of fear in PTSD patients, and inspired the most common psychotherapies that are currently used as first-line treatments. In particular, deficient extinction processes and safety learning deficits are thought to occur and are attributed to dysfunctions at the level of the amygdala and the ventromedial prefrontal cortex (vmPFC), which have been repeatedly reported to be hyperactive and hypoactive, respectively, in PTSD (Hayes et al., 2012). While in physiological conditions the vmPFC inhibits fear expression by constraining amygdala activation, the fear learning model of PTSD hypothesizes that the vmPFC fails to inhibit the amygdala, thereby leading to increased fear responses and impaired extinction in patients (Shalev et al., 2017).

Failure in the proper processing of contextual information is also thought to account for the generalization of fear that occurs in patients, and probably lies at the basis of intrusive memories, core features of PTSD symptomatology, during which internal or external trauma reminders trigger involuntary recalls of trauma-related fragmented images, with incredibly vivid sensory details. Inappropriate contextualization of salient cues, that are perceived as threatening even in safe situations, also accounts for persistent hypervigilance and overreactivity to salient stimuli (Acheson et al., 2012; Shalev et al., 2017). The correct elaboration of contextual information appears to depend on the functionality of the hippocampus, and its connections with the amygdaloid nuclei. According to the *dual representation theory*, in the fear circuitry physiology the strong hippocampal-amygdalar relationship allows hippocampus to act as a modulator during amygdala-dependent encoding of fear memory, providing it with contextual specificity and integration in the declarative memory system (Desmedt et al., 2015). Amygdala hyperactivation, together with reduced hippocampal dimensions, which have been consistently reported in multiple PTSD cohorts, would account for deficits in

hippocampal-amygdalar interaction leading to the absence of a proper contextual indexation of the traumatic event in patients (Liberzon & Ressler, 2016).

Dysfunctional threat detection is also attributed to alterations at the level of the salience network, a large-scale brain network of interconnected regions, primarily composed by the insula and the dorsal anterior cingulate cortex (dACC) that are involved in filtering and detection of salient stimuli. The hyperactivation of these areas in the brain of PTSD patients may account for the increased attentional bias towards threat that is likely to drive hyperarousal (Shalev et al., 2017). The prioritization of sensory events appears to be the result of the tight communication occurring between the salience network and the norepinephrine-locus coeruleus (NE-LC) system (Lee et al., 2020). Consistently, salient stimuli were found to trigger LC hyperactivation in PTSD patients, and atypically high noradrenergic phasic responses are thought to drive behavioral and autonomic hyperreactivity to unexpected stimuli (Naegeli et al., 2018).

Norepinephrine signaling is known to be altered in people with a PTSD diagnosis, who display increased NE levels in both the blood and the cerebrospinal fluid (CSF), which, importantly, positively correlate with symptom severity (Schmidt & Vermetten, 2018). Beyond its central function in gating sensory stimuli, peripheral noradrenergic transmission is also profoundly involved in the immediate response to potential threats. Norepinephrine is indeed the main effector hormone of the sympathetic nervous system (SNS), and is released within few seconds from threat detection in the periphery, triggering a series of autonomic reactions overall known as the figh-or-flight response (Merz & Wolf, 2017). Consistent with an increased activation of the SNS in patients, the therapeutic use of adrenergic blockers was shown effective in the treatment of PTSD-associated psychopathology (Liberzon & Ressler, 2016).

Alongside the alterations found at the level of the SNS, the hypotalamic-pituitary-adrenal (HPA) axis is profoundly dysregulated in PTSD pathophysiology (Figure 2). As the SNS, the HPA axis is also rapidly activated upon the presentation of a stressor at the level of the paraventricular nucleus of the hypothalamus (PVN). Here, specialized neurons secrete the neurohormones corticotropin releasing hormone (CRH) and arginine vasopressin (AVP), starting a cascade of events ultimately triggering a slightly delayed rise in circulating glucocorticoids (GC), which peaks 20-30 min after the stress onset. Predictably, increased CSF concentration of CRH was described in PTSD patients, that, however, do not show a consistent profile with regard to GC levels alteration (Liberzon & Ressler, 2016; Schmidt & Vermetten, 2018). It is worth noting that, in order to restore the homeostasis in the aftermath of a threat, the HPA axis exerts a tight self-regulatory activity through the negative feedback action of circulating GC. By binding to mineralcorticoid (MR) and glucocorticoid receptors (GR)

throughout multiple brain regions, with the help of the co-chaperone FK506 binding protein 51 (FKBP5), GC mediates HPA axis shut down. This mechanism is likely altered in PTSD pathophysiology, which has been traditionally associated with GR hypersensitivity (Banerjee et al., 2017; Sarapas et al., 2011; van Zuiden et al., 2013). Recent evidence, however, suggests the existence of biologically distinct subsets of PTSD patients, with opposite endocrine profiles (hyper- and hypocortisolism), explaining the lack of consistent finding on HPA dysregulation (Castro-Vale et al., 2016; Mehta et al., 2011; Zaba et al., 2015).

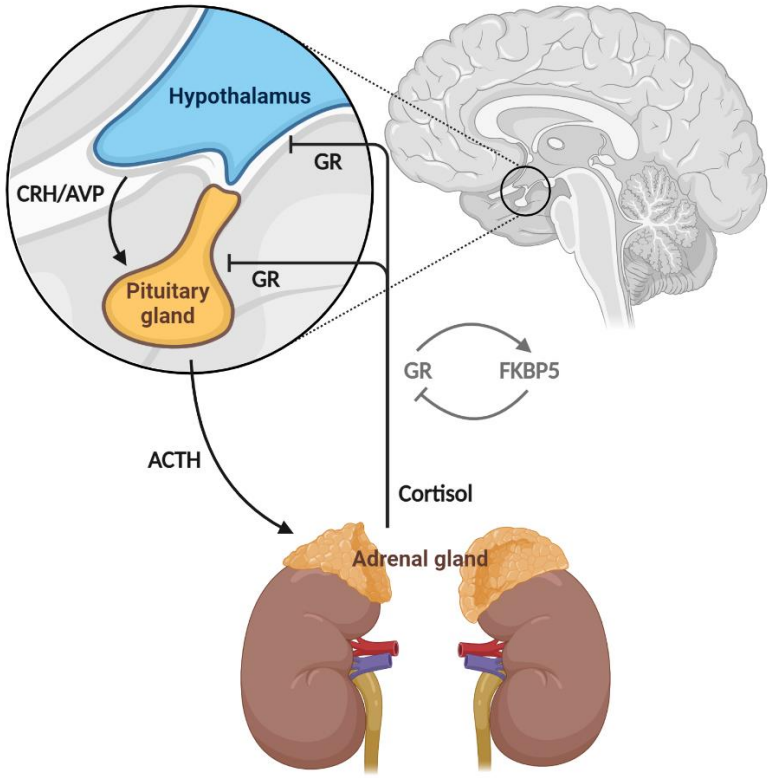

Figure 2 – **The hypothalamic-pituitary-adrenal axis (HPA).** Corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) are released from the paraventricular hypothalamic area in the hypophyseal portal system within few seconds from stressor presentation and stimulate the release of adrenocorticotrophin hormone (ACTH) from the anterior pituitary. This in turn triggers the release of the glucocorticoid hormone cortisol (corticosterone in rodents) from the adrenal cortex. After stress termination, glucocorticoids, via their receptors (GR), exert a negative feedback on the central axis components (hypothalamus and pituitary gland), which is responsible for the termination of the axis activation. GR activity is tightly regulated by the co-chaperone FKBP51 that inhibits GR binding affinity with glucocorticoids. Created with BioRender.com.

Besides the stress systems and fear and arousal brain circuitries, a plethora of other networks have been extensively studied for their role in PTSD, and are thought to be involved in the etiology of the disorder (Bandelow et al., 2017; Shalev et al., 2017; Southwick et al., 1999; Yehuda, Hoge, et al., 2015), thus contributing to a complex portrait of the psychobiology underlying this condition. These include different neurotransmitters and neuropeptides, comprising monoamines, such as dopamine and serotonin, and neurotrophins such as the brain-derived neurotrophic factor (BDNF).

1.1.3 An etiological hypothesis for post-traumatic stress: vulnerability to traumas

Given its conditional nature, PTSD is the only mental disorder in which the triggering factor, the trauma, can be easily identified (Almli et al., 2014). As stated above, however, there is a conspicuous variability in the tendency of trauma-exposed individuals to develop PTSD symptoms. Traumatic stressor characteristics play a role in increasing the propensity to experience PTSD: interpersonal assault-involving traumas, such as rape and combat, are indeed associated with major risk compared with natural disasters or motor vehicle accidents (Kremen et al., 2012; Stein et al., 2002). Other environmental load factors, such as previous exposure to traumatic stress, particularly during childhood, account for the development of PTSD following the triggering trauma (Lanius et al., 2010). However much of the variance in trauma vulnerability relies on individual differences rather than on external causes. Few recognized familial risk factors include impaired general intellectual ability, represented by lower intelligence quotient (IQ), attention and executive function deficits, as well as female gender, personality traits (neuroticism) and pre-existing mental conditions (depressive or anxiety disorders) (Kremen et al., 2012; Stein et al., 2002). Several physiological parameters have also been proposed as potential vulnerability markers, including startle hyperresponsivity during provoked fear, slower post-stress cardiovascular recovery and decreased heart rate variability, as well as increased cytokine reactivity following laboratory stressors (Walker, F.R. et al., 2017).

These neurobehavioral and physiological traits have to be interpreted as intermediate phenotypes indexing the underlying genetic susceptibility to the negative outcomes of trauma exposure (Jakovljevic, 2019; Lebois et al., 2016). As for other mental disorders, indeed, a component of the interindividual differences in vulnerability is genetically determined, giving to PTSD the aspect of a multifactorial polygenic disorder (Almli et al., 2014; Liberzon & Ressler, 2016). Support for genetic influence on PTSD susceptibility comes from classical twin studies, which estimated a heritability of ~30% in men and up to 70% in women for the probability to develop the disorder after trauma exposure, net of the genetic, personality-driven, influence on risk of experiencing assaultive traumas (Kremen et al., 2012; Sartor et al., 2011; Stein et al., 2002; True et al., 1993).

Importantly, it is now increasingly evident that inborn predisposition is continuously modulated by environmental challenges by the means of epigenetic mechanisms, which account for the development of acquired vulnerability. The epigenetic outcome of such interplay is often strictly dependent on the genetic substrate, with specific alleles being differentially modulated, and on stressor timing, since various time windows may trigger specific outcomes, giving rise to a complex link between genes and environment (Kremen et al., 2012; Zannas et al., 2015).

1.1.3.1 Genetic factors

The heritable nature of PTSD has encouraged research on genes implicated in risk for the development of the disorder after trauma exposure. The individuation of genetic variants associated with PTSD can contribute in many different ways to our understanding of the disease. Indeed it would help identifying strong biomarkers of vulnerability (DNA remains genetically unmodified over the life course) and disentangling the biological mechanisms underlying the disorder, two important aspects to address in order to develop preventive or acute interventions.

Plenty of research on PTSD genetics has so far been based on candidate gene approaches. Through this methodology, genetic variation within pre-specified genes of interest is associated with trauma vulnerability, in terms of higher frequency among PTSD cases or correlation with typical disease endophenotypes. Using this methodology a number of genes has been identified as being involved in the etiology of PTSD.

Due to its role in PTSD pathophysiology, and its significance as therapeutic target, the serotoninergic system has been one of the most explored systems for disease-risk variants. The most studied variant is located within the serotonin transporter gene (*SLC6A4*/5HTT), where the promoter variable number tandem repeat (VNTR) polymorphism (5-HTTLPR) has been repeatedly associated to PTSD risk. Findings are, however, conflicting with regard to the vulnerability-associated alleles. Indeed, although several studies reported the short (S) allele to be risky, few studies have found reversed associations (Ryan et al., 2016). Significant links have been also reported between PTSD and other neurotransmitter- and neuropeptide-related genes. However, many of these findings could not be replicated in different studies (see Table 1 for further details). Interestingly, genetic variation within the gene encoding for the brain-derived neurotrophic factor (*BDNF*), whose plasma levels have been proposed to mark past-trauma load (Nees et al., 2018), has been associated with PTSD prognosis. Indeed, carriers of the minor *BDNF* allele variant (Methionine, Met) at rs6265 (Val66Met polymorphism) displayed increased symptom severity, particularly with regard to intrusions, compared to Valine/Valine (Val/Val) carriers (Pitts et al., 2019). *BDNF* allelic variants have also been shown to influence the efficiency of recovery processes, with the Val/Val genotype being associated with PTSD symptom improvement, thus representing a potential protective factor (Lebois et al., 2016). However, results from a recent meta-analysis provide little support for rs6265 genetic variance as a predictor of PTSD (Bountress et al., 2017).

Table 1 – Summary of the major findings of PTSD risk genetic variation

Gene		Polymorphism			Ethnicity	References
Function	**name**	**name**	**type**	**alleles**		
Candidate gene studies						
neurotransmitter systems	*SLC6A4*	5-HTTLPR	VNTR	Long>Short*	USA Eur & AA & other, Eur, Korean, Rwandan, Turkish, Israeli, Armenian	***Reviewed in:*** *Ryan et al., 2016; Voisey et al., 2014;* ***Meta-analysis:****Zhao et al., 2017*
	5HTR2A	rs6311	SNP	G>A	USA Eur & AA, Eur, Korean	***Reviewed in:*** *Ryan et al., 2016; Voisey et al., 2014*
	SLC6A3	rs28363170	VNTR	10 rpt>9 rpt	USA Eur & AA & other, Eur, Israeli, Hispanic	***Reviewed in:*** *Ryan et al., 2016; Voisey et al., 2014;* ***Meta-analysis:*** *Li et al., 2016*
	DRD2	rs1800497	SNP	C>T	USA Eur, Eur, Aus Eur & mixed	
	COMT	rs4680 (Met158Val)	SNP	G>A(Met)	USA Eur & other, Hispanic, Eur, Rwandan	
	GABRA2	rs279836	SNP	T>A	Eur, Aus Eur	*Nelson et al., 2009*
		rs279826	SNP	A>G		
		rs279871	SNP	T>A		
neuropeptide systems	*NPY*	rs16147	SNP	T>C	USA Eur, Eur	*Watkins et al., 2017*
neurotrophic factors	*BDNF*	rs6265 (Met66Val)	SNP	G>A(Met)	USA Eur, Eur, Aus Eur, Korean	*Hori et al., 2020; Pitts et al., 2019;* ***Meta-analysis:*** *Bountress et al., 2017*
stress	*ADCYAP1R1*	rs2267735	SNP	C>G	USA AA & Eur & other, Chinese	***Meta-analysis:*** *Lind et al., 2017*
	CRHR1	rs12938031	SNP	A>G	USA Eur, Eur	***Reviewed in:*** *Ryan et al., 2016; Voisey et al., 2014;* ***Meta-analysis:*** *Sheerin et al., 2020*
	FKBP5	rs9296158	SNP	A>C	USA AA & Eur, Eur	
		rs3800373	SNP	C>A		
		rs1360780	SNP	T>A		
		rs9470080	SNP	T>A		
	NR3C1	rs258747	SNP	A>G	USA Eur, Eur, Chinese	
immune system	*CRP*	rs1130864	SNP	C>T	USA AA	*Michopoulos et al., 2015*
GWAS studies						
chronobiology	*RORA*	rs8042149	SNP	T>C	USA AA & Eur, Eur	*Amstadter et al., 2013; Loguel et al., 2013*
	NLGN1	rs6779753	SNP	G>T	USA AA	*Kilaru et al., 2016*
osteogenesis	*TLL1*	rs6812849	SNP	C>A	USA AA & Eur, Eur	*Xie et al., 2013*
ncDNA	*LINC01090*	rs10170218	SNP	A>C	USA AA & Eur, Eur	*Guffanti et al., 2013*
	upstream ZDHHC14	rs34517852	SNP	T>A	USA Eur, Eur	*Nievergelt et al., 2019*
	MIR5007	rs115539978	SNP	C>T	USA AA, African	*Nievergelt et al., 2019*
neurotransmitter systems	*PARK2*	rs9364611	SNP	C>T	USA Eur, Eur	*Nievergelt et al., 2019*

Abbreviations: ncDNA – non-protein coding DNA; SLC6A4 – serotonin transporter gene; 5HTR2A – serotonin receptor 2a; SLC6A3 – dopamine transporter gene; DRD2 – dopamine receptor 2; COMT - catechol-O-methyltransferase; GABRA2 – GABA type A receptor subunit α2; NPY – neuropeptide Y; BDNF – brain-derived neurotrophic factor; ADCYAP1R1 - ADCYAP receptor type I; CRHR1 – CRH receptor 1; FKBP5 - FK506 binding protein 5; NR3C1 – glucocorticoid receptor gene; CRP - C-reactive protein; GWAS – genome-wide association study; RORA - retinoid-related orphan receptor A; NLGN1 – neurologin 1; TLL1 – tolloid like 1; LINC01090 - long intergenic non-protein coding RNA 1090; ZDHHC14 - zinc finger DHHC-type palmitoyltransferase 14; MIR5007 - microRNA 5007; PARK2 – parkinson protein 2; VNTR – variable number tandem repeat; SNP – single nucleotid polymorphism; rpt – repetitions; G – guanine; A – adenine; T – thymidine; C – cytosine; Met – methyonine; Val – valine; AA - African American; Aus - Australian; Eur - European descent (excluding Hispanic descent for USA populations). Symbols: underlined – risk allele; * - conflicting results.

Most importantly, numerous genetic *loci* encoding key modulators of the stress response have been addressed in candidate gene studies for PTSD. Compelling results have been obtained for the gene encoding the pituitary adenylate cyclase-activating polypeptide receptor 1 (*ADCYAP1R1* – PACAPR1), which is highly expressed in fear brain circuitries, where it modulates neuroendocrine (including CRH) release in response to stressors (Lebois et al., 2016). A SNP disrupting a putative estrogen response element (rs2267735) has been consistently associated with PTSD diagnosis and symptoms in traumatized women only, preserving significance also after controlling for ancestry (Voisey et al., 2014). A further promising candidate, which received much attention and has been comprehensively studied, is the *FKBP5* gene, encoding for the homonymous GR chaperone. Genetic variation within this *locus* revealed four functional, highly linked SNPs (rs9296158, rs9470080, rs3800373, and rs1360780) strongly associating with PTSD susceptibility or resilience in case of history of childhood abuse (Lebois et al., 2016), although findings were mixed with varying ethnicity (Ryan et al., 2016).

Since the hypothesis-driven selection of target genes is an important limitation for the detection of genetic *loci* associated with a disorder whose biological underpinnings have not been elucidated yet, genome-wide association studies (GWAS) have recently grown in popularity as promosing unbiased methods to uncover the polygenic nature of PTSD. Unfortunately, even the most promising findings from candidate gene approaches failed replication in GWAS studies, that instead identified novel potential genes of interest for PTSD etiology (see Table 1). For instance, the identification of genes encoding for the retinoid-related orphan receptor (*RORA*), a cross-tissue cycling protein regulating the cyrcadian expression of downstream clock genes (Sato et al., 2004), and neuroligin 1 (*NLGN1*), a synaptic adhesion molecule involved in sleep-wake regulation (El Helou et al., 2013), highlighted the key role of chronobiology in trauma susceptibility (Banerjee et al., 2017). Moreover, GWAS approaches underlined the possible significance of the non-protein coding genome in PTSD development, uncovering disease-associated genetic variance at the level of multiple non-coding RNA *loci* (Daskalakis, Provost, et al., 2018; Daskalakis, Rijal, et al., 2018). As for candidate gene associations, however, GWAS findings failed replication in larger or independent cohorts, probably due to sample heterogeneity and moderate statistical power. Recent well-powered trans-ethnic GWAS meta-analyses from the Psychiatric Genomic Consortium for PTSD (PGC-PTSD) failed to identify specific *loci* significantly related to *PTSD* risk in the overall sample (Duncan et al., 2018; Nievergelt et al., 2019). However, analyses stratified by ancestry and sex revealed up to 6 independent genome-wide significant *loci*, spanning the non-coding genome and novel dopamine-related genes

(Nievergelt et al., 2019 and Table 1). Of major importance, these studies were for the first time able to construct a polygenic risk score (PRS) for PTSD, which demonstrated predictive value for re-experiencing symptoms and showed high positive correlation with polygenic risk from other mental disorders, including major depression and schizophrenia (Duncan et al., 2018; Nievergelt et al., 2019).

Although these recent findings provide hope that analysis of larger samples might shed light on PTSD genetic susceptibility, a clear and consistent association between genetic variability and PTSD risk has yet to be confirmed (Nievergelt et al., 2019). Contributions to conflicting results have been suggested to derive from the heterogeneous nature of the disease, which reduces the detectable effect of allele variant liability. A possibility to overcome this issue relies on candidate genes or GWAS studies focusing on the genetic architecture of basic psychological and neurobiological dimensions, which are shared among different diagnostic categories, as suggested by the Research Domain Criteria (RDoC) initiative (Nees et al., 2018; Schmidt & Vermetten, 2018).

Another aspect that should not be underestimated, however, is that genetic approaches completely lack information regarding the environmental contribution to trauma susceptibility, which has been demonstrated to play an important role in the etiology of the disorder (Daskalakis, Rijal, et al., 2018). This lack of perspective may also account for the inconsistent results of genetic studies, supporting the need for research on gene x environment interaction mechanisms at the basis of PTSD risk.

1.1.3.2 Epigenetic signatures

Epigenetic mechanisms mediate the effects of trauma exposure on the genome, influencing gene expression and consequently affecting brain function up to symptom onset, thus integrating the two main etiological factors of PTSD: allelic predisposition and traumatic events. Importantly, being sensitive to the environment in general, epigenetic signatures represent a portrait of an individual's experiences and lifestyle, which are known to modulate and influence vulnerability traits (Zovkic et al., 2013). In this view, epigenetic marks are closer to define an integrative PTSD risk profile than gene variants *per se*. However, their environmental responsivity, itself, challenges the finding of consistent associations in the clinical framework, given that every subject is likely to have lived unique experiences that may influence their epigenetic profile.

Epigenetic regulation of gene expression takes place through multiple mechanisms of which DNA methylation is the most investigated in PTSD research. Comparable to candidate gene studies, differential methylation in targeted *loci* has been implicated in the pathogenesis of the disorder (Table

2). For instance, *SLC6A4* methylation levels were found to modulate the outcome of multiple traumatic events, with higher methylation protecting from PTSD onset in case of major trauma load (Koenen et al., 2011). Similarly, the degree of methylation of the glucocorticoid-induced leucine zipper (*GILZ*) *locus* was recently found to quantify lifetime traumatic events and to associate with PTSD diagnosis (Lebow et al., 2019). Moreover, although polymorphisms in the GR gene failed to be consistently associated with PTSD outcome, decreased methylation of the promoter region, leading to heightened GR expression, was found to correlate with symptom severity in multiple studies (Labonté et al., 2014; Vukojevic et al., 2014; Yehuda, Flory, et al., 2015). Epigenetic signatures at the *ADCYAP1R1 locus* have also been linked to PTSD, with higher methylation levels associating with PTSD diagnosis regardless of gender (Ressler et al., 2011).

Table 2 – Summary of the major findings of candidate epigenetic association studies

Gene		Allele specificity	Methylation	Ethnicity	References
Function	**Name**				
neurotrasmitter systems	*SLC6A4*	no	↓	USA AA	*Koenen et al., 2011*
	SLC6A3	rs28363170 (9rpt)	↑	USA AA	*Chang et al., 2012*
	COMT	rs4680 (Met)	↑	USA AA	*Norrholm et al., 2013*
neurotrophic factors	*BDNF*	no	↑ and ↓*	Korean, Australian	*Kim et al., 2017; Voisey et al., 2019*
stress	*ADCYAP1R1*	rs2267735 (C)	↑	USA AA	*Ressler et al., 2011*
	FKBP5	rs1360780 (T)	↓ (childhood trauma)	USA AA	*Klengel et al., 2013*
	NR3C1	no	↓	Rwandan, Eur, USA Eur	***Reviewed in:*** *Zannas et al., 2015*
	TSC22D3	no	↓	USA AA	*Lebow et al., 2019*
immune/inflammatory system	*AIM2*	no	↓	USA Eur & AA	*Miller et al., 2018*
	IL18	no	↑	USA Eur & AA	*Rusiecki et al., 2013*
ncDNA	*H19*	no	↑	USA Eur & AA	*Rusiecki et al., 2013*
	LINE1	no	↓	USA Eur & AA	*Rusiecki et al., 2012*
	Alu	no	↑	USA Eur & AA	*Rusiecki et al., 2012*

Abbreviations: ncDNA – non-protein coding DNA; $SLC6A_4$ – serotonin transporter gene; $SLC6A_3$ – dopamine transporter gene; COMT - catechol-O-methyltransferase; GABRA2 – GABA type A receptor subunit α_2; BDNF – brain-derived neurotrophic factor; ADCYAP1R1 - ADCYAP receptor type I; FKBP5 - FK506 binding protein 5; NR3C1 – glucocorticoid receptor gene; TSC22D3 – glucocorticoid-induced leucine zipper gene; AIM2 - absent in melanoma 2; IL18 - interleukin 18; LINE1 - long interspersed nuclear element 1; rpt – repetitions; Met – methyonine; C – cytosine; T – thymidine; AA - African American; Eur - European descent (excluding Hispanic descent for USA populations). Symbols: ↑ - increased; ↓ - decreased; * - conflicting results.

Of particular interest for a mechanistic explanation of the interaction between genetic and environmental factors driving vulnerability, are findings of genotype-specific epigenetic changes at the level of PTSD risk-associated *loci*. Indeed, the increased PTSD risk attributed to the 9 repeat allele of the DAT_1 gene (*SLC6A3*) was found to depend on an allele-specific increase in promoter methylation, which doubled the likelihood of symptom development in the aftermath of traumatic experiences (Chang et al., 2012). Similarly, higher methylation levels at the promoter of *COMT*

rs4680 Met alleles appear to mediate the impairments in fear inhibition outlined in Met/Met patients (Norrholm et al., 2013).

Importantly, life events appear to exert different effects on the epigenome depending not only on their nature, but also on the timing of their occurrence. It is indeed possible to discriminate different time windows of genome susceptibility to changes of especially lasting nature (Lupien et al., 2009). Among them, childhood experiences appear to trigger particularly durable epigenetic modulation of gene expression patterns, whose effects become evident only later in life, in terms of increased susceptibility to stressors. Not surprisingly, data on many putative risk alleles, which failed to reach a strong correlation with PTSD outcome for inconsistency across studies, when corrected for childhood traumas, showed a clear association with disease susceptibility, symptomseverity or diagnosis. Only in combination with early life adversities *NR3C1* risk alleles carriers showed a higher number of GR in peripheral blood mononuclear cells (PBMs) and appeared to be more prone to develop PTSD symptoms (van Zuiden et al., 2013). Furthermore, genetic variation at the *ADCYAP1R1* and GABA type A receptor subunit α_2 (*GABRA2*) genetic *loci* interacts with childhood adversities to shape the risk for PTSD in adulthood (Almli et al., 2014; Nelson et al., 2009). These effects are likely mediated by allele-specific epigenetic modulation following stress in early life. In this line, epigenetic mechanisms have been proposed to explain the moderating effects that childhood adversities exert on the association between specific *FKBP5* genetic variants and PTSD susceptibility. For instance, Metha et al. (2011) found that the clinical condition (healthy or PTSD) of trauma-exposed rs9296158 risk allele carriers was paralleled by an opposite regulation of *FKBP5* expression. Similarly, homozygosity for the risk allele within the rs9470080 polymorphism was found to trigger resilience or vulnerability to PTSD symptoms onset depending on childhood trauma exposure (Xie et al., 2010). This framework led Klengel et al. (2013) to postulate an epigenetic mechanism for the interaction between specific *FKBP5* allelic variants and early life adversities. Risk alleles within the *FKBP5* SNP rs1360780 were found to display higher protein expression in stressful circumstances, leading to excessive cortisol release following early life stress exposure. Notably, glucocorticoid receptor activation has been shown to induce local changes in DNA methylation, thus providing an explanation for the allele-specific de-methylation of the *FKBP5 locus* occurring following childhood adversities.

An intriguing aspect outlined by epigenetic research on PTSD regards the intergenerational transmission of trauma effects, which appear to be mediated by epigenetic mechanisms (Jawaid et

al., 2018; Yehuda & Lehrner, 2018). For instance, PTSD in mothers exposed to the Tutsi genocide while pregnant, was associated with epigenetic modifications in the GR gene that were similarly found in their children (Perroud et al., 2014). Moreover, the first association of preconception parental trauma with epigenetic alterations in the offspring has been provided by Yehuda et al. (2016), who found opposite methylation levels at the *FKBP5 locus* in Holcaust survivors and their offspring. However, the present evidence does not allow to infer any mechanistic explanation for these phenomena; it demonstrates that the influence of trauma exposure on the epigenetic apparatus passes through generations.

Unbiased approaches are also available for interrogating the PTSD epigenome. Epigenome-wide associationstudies (EWAS) analyzing methylated CpG sites throughout the DNA are growing, and findings generally conform with current knowledge regarding PTSD etiology, with inflammatory and immune systems, endocrine, neuropeptide and synaptic plasticity pathways being principally represented among differentially methylated genes (Daskalakis, Rijal, et al., 2018). Epigenome-wide approaches have recently also been shown to be helpful for interrogating the phenotypical heterogeneity of PTSD patients in order to characterize distinct disease-associated biotypes on the basis of their DNA methylation profiles (Yang et al., 2020). In this line, a former EWAS study already evidenced the profound differences in the epigenetic profile of PTSD patients exposed or not exposed to childhood maltreatment (Mehta et al., 2013), confirming that PTSD subgroups may be distinguished by investigating DNA methylation.

However, sample sizes examined to date in EWAS studies are rather small, accounting for underpowered analyses. To address this issue, an EWAS working group has recently been formed within the Psychiatric Genomics Consortium for PTSD, with the goal to elucidate the epigenetic alterations underlying PTSD pathophysiology by means of well-powered, multi-site meta-analyses (Ratanatharathorn et al., 2017).

1.1.3.3 Experimental designs for vulnerability studies

When searching for PTSD vulnerability markers, the environmental responsivity of epigenetic mechanisms may represent both a blessing and a curse. A blessing because, as mentioned above, epigenetic signatures integrate inherited and acquired risks, thus providing an exhaustive profile of biological susceptibility. A curse, since traumatic events are likely to introduce changes that prevent the possibility to distinguish vulnerability factors from PTSD correlates, when dealing with patients. Besides, trauma unpredictability further challenges the detection of markers of susceptibility, which

is paramount to reduce the burden associated to PTSD by the means of preventive interventions. To this aim, specific experimental designs need to be applied, which allow to disentangle pre- from post-trauma signatures.

1.1.3.3.1 Childhood adversity, longitudinal & twin studies

Three main strategies are available to point out biological abnormalities predisposing to PTSD: childhood adversity, twin and prospective studies (Figure 3).

Monozygotic twin samples represent a highly valuable tool to discriminate pre-existing features from correlates of the disorder by means of the co-twin control design (Kremen et al., 2012). Unlike classical twin studies, which compare monozygotic (MZ) and dizygotic pairs (DZ) and are mainly used to study heritability, the co-twin control design exploits comparisons within MZ pairs discordant for trauma exposure. The identification of susceptibility factors is achieved by comparing four groups: trauma-exposed twins who developed PTSD with their non-exposed co-twins, and healthy, trauma-exposed twins with their non-traumatized co-twins. Biological markers that are more prevalent in MZ couples with one PTSD case, and equally occur in both the PTSD and the unexposed, healthy co-twin, can be considered predisposing factors, while signatures evidenced only in the affected twin have to be considered disease correlates.

Longitudinal designs are also essential for uncovering markers of PTSD susceptibility without recurring to twins. These studies usually focus on high risk subpopulations that are prospectively monitored starting before trauma exposure, such as soldiers before and after deployment, or rescue workers when still in training schools and, later, while practicing the profession.

Since longitudinal monitoring and large cohorts of discordant twins are both complex requirements for clinical study designs, multiple studies exploited the fact that early adversities have been demonstrated to increase vulnerability to later traumatic events (Lanius et al., 2010) for studying susceptibility marks. Indeed, while cross-sectional comparisons between PTSD patients with and without childhood maltreatment do not allow the isolation of pre-trauma vulnerability factors, the investigation of healthy adults with and without childhood adversities may help identifying the long-term predisposing components contributed by the early environment (Admon et al., 2013).

By applying these designs, multiple studies showed that several neurobiological signatures found in PTSD patients are actually pre-existing features. For instance, smaller hippocampal volume and deficits in contextual cognitive tasks have been found to precede disease onset and to cause increased susceptibility to traumatic events (Kremen et al., 2012). Also, amygdala and dACC hyperactivity in

response to emotionally negative stimuli are evident before exposure to trauma, suggesting that hyperarousal symptoms, unlike re-experiencing and avoidance, may be a predisposing phenotype (Admon et al., 2013). Numerous peripheral markers of vulnerability, particularly within the stress pathway, have also been uncovered, including lower *FKBP5* and higher *GILZ* mRNA expression in the blood of people vulnerable to PTSD (van Zuiden et al., 2013).

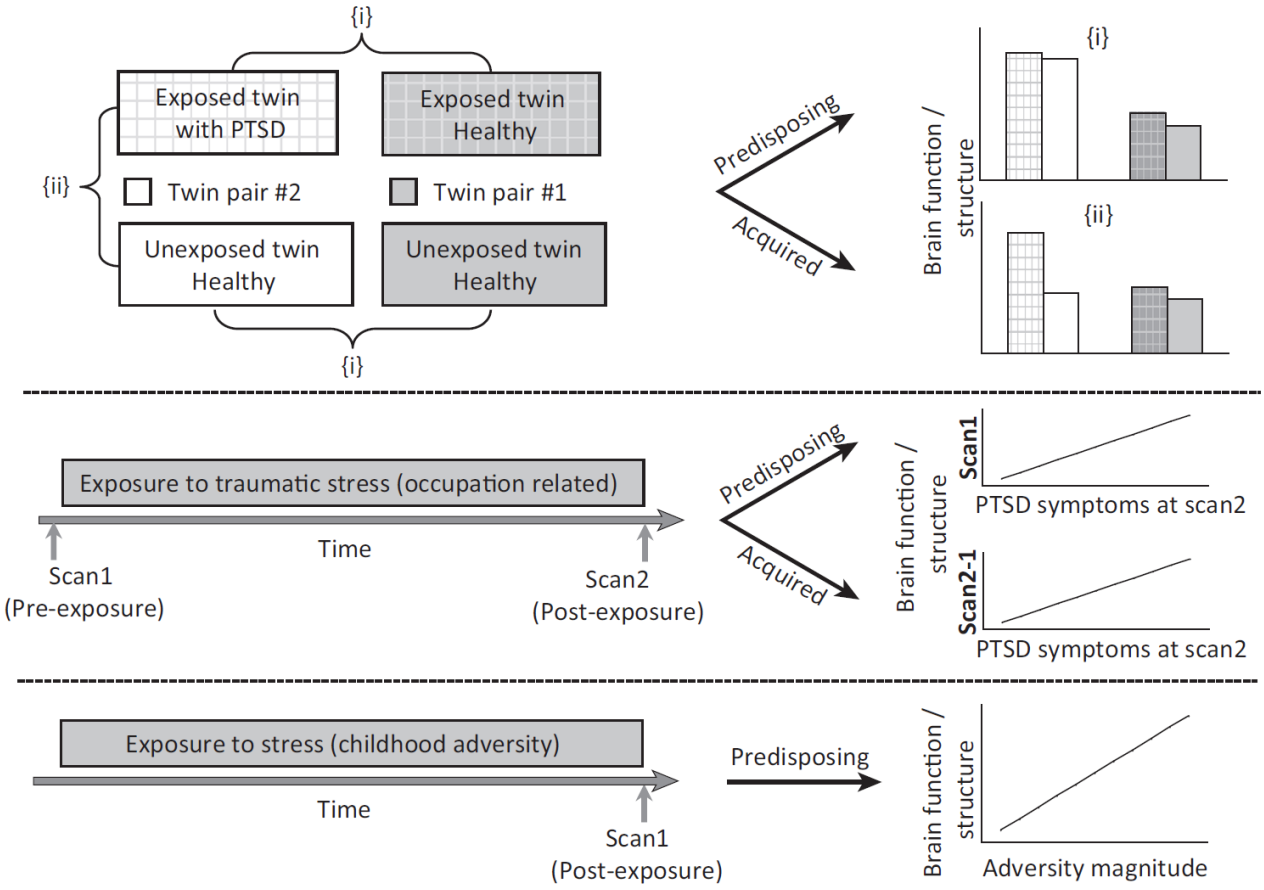

Figure 3 - Research strategies to disentangle predisposed from acquired neural abnormalities in post-traumatic stress disorder (PTSD). (Top) Co-twin control studies can identify predisposing factors that are present in both the trauma-exposed twin with PTSD and their unexposed identical co-twin, but not in a different pair of healthy monozygotic twins where one twin was exposed to trauma {i}. Acquired alterations can be found by comparing the trauma-exposed twin with PTSD to their own unexposed co-twin {ii}. (Middle) Longitudinal studies can identify predisposing factors, existing prior to trauma exposure and predicting post-trauma PTSD, whereas abnormalities that become evident only after PTSD onset may represent acquired deficits. The indication "Brain function/structure" may be substituted with "mRNA level" for studies assessing gene expression. (Bottom) Studies of early life stress exposure examine biological abnormalities in healthy adults exposed to childhood adversities, which likely represent factors predisposing to disease development upon future re-exposure to trauma. Modified from Admon et al., 2013.

Although fundamental to gain new insights into the biological underpinnings of PTSD susceptibility, the aforementioned designs take advantage of rather specific subpopulations, which challenges the generalizability of the results obtained to different people and contexts. Furthermore, despite of paramount importance to embrace the complexity of this syndrome, studying humans entails some limitations. The incidental and highly variable nature of traumas strongly increases the variability of the disease clinical manifestation and limits the feasibility of prospective studies. Moreover the confinement of molecular assessments to the periphery or to post-mortem brain

dissection reduces the ability to discern the pathophysiological mechanisms underlying risk or resilience to traumas. To overcome these drawbacks, it is crucial to acquire a translational perspective and to complement clinical surveys with studies on laboratory animals, primarily rodents.

1.1.3.3.2 Rodent models of trauma vulnerability

The first challenge to be met when studying the biological correlates of trauma vulnerability in rodents is to figure out how to establish whether or not an animal is vulnerable to traumatic events. This may be achieved by assessing putative similarities between the neurobehavioral profile of an animal following a highly stressful event and the symptomatology of patients, enabling to to evaluate the model's *face validity* (Blanchard et al., 2013). Given the complex nature of PTSD, this point is challenging, and it is important to remember that rodent models are not supposed to reproduce the exact clinical picture of human pathologies. For instance, core PTSD symptoms, such as intrusive memories or affective numbing, cannot be measured in animals. Nevertheless hypervigilance and hyperarousal, avoidance of trauma reminders, dissociative amnesia or anhedonia as well as specific endophenotypes such as altered physiological states or brain activation, can be mimicked and objectively evaluated in rodents (Cohen et al., 2014; Whitaker et al., 2014). Thus, with a good knowledge of the ethogram and physiology of laboratory animals, it is possible to establish strong, translational parallels between human and rodent phenotypes referring to selected groups of symptoms (Borghans & Homberg, 2015). Most importantly, to be considered a good PTSD model, the outlined alterations should persist or even become more pronounced over time (Cohen et al., 2014; Yehuda & Antelman, 1993). Besides, it is important to take into account key aspects of disease etiology when modeling PTSD, as proposed by Yehuda and Antelman (1993). For instance, it is generally suggested that even a brief stressor should be able to induce biological or behavioral features of PTSD, and that stressor intensity should be controllable, thus predicting the severity of the outcome in a dose-dependent manner. Different kind of stressors have been validated to mimic traumatic events in animal models of PTSD, which, depending on their nature, can be grouped into psychological, social and physical stressors (see Table 3). Of major importance, the variability of biobehavioral changes induced by the stressor as a function of experience or genetics, is considered compulsory to mimic the interindividual differences in trauma vulnerability outlined in the general population (Borghans & Homberg, 2015; Cohen et al., 2014).

Particular emphasis has been placed on the last aspect by studies designed to outline the biological basis of trauma vulnerability. In rodents, interindividual differences can be investigated by

grouping animals according to natural variations in the outcomes of trauma exposure, or by using environmental or genetic predictors of risk (Zovkic et al., 2013) (Table 4).

Table 3 – Stressors used to model PTSD in rodents

Stressors			Behavioral outcomes	References
Category	**Type**	**Description**		
Physical	SPS	2-h RS + 20-min forced swim + ether until unconsciousness	↑fear acquisition, contextual fear, anxiety-like behaviors, behavioral despair, ASR, sleep alterations ↓fear extinction, spatial memory	***Reviewed in:*** *Borghans & Homberg, 2015; Flandreau & Toth, 2018; Schöner et al., 2017*
	RS	15-min to 2-h	↑fear acquisition, anxiety-like and compulsive behaviors, avoidance of trauma-related cues, sleep alterations ↓spatial memory	
	FS	classical fear conditioning procedure	↑cued fear, anxiety-like and aggressive behaviors, avoidance of trauma-related cues, social withdrawal, ASR, sleep alterations ↓fear extinction	
	SEFL	unpredictable FS on day 1 + FS in novel environment on day2	↑ non-associative fear, vigilance, sleep alterations ↓risk assessment, attention	
	UT	30-s forced swim + 30-s submerging	↑contextual fear, anxiety-like behaviors, ASR ↓spatial memory	
Social	HI + PPS	daily cage switching of single animals + immobilization in close proximity of a predator	↑contextual fear, anxiety-like behaviors, avoidance of trauma-related cues, ASR ↓social interaction, spatial memory	***Reviewed in:*** *Borghans & Homberg, 2015; Flandreau & Toth, 2018; Schöner et al., 2017*
	SI	3-4 weeks of isolation	↑contextual fear, anxiety-like and aggressive behaviors ↓fear extinction	
	SD	5- to 10-day exposure to a cage-within-cage resident-intruder protocol	↑fear, anxiety-like behaviors, avoidance of trauma-related cues, anhedonia ↓social interactions	
Psychological	PSS	5-min exposure to soiled cat litter	↑cued fear, anxiety-like and defensive behaviors, avoidance of trauma-related cues, ASR	***Reviewed in:*** *Borghans & Homberg, 2015; Flandreau & Toth, 2018; Schöner et al., 2017*

Abbreviations: SPS - single prolonged stress; RS - restraint stress; FS - footshock; SEFL - stress-enhanced fear learning; UT - underwater trauma; HI+PPS - housing instability + predator-based psychosocial stress; SI - social isolation; SD - social defeat; PSS - predator scent stress; ASR - acoustic startle response. Symbols: ↑ - increased; ↓ - decreased.

Different models for classifying rodents according to natural variations in trauma responses have been developed. Richter-Levin's *cut-off behavioral criteria* (CBC) has been the first classification system proposed to distinguish vulnerable and resilient individuals among rats subjected to a traumatic stress, based on *a priori* cut-off values for behavioral measures significant to PTSD. According to CBC, 25% of rats exposed to a predator showed enduring maladaptive responses, such as increased anxiety and hyperarousal (Cohen et al., 2004). This model has been further developed and repurposed with the name of *behavioral profiling* (BF) in later work (Ardi et al., 2016; Horovitz et al., 2014). The main modification provided within the BF method is that the reference values indicating resilience were empirically established for each measured variable based on the averaged

performance of the non-traumatized control group. A different method, inspired by the CBC, was also proposed, which focuses on single behavioral domains critical for PTSD such as arousal or avoidance. A comprehensive score is calculated integrating different behavioral measures, which is then used to segregate rodents in susceptible and resilient groups, applying a threshold, which is based on the distribution of the non-traumatized controls (Anacker et al., 2016; Torrisi et al., 2020).

Table 4 – Rodent models of stress vulnerability

Method	Model	Stressor type	Behavioral outcomes	References
Subpopulations	outbred strains	PSS, UT, FS+SI	↑ anxiety, arousal, contextual fear, fear generalization, social withdrawal ↓ fear extinction	*Ardi et al., 2016; Cohen et al., 2004; Colucci et al., 2020*
	inbred strains	FS, RS, SD, SEFL	↑ avoidance, anxiety, arousal, fear generalization ↓ fear extinction	*Anacker et al., 2016; Hager et al., 2014; Sillivan et al., 2017; Torrisi et al., 2020*
Breeding lines & strains	F-S vs F-R	FS	↑ cued fear, fear generalization (F-S)	*McGuire et al., 2013*
	BALB vs B6	CUMS	↑ anhedonia, behavioral despair, social withdrawal ↓ fear extinction (BALB)	*Uchida et al., 2011*
	B6N vs B6JOla	FS	↑ social withdrawal, arousal, neophobia, behavioral despair ↓ fear extinction (B6N)	*Siegmund & Wotjak, 2007*
Early life stress models	licking, grooming and arched back nursing (low vs high)	FS	↑ anxiety, contextual fear ↓ cognitive functions (low)	***Reviewed in:*** *Krugers & Joëls, 2014; Murthy & Gould, 2018; C. Walker C. et al., 2017; Zovkic et al., 2013*
	maternal separation (≥3h vs 15min/day)	FS	↑ anxiety, anhedonia, behavioral despair, cued and contextual fear ↓ self-care, cognitive functions (≥3h)	
	limited bedding & nesting	FS	↑ anxiety, behavioral despair ↓ cognitive functions	
	Juvenile stress	UT	↑ anxiety, fear generalization	*Ardi et al., 2016*
Transgenic models	*5-Htt* KO	FS, SD	↓ fear extinction	*Narayanan et al., 2011; Nonkes et al., 2012; Shan et al., 2014; Wellman et al., 2007*
	Fkbp5 KO	FS, RS, SD	↔ fear, working memory, anxiety ↑ active coping	*Hartmann et al., 2012; O'Leary et al., 2011; Touma et al., 2011*
	BDNF Val66Met	FS	↓ fear extinction (Met carriers)	*Soliman et al., 2010*
	COMT Val158Met	PPS	↑ avoidance ↓ working memory (Val/Val)	*Deslauriers et al., 2019*

Abbreviations: F-S – fear susceptible; F-R – fear resilient; BALB – BALB/c mice; B6 – C57BL/6J mice; B6N - C57BL/6NCrl mice; B6JOla - C57BL/6JolaHsd mice; KO – knockout; 5-Htt – serotonin transporter; Fkbp5 - FK506 binding protein 5; BDNF – brain-derived neurotrophic factor; Val – valine; Met – methionine; COMT - catechol-O-methyltransferase; RS - restraint stress; FS - footshock; SEFL - stress-enhanced fear learning; UT - underwater trauma; SI - social isolation; SD - social defeat; PSS - predator scent stress; CUMS – chronic ultramild stress; PPS - predator-based psychosocial stress. Symbols: ↑ - increased; ↓ - decreased; ↔ - unchanged.

Although mainly applied for understanding the behavioral and molecular post-trauma adaptations that distinguish vulnerable and resilient individuals (Anacker et al., 2016; Ardi et al., 2016; Benatti et al., 2012; Colucci et al., 2020; Sillivan et al., 2017; Torrisi et al., 2020), if employed in longitudinal designs, this classification system might also help dissecting the biological correlates of PTSD susceptibility before trauma exposure. Importantly, the demonstration that this approach can be successfully applied to underline interindividual differences within inbred rodent strains (Anacker et al., 2016; Hager et al., 2014; Larrieu et al., 2017; Sillivan et al., 2017; Torrisi et al., 2020) opens also the possibility of isolating and investigating the epigenetic mechanisms that regulate trauma vulnerability and resilience. Unfortunately, since the classification in susceptible and resilient groups is achieved through post-trauma phenotyping, this method is not suitable for providing information on the molecular source of vulnerability at the brain level.

To overcome this issue, selective breeding of vulnerable and resilient individuals may help segregating two phenotypically divergent lines, allowing the assessment of differences existing prior to trauma that are linked to interindividual variability in stress susceptibility (Scharf & Schmidt, 2012). In this line, fear susceptible (F-S) and fear resistant (F-R) mouse lines have been developed based on interindividual variability in conditioned fear (Parker et al., 2012). Importantly, F-S mice were found to display greater HPA axis drive and excessive limbic circuitry activity in basal conditions compared to the F-R line, which may result in the observed differences in stress susceptibility (McGuire et al., 2013). Similarly, strain divergences in trauma *sequelae* have also been outlined. The BALB strain has been demonstrated to be more susceptible to the negative outcomes of stress exposure than the B6 strain (Uchida et al., 2011). Furthermore, within the B6 strain, B6N mice were found to be more susceptible to develop PTSD-like symptoms than B6JOla mice, a phenotype that was accompanied by signs of blunted affect and social withdrawal before trauma exposure (Siegmund & Wotjak, 2007).

Hypothesis-driven approaches can also be applied to test the contribution of specific experiences or genetic variations to trauma vulnerability and to biologically characterize pre-trauma vulnerable and susceptible animals. For instance, history of stress exposure in early life is a widely recognized factor predisposing individuals to develop PTSD in adulthood, and rodent studies of perinatal or juvenile stress might be of great help to address the biological basis of stress and trauma sensitization (Zovkic et al., 2013). Several protocols are used to model neglect in infancy that induce increased anxiety and depressed-like mood as well as cognitive impairments at adulthood (reviewed in: Krugers & Joëls, 2014; Murthy & Gould, 2018; Walker C. et al., 2017). Furthermore, juvenile stress, a model of childhood adversities, has been demonstrated to exacerbate the effects of adult trauma exposure in

outbred rats (Ardi et al., 2016). Transgenic models can also be used to investigate the role of specific genes in shaping vulnerability to traumas. Multiple genetic *loci* have been so far interrogated by creating knock-in (KI) rodents, carrying human risk variants such as the COMT Val158Met and the BDNF Val66Met KI mouse lines, or candidate gene knockout (KO) lines such as the *5-Htt* KO and *Fkbp5* KO models (Table 4).

1.2 Methyl-CpG binding protein 2

The Methyl-CpG binding protein 2 (MECP2) is an important member of a family of proteins recognizing and binding to methylated *loci* within the DNA. As the other representatives of the methyl binding protein family, MECP2 acts as a key regulator of genes transcription, by exerting a wide number of different functions within the cell nucleus. Although MECP2 is ubiquitously expressed throughout the body, the protein is predominantly represented in the brain, where it plays fundamental roles in activity-dependent regulation of gene expression within neurons. In fact the timing of MECP2 expression in mouse and human correlates with the maturation of the central nervous system (CNS), suggesting that MECP2 is mostly involved in neuronal maturation and maintenance (Chahrour & Zoghbi, 2007; Zimmermann et al., 2015) (Figure 4).

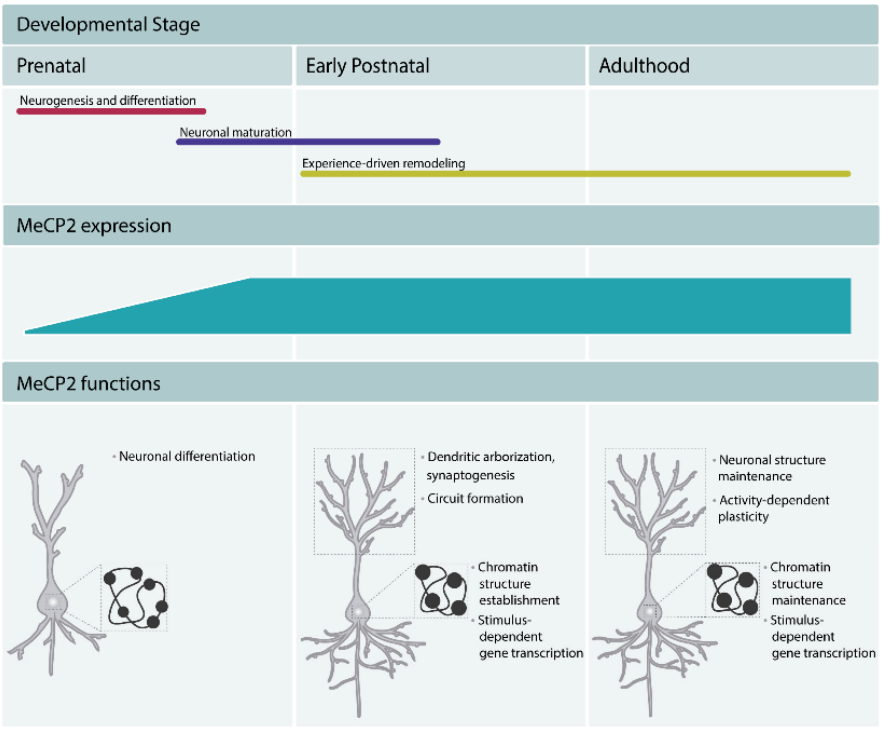

Figure 4 – Timeline of MeCP2 expression and function. MeCP2 expression levels rise together with brain development, suggesting that MeCP2 function is needed to regulate neuronal differentiation and maturation. To this aim, MeCP2 promotes chromocenter (represented as black dots in the nucleus) clustering, and the establishment of the typical chromatin structure of mature neurons. At adulthood, MeCP2 is critical for the maintenance of proper neuronal function. By regulating the neuronal transcriptomic profile and maintaining a permissive state for stimulus-dependent gene transcription. From Karaca et al., 2019.

1.2.1 Structure and function

MECP2 is encoded by a gene located in the *Xq28* region of the human X chromosome, and a homologous region in the mouse and has been demonstrated to go through X inactivation (XCI) in both species (D'Esposito et al., 1996). The gene spans four exons, and alternative splicing of exon 2 in the primary transcript generates two different isoforms differing in their N-termini: MECP2-E1 and MECP2-E2. MECP2-E1 is the most abundant isoform in various regions of the brain, while

MeCP2-E2 is more prevalent in fibroblast and lymphoblast cells (Kriaucionis & Bird, 2004; Mnatzakanian et al., 2004).

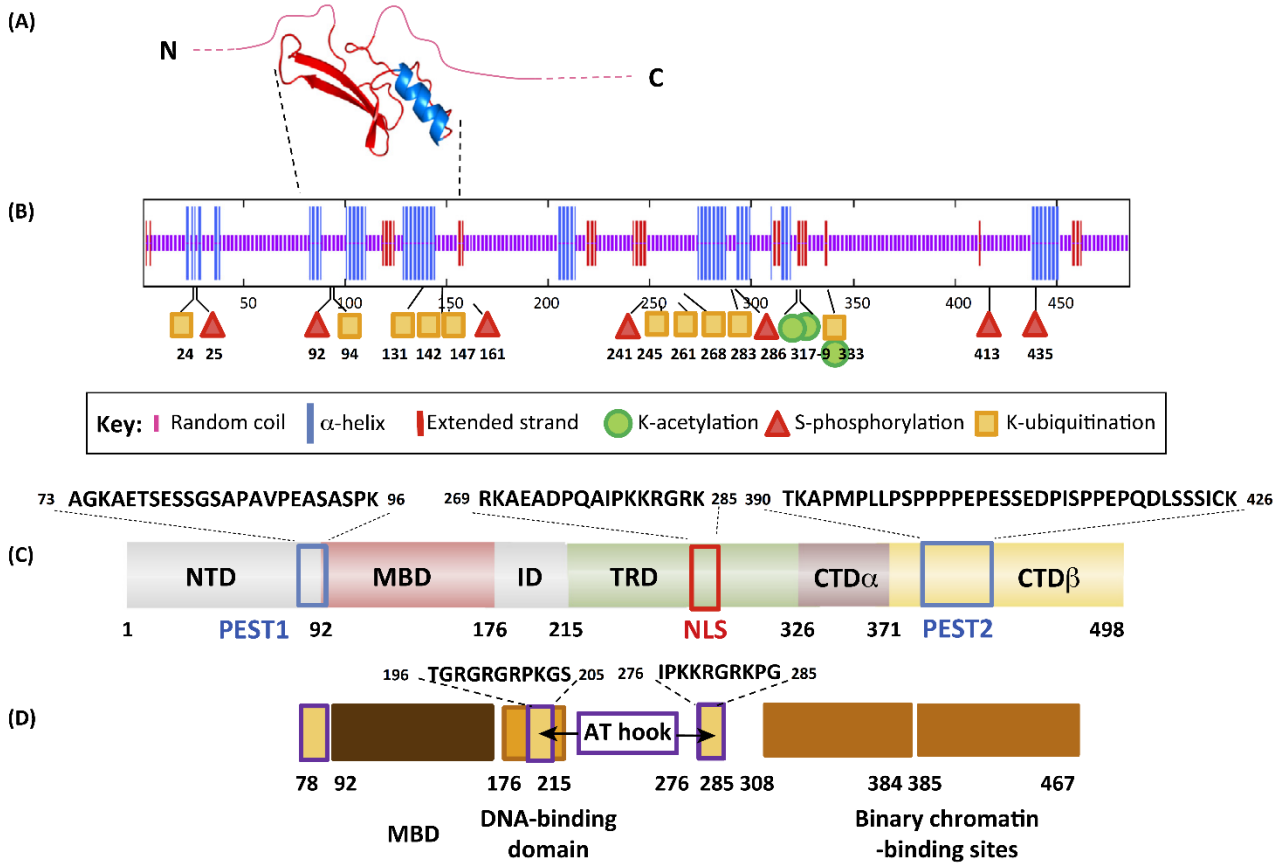

Figure 5 – Structure and domains of methyl-CpG binding protein 2 (MECP2). (A) Tertiary structure of MECP2. Except for the methyl-binding domain (MBD), MECP2 has long regions that do not fold into α-helices or β-sheets. (B) MECP2 post-translational modifications (PTMs) are shown along the predicted secondary structure. (C) MECP2 functional domains are illustrated along with the corresponding amino acid numbers. The sites and sequences of the regions enriched in proline–glutamic acid, serine, and threonine (PEST) and the nuclear localization signal (NLS) are also reported. (D) MECP2 DNA- and chromatin-interacting domains are reported, with the relative amino acid numbers. From Ausió et al., 2014.

MECP2 is an intrinsically disordered protein, as it lacks a fixed three-dimensional structure (Figure 5A, B). This peculiarity enables the protein to interact with a wide variety of complexes and nucleic acids and thus to exert multiple functions. MECP2 protein is composed by 5 functional domains: the N-terminal domain (NTD), the Methyl-CpG binding domain (MBD), the intervening domain (ID), the transcriptional repressor domain (TRD), and the C-terminal domain (CTD, Yang et al., 2011) (Figure 5C). The MBD is a short, highly conserved α/β tertiary structure that specifically binds to symmetrically methylated CpG dinucleotides (mCG). DNA binding is coadiuvated by two consensus A/T hook motifs localized within the ID (Figure 5D), which recognize AT-rich regions. Hence, MeCP2 affinity for mCG residues is strongly heightened by adjacent A/T-rich sequence motifs. Importantly, MeCP2 has been also localized at the level of methylated or hydroxymethylated cytosines in the non-CG context (mCH and hmCH where H = A, C, or T), two epigenetic marks that are enriched in the brain and increase postnatally, as MECP2 protein levels rise (Chen et al., 2015;

Gabel et al., 2015; Mellén et al., 2012). Upon binding to DNA, MeCP2 mainly exerts transcriptional repression functions, acting as a platform for recruitment of either corepressors, chromatin remodeling complexes or DNA methyltransferases through its TRD. Interestingly, the MeCP2 binding profile also spans regions devoid of DNA methylation, and direct interaction between MeCP2 MBD/CTD and nucleosomes has been described that mediate chromatin compaction and regulate gene expression (Nikitina et al., 2007). Intriguingly, specific histone post-translational modifications, namely methylation of histone 3 at lysine 9 and 27 (H3K9me2 and H3K27me3), were shown to enhance MeCP2 binding affinity to nucleosomes (Lee et al., 2020; Thambirajah et al., 2012). MeCP2 also interacts with RNA at RNA-binding regions within the MBD and the TRD, and is involved in alternative splicing of mRNA and miRNA processing regulation via the recruitment of splicing factors and ribonuclease complexes (Good et al., 2021; Lyst & Bird, 2015) (Figure 6).

Mouse studies revealed that, rather than being a global repressor, MeCP2 regulates selected genes in specific neuronal subpopulations, modulating their expression in dependence on the physiologic state of the organism, and sometimes even triggering gene activation (Chahrour & Zoghbi, 2007; Zimmermann et al., 2015). The high versatility of MECP2 function is attributed to both its disorganized structure and its modulation through posttranslational modifications (PTMs) (Figure 5A,B). In line with the primary role of the protein in the CNS, neuronal activity is acknowledged to control MeCP2 functions by inducing changes at the protein level. Several reports have in fact demonstrated that MeCP2 phosphorylation is a key mechanism by which active neurons modulate MeCP2 affinity for its partners and targets, thus triggering cellular adaptations to different stimuli (Bellini et al., 2014).

Notably, synapse formation and function require activity-dependent gene expression, which is consistent with the critical role of MeCP2 in maturing and adult neurons. Indeed, MeCP2 functions are crucial for neural circuit development as well as maintenance of synaptic plasticity (Karaca et al., 2019). Consistently, long-term depression (LTD) and potentiation (LTP) are impaired in the absence of proper MeCP2 functionality, pointing at a fundamental role of MeCP2 activity in learning and memory and adaptation to changing environments (Boggio et al., 2010; Moretti et al., 2006; Robinson & Pozzo-Miller, 2019). Deficits in glutamatergic neurotransmission have been proposed to account for the outlined alterations in activity-dependent synaptic plasticity of neural circuits, pointing at MeCP2 as a key regulator of the brain excitatory/inhibitory balance, which is essential to maintain neuronal network stability (Goffin & Zhou, 2012). Furthermore, MeCP2 functions have been shown to be fundamental to multiple neurotransmitter systems, with compelling evidence for

monoaminergic and inhibitory neurons (Chao et al., 2010; Gantz et al., 2011; Philippe et al., 2018; Samaco et al., 2009; Santos et al., 2010; Taneja et al., 2009).

Noteworthy, besides its critical role in neurons, MeCP2 emerged as an important regulator of neuroglia differentiation and functionality (Jin et al., 2017; Sharma et al., 2018). Astrocytic MeCP2 was shown to exert multiple non-cell autonomous effects ultimately affecting neuronal morphology and functions, spanning synaptic plasticity, neurotransmitter homeostasis and metabolic support (Kahanovitch et al., 2019). MeCP2 is indeed involved in brain mitochondria functionality and its functional alterations are associated with hypoxia hypersensitivity and oxidative stress (Grosser et al., 2012; Valenti et al., 2017; Zuliani et al., 2020). Furthermore, although lower than in neurons and astrocytes, microglia express detectable levels of MeCP2 that is involved in the regulation of its activation and the initiation of inflammatory immune responses within the brain (Cortelazzo et al., 2017; Jin et al., 2017; Kahanovitch et al., 2019). MeCP2 is also expressed in oligodendrocytes, where it negatively regulates myelin gene expression, ultimately influencing axonal functionality and white matter formation (Kahanovitch et al., 2019).

Overall, MECP2 emerges as a complex and dynamic regulator of cell functions across the CNS that is fundamental for its proper development and for the organization and maintenance of functional networks, responsive to changing environments.

1.2.2 MECP2 involvement in neurologic and mental disorders

In line with the evidence highlighting the critical role played by MECP2 in synaptic plasticity and brain function, alterations in this protein have been found in numerous neuropathological disorders, from neurologic and neurodevelopmental pathologies to complex mental disorders, providing support to the importance of MECP2 for ensuring a proper functioning of the CNS (Bach et al., 2020).

Initially identified as an oncogene, *MECP2* has been first linked to neurodevelopment in 1999, when Huda Zoghbi's team demonstrated that *MECP2* mutations, arising *de novo*, mainly in the paternal germline, are the main cause of Rett Syndrome (RTT), a rare X-linked neurologic disorder affecting exclusively girls (Amir et al., 1999). Severe cognitive, social and physical impairments characterize RTT, whose symptoms typically develop around 6-18 months of age (Cosentino, Vigli, Franchi, et al., 2019). Distinct *MECP2* mutations have been associated to RTT diagnosis, ranging from large deletions to frameshift and point mutations affecting important MECP2 functional

domains. As a consequence, MECP2 protein is usually either unexpressed (or severely underexpressed) or non-functional (Huppke et al., 2002; Zimmermann et al., 2015). Few cases are linked to hypofunctional or hypomorphic mutations, resulting in milder phenotypes (Smeets et al., 2005). Importantly, patients are mainly heterozygous for the mutation and, given the random process of XCI, the mutated allele displays a mosaic expression throughout the body, resulting in different degrees of clinical severity (Young & Zoghbi, 2004).

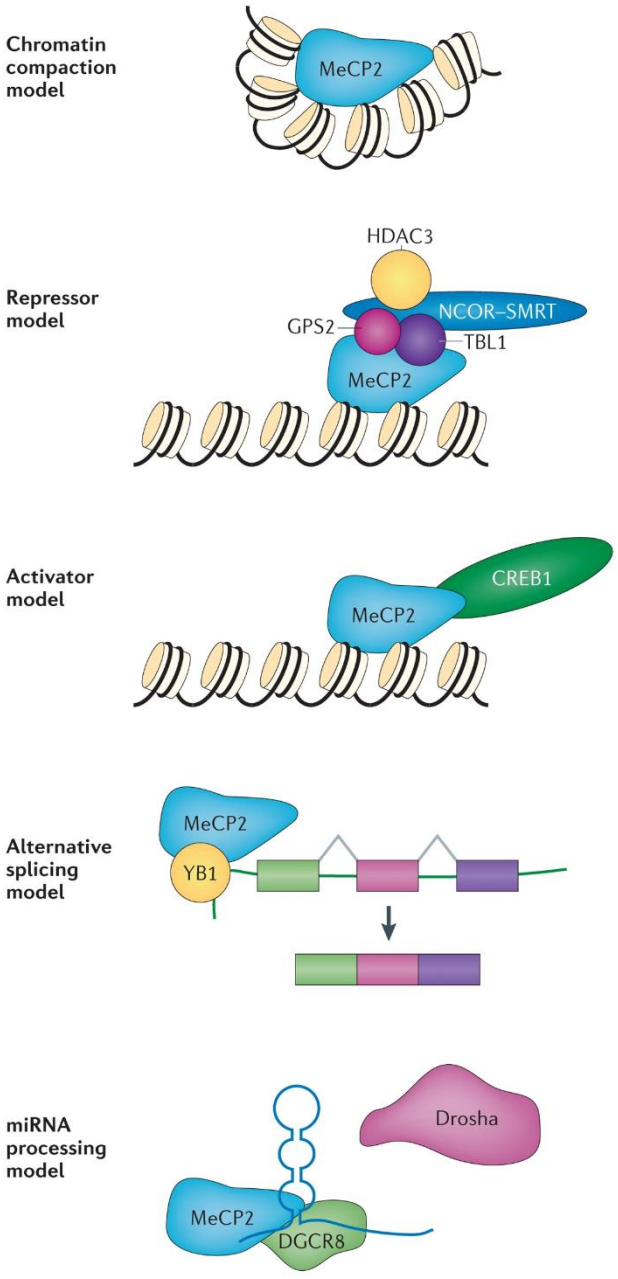

Figure 6 – Methyl-CpG binding protein 2 (MeCP2) functions. MeCP2 compacts chromatin structure and represses the transcription of target genes by recruiting the nuclear receptor co-repressor (NCOR)– silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) co-repressor complex. It has been also found to activate transcription by recruiting the co-activator cyclic AMP-responsive element-binding protein 1 (CREB1). Morerover, MeCP2 was found to regulate alternative splicing via its interaction with Y-box binding protein 1 (YB1), and to modulate microRNA (miRNA) processing by interacting with DiGeorge syndrome critical region 8 (DGCR8) and preventing the formation of the Drosha–DGCR8 complex. GPS2, G protein pathway suppressor 2; HDAC3, histone deacetylase 3; TBL1, transducin β-like protein 1. From Lyst & Bird 2015.

Of note, *MECP2* mutations, which were initially thought to be lethal in hemizygosity, have been attributed to a wide range of neurologic conditions, ranging from mild mental retardation to neonatal encephalopathy, in male patients (Villard, 2007). *MECP2* was also found to be involved in autism-spectrum disorder (ASD), a complex developmental condition characterized by a strong male:female bias (3:1) (Werling & Geschwind, 2013), with evidence of either increased or decreased *MECP2* expression in patients' samples (Ausió et al., 2014; LaSalle & Yasui, 2009; Samaco et al., 2004). Also, mutations at the level of both coding and non-coding regulatory regions within the *MECP2 locus* have been associated to autism vulnerability (Loat et al., 2008; Wen et al., 2017), pointing at MECP2 functionality as a critical factor for a proper development of the CNS.

In this line, *MECP2* duplication syndrome (MDS) is a progressive neurodevelopmental condition that occurs almost exclusively in males

and is characterized by moderate-to-severe intellectual disability, infantile hypotonia and recurrent pulmonary infections (Van Esch, 2012). It is caused by duplications spanning the *MECP2 locus* which are usually inherited from heterozygous mothers with favourably skewed XCI. Interestingly, although asymptomatic for MDS, carrier mothers usually show generalized anxiety, depression, and compulsions (Sandweiss et al., 2020). Therefore, while a tight control of MECP2 levels emerges as essential to ensure appropriate brain development, alterations at the level of the *MECP2 locus* appear also involved in the onset of symptoms of mental disorder (Ausió et al., 2014).

Consistently, *MECP2* mutations as well as alterations in gene or protein expression have been described in numerous mental disorders. For instance, reduced MECP2 functionality has been linked to major depression, with patients showing decreased MECP2 expression levels in peripheral blood (Ausió, 2016; Su et al., 2015). In line with this, treatment with SSRIs, which are commonly prescribed as antidepressants, stimulates *MECP2* transcription and triggers protein PTMs in rodents brain (Cassel et al., 2006; Hutchinson, Deng, Aryal, et al., 2012; Hutchinson, Deng, Cohen, et al., 2012). Conversely, *MECP2* up-regulation is observed in the peripheral blood of patients diagnosed with type II bipolar disorder, which is characterized by major depressive states and hypomania (D'Addario et al., 2018).

Of major importance, MECP2 alterations have also been associated with substance abuse disorders, including addiction to cocaine and alcohol (Ausió et al., 2014). MeCP2 expression was significantly increased in the brain of mouse models developing alcohol dependence following chronic intermittent ethanol exposure (CIE), and *MeCP2* mutated mice display increased ethanol sensitivity, which is usually associated with reduced voluntary intake (Repunte-Canonigo et al., 2014). In a way similar to alcohol, cocaine intake was shown to induce MeCP2 protein expression and phosphorylation in reward-processing regions of rodent brains (Bodetto et al., 2014; Mao et al., 2011), which was demonstrated to increase the motivation to consume cocaine, thus likely contributing to addiction vulnerability (Im et al., 2010).

1.2.2.1 MECP2 and stress: vulnerability to psychopathology

The aforementioned mental disorders share some similarities, let alone the fact that stressful events, particularly throughout childhood, represent a common risk factor (Spalletta et al., 2020; Wittchen et al., 2011). Of note, among the identified targets of MeCP2 transcriptional regulation there are plenty of genes involved in the stress response, including the prime-movers of HPA axis activation, *Crh* and *Avp*, and the negative feedback modulator *Fkbp5* (McGill et al., 2006; Nuber et al., 2005). Moreover, the timing of increased brain *MeCP2* expression critically overlaps with a key

maturational period for the HPA axis. Indeed, in rodents, early postnatal days represent a transitory state in which the HPA axis is maintained hyporesponsive by maternal care to undergo progressive maturation. Every perturbation in circulating glucocorticoids occurring in this period would interfere with such maturation, persistently lowering the threshold of HPA responsiveness and increasing vulnerability to future psychopathologies (Franklin et al., 2012).

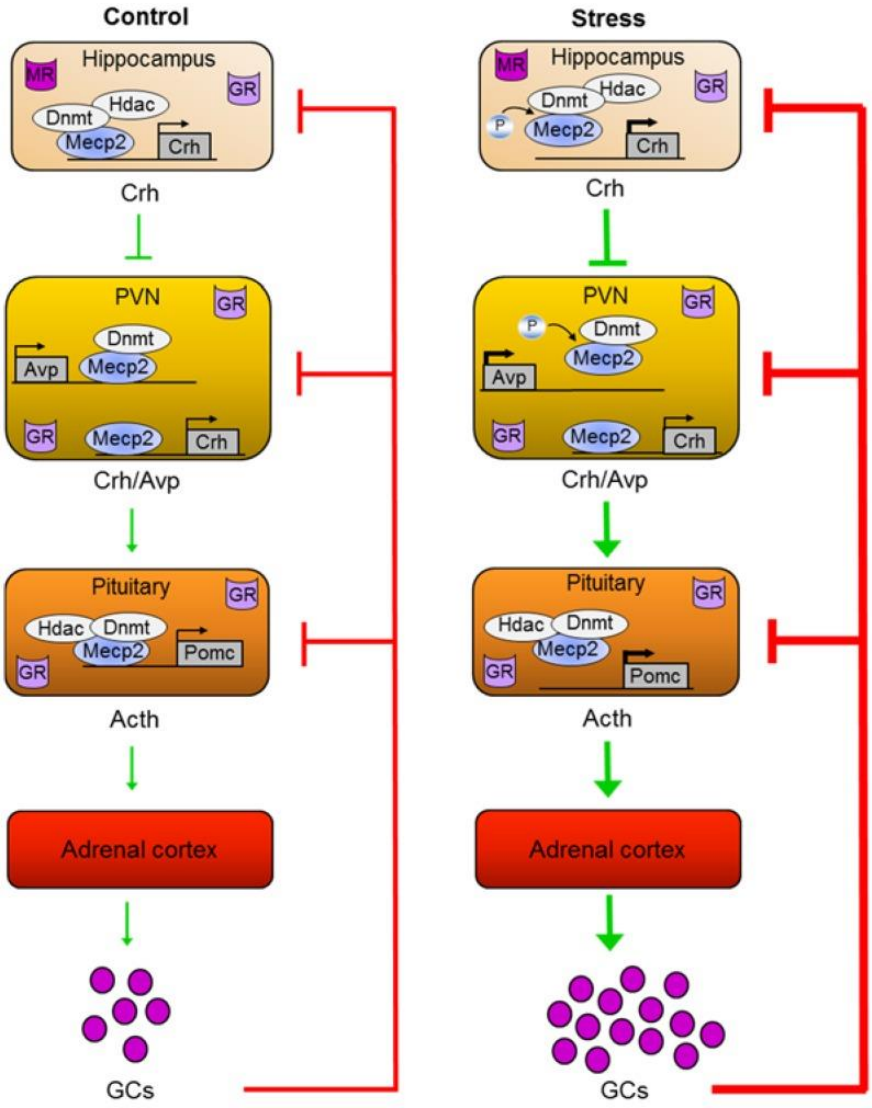

Figure 7 – Methyl-CpG binding protein 2 (MeCP2) is involved in the epigenetic programming of the hypothalamus-pituitary-adrenal (HPA) axis following early life stress (ELS). Under resting conditions, MeCP2 binds and represses corticotropin-releasing hormone (*Crh*) gene expression in the hippocampus. Upon ELS, MeCP2 is phosphorylated at S421 and dissociates from the DNA, relieving *Crh* repression. In the paraventricular nucleus of the hypothalamis (PVN), MeCP2 binding to *Crh* is unaffected by ELS. In contrast, MeCP2 occupancy at the arginine vasopressin (*Avp*) *locus* responds to ELS via S421-phosphorylation, and *Avp* is de-repressed. Due to its association with DNA methyl-transferases (Dnmts), Mecp2 occupancy maintains the DNA methylated. Thus, ELS-induced dissociation facilitates hypomethylation, giving rise to the lasting overexpression of *Avp*. Similarly, MeCP2 binds and represses Pro-opiomelanocortin (*Pomc*) expression in the pituitary gland under basal conditions. ELS induces Mecp2 dissociation, facilitating hypomethylation and increased *Pomc* expression. Hdac: hystone de-acetylase; GR; glucocorticoid receptor; GCs: glucocorticoid hormones. From Zimmermann et al., 2015.

Most importantly, MeCP2 has been found to critically contribute to the early programming of the HPA axis via epigenetic mechanisms (Chahrour & Zoghbi, 2007; Murgatroyd & Spengler, 2011) (Figure 7). Indeed, in their seminal paper, Murgatroyd et al. (2009) demonstrate that maternal

separation, an established rodent model of early life stress (ELS), induced MeCP2 protein phosphorylation and dissociation from its binding region on the *Avp* gene in the hypothalamus of stressed mice. This would expose key regulatory regions within the *Avp locus* to postnatal methylome reconfiguration, a development-associated demethylation process normally occurring in early life, allowing the long-lasting demethylation and persistent overexpression of *Avp* (Murgatroyd, 2014; Murgatroyd & Spengler, 2011; Murgatroyd et al., 2009).

MECP2 involvement in the long-lasting programming of the stress response during critical periods of development has been further acknowledged in other studies showing MeCP2-mediated *Crh* upregulation in the hippocampus of maternally-separated rats (Zimmermann et al., 2015). Such effects are likely to trigger the impairments in cognitive functions relevant to successful coping with stress displayed by ELS adults (Wang et al., 2014). These findings support a strong contextual specificity for the role of MeCP2 phosphorylation in mediating ELS acute and long-lasting consequences, depending on either tissues, circuits and genetic loci (Zimmermann et al., 2015).

In sum, early life stress seems to permanently impair behavioral and physiological strategies of coping with stress, leaving durable epigenetic marks on the genome. Given its greater stability, the main candidate epigenetic modification to cover such a role is DNA methylation. The above mentioned examples suggest that MECP2 protein, as the founding member of methylated GpGs readers, is an important mediator in turning a relatively time-limited adverse life experience into permanent alterations of brain circuits and the HPA axis, leading to stress susceptibility in adulthood.

1.3 Aims and hypotheses of the project

Considering the key role played by susceptibility in determining the outcome of traumatic experiences and the well-documented contribution of epigenetic mechanisms in the establishment of interindividual differences in PTSD risk and resilience (Rakesh et al., 2019), this thesis aimed at translationally addressing, in mouse models and human cohorts, the potential contribution of the X-linked epigenetic regulator MECP2 in shaping vulnerability to develop stress and trauma-related disorders.

Specifically, the first aim of this work was to take advantage of a preclinical model to explore whether disruptions in MeCP2 functionality may predispose to the onset of PTSD-like symptomatology in the aftermath of a traumatic event (paragraph 2.1). To this purpose, we exposed transgenic male mice constitutively carrying a truncated mutation, leading to the expression of a hypofunctional form of the protein (MeCP2-308, Shahbazian et al., 2002), to unescapable footshocks. We then evaluated whether mice developed phenotypes comparable to patients' symptomatology from a behavioral, physiological and molecular perspective. Because of the critical role that MeCP2 exerts on the regulation of the stress response, which is likely to affect the capacity of successfully coping with highly stressful situations (Zimmerman et al., 2015), we expected transgenic mice to show: i) alterations in the memory of the traumatic event, in term of exaggerated fear in response to trauma cues and overgeneralization of fear responses; ii) altered HPA axis responsivity to stress, measured as the stress-dependent surge in the glucocorticoid hormone corticosterone (CORT) release in peripheral blood; iii) peripheral modulation of gene expression patterns mirroring the alterations found in PTSD patients.

Furthermore, given the well-known gender bias in stress-related disorders (Hodes and Epperson, 2019), we aimed at evaluating whether MeCP2 dysfunction provides stress vulnerability in female mice (paragraph 2.2). To this aim, we exposed female mice homozygous and heterozygous for the MeCP2 mutation, and wt controls to unescapable electric shocks, and compared their behavioral profile to that of males. Stress physiology as well as central patterns of gene expression were evaluated in transgenic mice from either sex. Given the outlined contribution of MeCP2 in the establishment of sex differences throughout development (Kurian et al., 2008; Forbes-Lorman et al., 2012; 2014), we hypothesized that male and female mice may be differentially affected by the effects of MeCP2 truncation on trauma outcomes.

The second main goal of the present thesis was to assess whether MECP2 hypofunction confers increased stress vulnerability in human cohorts. This issue was addressed in two different studies. The first (paragraph 2.3) explored the link between blood *MECP2* expression and measures of stress susceptibility (e.g. depressed mood and anxiety) in non-traumatized healthy participants (Hautzinger & Bailer, 1993; Spielberger et al., 1970). The second (paragraph 2.4) focused on the assessment of blood *MECP2* levels in a human cohort of PTSD patients and healthy controls to study its correlation with the severity of PTSD symptomatology (Schnyder & Moergeli, 2002; Blake et al., 1995).

Based on the well-known contribution of childhood adversities towards increasing susceptibility to further stressors (McLaughlin & Lambert, 2017; Spalletta et al., 2020) and the preclinical evidence of MeCP2 involvement in the lasting effects of early stress (Murgatroyd et al., 2009), we also investigated whether MECP2 might be modulated by exposure to early adversities (Bernstein & Fink, 1998). We postulated that stressful experiences during childhood would associate with decreased expression of blood *MECP2*.

Furthermore, given the well documented responsivity of MeCP2 to environmental challenges and its critical role in memory formation (Bellini et al., 2014; Robinson & Pozzo-Miller, 2019), we hypothesized that *MECP2* expression would be modulated by traumatic experiences in adulthood, and that alterations in this process would associate with maladaptive trauma outcomes (e.g. increased PTSD symptom severity).

The effects of gender were also explored in both human studies with the hypothesis that *MECP2* expression may be differentially modulated by lifetime stressors in men and women, thus taking part in the mechanisms involved in the regulation of gender differences in susceptibility to trauma- and stress-related disorders.

EMPIRICAL STUDIES

Chapter 2

2.1 Study 1: Methyl-CpG binding protein 2 functional alterations provide vulnerability to develop behavioral and molecular features of post-traumatic stress disorder in male mice[1]

[1]Publication: Cosentino L, Vigli D, Medici V, Flor H, Lucarelli M, Fuso A, De Filippis B. *Methyl-CpG binding protein 2 functional alterations provide vulnerability to develop behavioral and molecular features of post-traumatic stress disorder in male mice*. Neuropharmacology. 2019; 160:107664.doi: 10.1016/j.neuropharm.2019.06.003.

Abstract

Post-traumatic stress disorder (PTSD) is a mental disorder characterized by symptoms of persistent anxiety arising after exposure to traumatic events. Stress susceptibility due to a complex interplay between genetic and environmental factors plays a major role in the disease etiology, although biological underpinnings have not been clarified. We hypothesized that aberrant functionality of the methyl-CpG binding protein 2 (MECP2), a master regulator of experience-dependent epigenetic programming, confers susceptibility to develop PTSD-like symptomatology in the aftermath of traumatic events. Transgenic male mice expressing a truncated form of MeCP2 protein (MeCP2-308) were exposed at adulthood to a trauma in the form of high-intensity footshocks. The presence and duration of PTSD-like symptoms were assessed and compared to those of trauma-exposed wild type littermates and MeCP2-308 mice subjected to a mild stressor. The effects of fluoxetine, a prime pharmacological PTSD treatment, on PTSD-like symptomatology were also explored. Trauma-exposed MeCP2-308 mice showed long-lasting hyperresponsiveness to both correct and incorrect predictors of the trauma and persistent increased avoidance of trauma-related cues. Traumatized MeCP2-308 mice also displayed abnormal post-traumatic plasma levels of the stress hormone corticosterone and altered peripheral gene expression mirroring that of PTSD patients. Fluoxetine improved PTSD-like symptoms in trauma-exposed MeCP2-308 mice. These findings provide evidence that MeCP2 dysfunction results in increased susceptibility to develop PTSD-like symptoms after trauma exposure, and identify trauma-exposed MeCP2-308 mice as a new tool to investigate the underpinnings of PTSD vulnerability.

Highlights

- MeCP2-308 mice traumatized at adulthood display long-lasting memory alterations
- Trauma-exposed MeCP2-308 mice develop persistent avoidance behavior
- Fluoxetine partially reverses behavioral aberrancies of traumatized MeCP2-308 mice
- MeCP2-308 mice showed aberrant increase of corticosterone acutely after trauma
- Traumatized mutant mice mirrored patients' peripheral gene expression alterations

Introduction

The X-linked methyl-CpG binding protein 2 (MECP2) is the founding member of a family of proteins recognizing and binding methylated DNA (1, 2). MECP2 mainly exerts transcriptional regulatory functions, directly interacting with nucleosomes or acting as a platform for recruitment of either corepressors, coactivators, chromatin remodeling complexes, DNA methyltransferases or splicing factors (3). MECP2 is highly expressed in the brain where it mediates gene expression changes in response to neuronal activity, ultimately influencing neuroplasticity (4).

In line with its critical role in the regulation of neuronal maturation and function, mutations in the MECP2 gene cause Rett syndrome (5, 6), a rare neurodevelopmental disorder primarily affecting girls, characterized by severe cognitive, social, affective, motor and physiological impairments. Since the identification of the MECP2 gene as the major genetic cause for this disorder, increasing reports have highlighted the presence of alterations in MECP2 expression levels in a number of neuropsychiatric conditions such as schizophrenia and bipolar disorder (7, 8). Recent evidence also reports the involvement of this multifunctional protein in setting up the stress response during critical periods of development (9, 10), suggesting an emergent role for MeCP2 as a master regulator of experience-dependent epigenetic programming and its potential involvement in shaping vulnerability to develop stress-related psychopathology (2, 7, 8).

Post-traumatic stress disorder (PTSD) is a chronic mental disorder that can arise in the aftermath of a traumatic event, characterized by a series of distressing and highly debilitating symptoms, such as re-experiencing, flashbacks, avoidance behavior, emotional numbing and hyperarousal (11).

The existence of an intrinsic individual susceptibility has been evidenced, with only 20-30 % of trauma-exposed people developing chronic PTSD symptomatology (12). A wide range of factors accounts for increased vulnerability to PTSD, including sex, intelligence, prior mental disorders, previous repetitive exposure to traumatic events or childhood traumas unrelated to the triggering event (13, 14). Support for genetic influences on PTSD susceptibility comes from classical twin studies which estimated heritability of up to 30-40% for the probability to develop the disorder after trauma exposure, even after taking into account the genetic influence on the risk of experiencing assaultive traumas that is mediated by personality traits (15, 16). Moreover, environment-driven epigenetic changes have been suggested to contribute to the differential risk of disease development following trauma exposure, suggesting that PTSD is a multifactorial polygenic disorder (17-19).

The importance of a complex interplay between genes and environment in PTSD etiology is further confirmed by multiple findings about interactions among haplotypes associated with disease

risk and specific experience-dependent methylation patterns differentially influencing the outcome of traumatic experiences (20-22).

In spite of the increasing literature suggesting a critical involvement of epigenetic processes in mediating risk and resilience to PTSD (21, 23, 24), the role of epigenetic factors in the etiology of PTSD has so far received little attention in transgenic animal models of this disorder. Moreover, the difficulty to study the neurobiological underpinnings of PTSD susceptibility in existing preclinical models has hampered the identification of biomarkers that can discriminate between susceptible and resilient individuals (25, 26), thus stressing the need for innovative tools to address this issue.

Based on these evidence, in the present study we reasoned that aberrant MECP2 functionality could be linked to PTSD susceptibility and that the outcome of highly distressing experiences, i.e. traumatic events, could be affected by MECP2 dysfunctions. In particular, we hypothesized that male mice carrying mutations in the MeCP2 gene (27) may develop behavioral and molecular alterations in the aftermath of a traumatic event that recapitulate those presented by PTSD patients.

To test this hypothesis, transgenic mice expressing a form of MeCP2 protein truncated at the level of aminoacid 308 (MeCP2-308) and wild type (wt) littermates (27) were exposed at adulthood to a trauma in the form of high-intensity footshocks and their behavioral and physiological responses were compared to those presented by MeCP2-308 and wt mice subjected to a mild stressor, i. e. shocks of low intensity. Given the X-linked location of the MeCP2 gene, to avoid the confounding effect of the X random inactivation, the study was conducted on males. Given the involvement of MECP2 in a number of disorders (7, 8, 28-30), to provide further support to the specificity of trauma-induced behavioral alterations for PTSD clinical condition, we also explored whether abnormal behaviors in trauma-exposed MeCP2-308 mice are reversed to normal by chronic treatment with the selective serotonin reuptake inhibitor fluoxetine (flx), one of the most frequently used treatments for PTSD (31, 32).

Methods

Animals

Experimental animals were 4–5 months of age MeCP2–308 hemizygous transgenic male mice and wt littermates (B6.129S-MeCP2tm1Hzo/J, Jackson Laboratories stock number: 005439, backcrossed to C57BL/6J mice for at least 12 generations) (33). All procedures were carried out in accordance with the European Communities Council Directive (10/63/EU) as well as the Italian law (26/2014) (see Supplement).

Experimental design

On the first day of the experimental schedule, during a fear conditioning protocol (34), experimentally naïve MeCP2–308 and wt mice received two footshocks of high intensity (0.8 mA, 3 s) to mimic the traumatic event, or footshocks of low intensity (0.3 mA, 3 s) as a control. Two days after conditioning, MeCP2-308 and wt mice receiving the high shock (trauma-exposed) were assigned to receive a chronic treatment with flx (wt, high flx; MeCP2-308, high flx; sample size (N) = 10; 6) or tap water, as a control (wt, high; MeCP2-308, high; N = 11; 8). Low-shocked mice received tap water only (wt, low; MeCP2-308, low; N = 7; 8). Experimental mice underwent a battery of tests designed to evaluate the effects of the manipulations (shock intensity and drug treatment) either on PTSD-like symptoms (fear conditioning task, active avoidance test and circadian activity) and on well-established phenotypic features of the transgenic mice under investigation, as control (general health status) (see Figure1 for the timeline of the experimental design, and supplement for detailed methods and results of the assessment of the general health status).

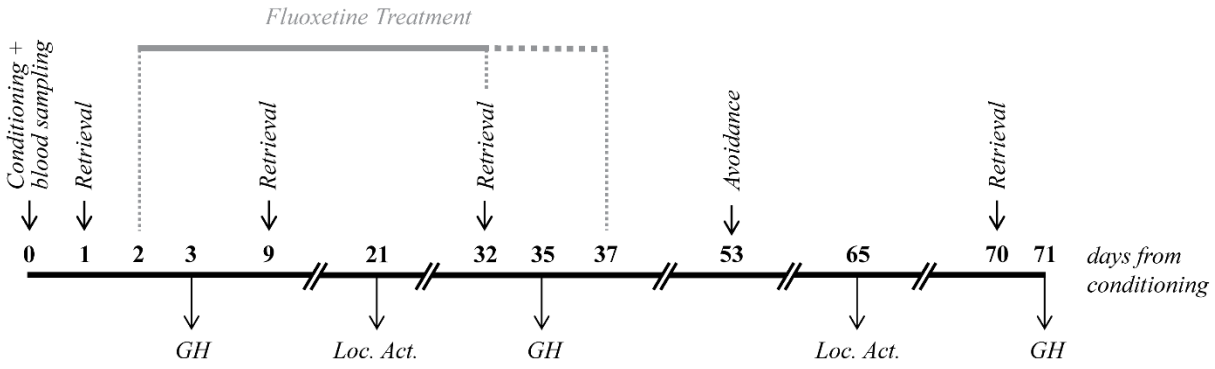

Figure 1 – Timeline of the experimental design. *Conditioning* with shock delivery, and *blood sampling* for CORT evaluation were conducted on the first day of the experimental schedule. Memory *retrieval* was repeatedly assessed 1, 9, 32 and 70 days after conditioning, while conditioned odor active avoidance (*Avoidance*) was tested only once, 53 days after conditioning. Fluoxetine treatment begun two days after conditioning, with a dosage of 20 mg/Kg/day for the first 30 days, followed by 5 days of gradual dose tapering, i.e. 10 and 5mg/Kg/day for 2 days each, and 2,5 mg/Kg/day for the last day of treatment. General health (*GH*) and locomotor activity (*Loc. Act.*) assessments were carried out during and after the end of the treatment. *Horizontal axis*: days from conditioning (shock delivery). ▬: full-dose fluoxetine treatment; ▪ ▪ ▪ :gradual fluoxetine dose tapering.

Fluoxetine Treatment

Flx (fluoxetine HCl, SantaCruz, USA) was dissolved in tap water and delivered ad libitum via light-proof drinking bottles in order to provide an average daily intake of 20 mg/Kg (35-37). The flx treatment started from 2 days after conditioning and lasted for 1 month. To avoid the occurrence of a discontinuation-like syndrome, the chronic treatment was followed by a 5 days period of gradual dose tapering (36) (see Supplement).

Fear conditioning

Conditioning procedure. To assess the presence and duration of fear memory alterations that resemble those found in PTSD patients, a previously published fear conditioning paradigm was used (34). We used a fear conditioning protocol in which the presentation of a tone is never associated with the delivery of the shocks (see Supplement and Figure 2), in order to simultaneously evaluate two typical PTSD symptoms: 1) an abnormal memory of the context where the traumatic event occurred; 2) the inability to restrict fear to stimuli predicting the threat. With this schedule conditioned fear is in fact expected to occur when mice are re-exposed to the conditioning context alone (correct predictor of the threat), but not to the tone presented in a novel context (which does not predict the threat), allowing the detection of trauma-triggered fear generalization and impaired coding of safety cues (the tone, in this paradigm), features typically displayed by PTSD patients (38).

Plasma corticosterone levels after shock. To evaluate the presence of abnormalities in the acute response to stress of the hypothalamic-pituitary-adrenal axis (39), plasma levels of corticosterone (CORT) were evaluated from blood samples collected from tail bleeding 15 minutes after the exposure to the last footshock (40). Samples were collected into potassium ethylenediaminetetraacetic acid (EDTA)-coated tubes and processed using a commercial radioimmunoassay (RIA) kit (ImmunChemTM 125I Corticosterone RIA, MP Biomedicals, Orangeburg, NY) (41) (see Supplement).

Fear memory retrieval. Fear responses to the conditioning context and the tone were assessed 24 hours and 9, 32 and 70 days after conditioning (see Figure 1) to evaluate on the same subject the intensity and persistence of fear response to the external reminders of the traumatic event and the ability to discriminate the correct predictors of the threat (37). For auditory fear memory retrieval, mice were placed in a new context for 120 s of acclimatization after which they were presented with the tone for 120 s and then returned to the home cages. Two hours later, mice were placed in the conditioning chamber and left there for 120 s, to assess contextual fear memory recall. An index of fear memory was obtained by subtracting the percent time spent freezing during baselines from percent time spent freezing during retrieval sessions (Δ freezing) (see Supplement and Figure 2).

Unconditioned response to tone cues. To control for genotype-driven differences, an additional cohort of MeCP2–308 and wt mice were subjected to the auditory fear test without being previously conditioned (N=31; wt=16, MeCP2-308=15).

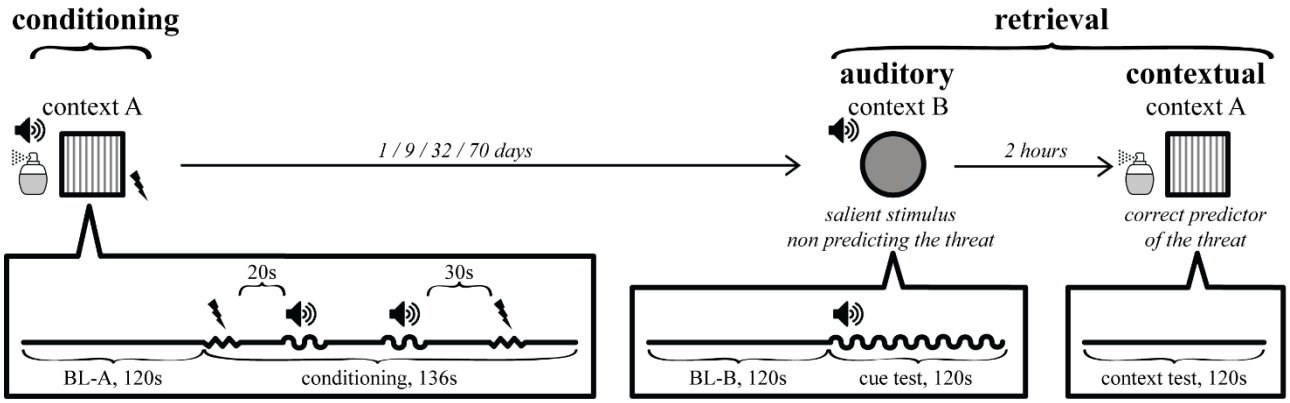

Figure 2 – Fear conditioning and retrieval protocols. *Conditioning* was conducted in Context A, a Plexiglas cubicle with grid floor scented with 4% acetic acid solution. A tone-shock unpairing procedure resulting in a mixed backward trace conditioning/trace conditioning schedule was applied. Specifically, 120 s after being placed into the chamber for animal acclimatization (BL-A), animals received a shock that was followed, after a 20 s delay, by a tone; after a 30 s delay, the same tone and the same shock spaced by a 30 s interval were again presented; then, after 20 s the animal was placed back in its home cage. Fear *retrieval* in response to the tone and the context were tested repeatedly on the same animals either 24 hours (recent memory) and 9, 32 and 70 days (remote memory) after conditioning. Briefly, for the auditory fear recall test mice were placed in a novel context (Context B), consisting in a Plexiglas cylinder with sawdust bedding scented with 70% ethanol solution, for a 120 s acclimatization period (BL-B) after which the tone was delivered continuously for 120 s (cue test). Mice were then placed back in their home cages for two hours, after which they were re-exposed to Context A for 120 s (context test), to assess contextual fear recall. The order or fear recall testing (first auditory and then contextual) was the same for all the animals and all the testing days. : footshock of 0.8 (high) or 0.3 (low) mA, 50 Hz, 3s; : tone of 65 dB, 1 kHz, 15s (*conditioning*) or 120s (*auditory fear retrieval*); : 4% acetic acid solution scent.

Conditioned odor active avoidance

To verify the presence of long-lasting avoidance symptoms, additional and highly specific features for PTSD chronic condition (11), the conditioned odor active avoidance test was applied 53 days after conditioning. The testing protocol was inspired by the conditioned odor avoidance task described in (42). Briefly, a 2-chambered apparatus was cleaned with 2 differently scented solutions: the starting chamber (aversive) with 4% acetic acid (the same as used for the conditioning chamber), while 70% ethanol was used for the opposite chamber (neutral). Mice were given 10 minutes of free exploration. Percent time spent in the aversive compartment and counts of head out behavior towards the aversive compartment were considered as indices of odor avoidance (see Supplement).

Circadian locomotor activity

To control for the presence of disruptions in the circadian rhythm, characteristic of PTSD symptomatology (11), spontaneous locomotor activity in the home cages was monitored continuously for 24 hours either at 21 and 65 days distance from the shock by means of an automatic device. No movements were detected when mice were sleeping, inactive, or performed moderate self-grooming.

Scores were obtained during 1 hour intervals and expressed as counts per minute (cpm) (see Supplement for further details).

Gene expression in the blood

To check for the presence of alterations in the expression of a subset of genes known to be deranged in the blood of PTSD patients (23, 43, 44), real-time PCR was performed on whole blood collected 9 days after the shock delivery, when PTSD symptoms are supposed to be well-established (45, 46). Blood was collected from a separate cohort of animals (N=6) by retro-orbital sampling and RNA was extracted with the Quick RNA MiniPrep Plus kit (Zymoresearch, Irvine, CA, U.S.A.) according to manufacturer's instructions. The expression of the following genes was analyzed in high-shocked wt and MeCP2-308 mice: serum and glucocorticoid regulated-kinase 1 (Sgk1), Fk506 binding protein 5 (Fkbp5), tetraspanin 32 (Tspan32), CAMP responsive element binding protein 1 (Creb1) and arginine vasopressin (Avp). One µg of total RNA was used for cDNA synthesis, and 1 µL of total cDNA was used for each Real-Time PCR reaction in triplicate for each sample (47) (see Supplement).

Statistical analyses

Data were analyzed with either parametric or nonparametric analysis of variance, depending on distribution of the response variable considered. Unpaired t-test, 2-way analyses of variance (ANOVAs) and Kruskal-Wallis test were performed to compare experimental groups. Unpaired t-test included genotype as an independent variable. ANOVAs included genotype and shock or genotype and manipulation (shock intensity plus drug treatment) as between-subject factors. Repeated measurements (e.g. delta percent time freezing at 9, 32 and 70 days after conditioning) were considered as within-subject factors. Post hoc comparisons were performed using Tukey's test, which can also be used in the absence of significant ANOVA results (48), or Mann-Whitney test with Bonferroni's correction. Animals were excluded from the analyses when identified as outliers by the use of the Grubbs' test, which is among the most appropriated methods in case of small samples (49, 50). The alpha level was set at 5%.

Results

Aberrant contextual fear recall in high-shocked MeCP2-308 mice is normalized by fluoxetine

As expected, high-shocked mice froze longer compared to low-shocked animals 24 hours after conditioning (shock: $F_{(1,45)} = 15.425$, $p < 0.001$). Exaggerated fear responses to the external reminders of the trauma were found in high-shocked MeCP2-308 mice that presented higher freezing durations compared to all the other groups [Figure 3A; genotype*shock interaction: $F_{(1,45)} = 0.619$, $p = 0.436$; *post hoc*: $p < 0.05$ (MeCP2-308, high vs wt, high and MeCP2-308, high vs MeCP2-308, low); $p < 0.01$ (MeCP2-308, high vs wt, low)].

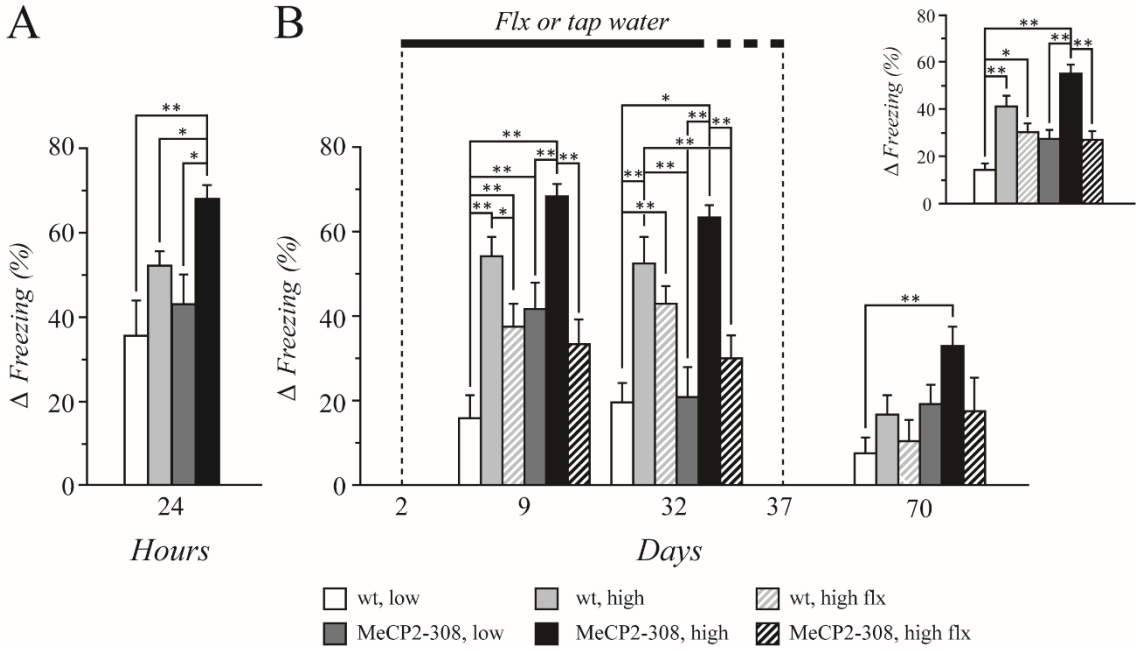

Figure 3 – *Aberrant contextual fear recall in high-shocked MeCP2-308 mice is normalized by fluoxetine*. A) *Contextual fear recall 24 hours after shock delivery*. Trauma-exposed MeCP2-308 mice (MeCP2-308, high) performed significantly higher levels of freezing behavior compared to wild type littermates (wt) and MeCP2-308 mice subjected to low shocks (MeCP2-308, low), depicting an exaggerated emotionality when re-exposed to predictors of the threat. Mice for each condition were as follows: wt, low = 7; wt, high = 21; MeCP2-308, low = 7; MeCP2-308, high = 14. **B)** *Long-lasting contextual fear recall and effects of fluoxetine (flx)*. Trauma-exposed MeCP2-308 mice showed long-lasting abnormal fear response, as demonstrated by increased context-related freezing compared to wt, low controls at 9, 32 and 70 days after shock delivery. Flx reduced freezing to the levels of low-shocked animals. These findings are confirmed on the average measure of the testing days (see inset). *Δ freezing* was obtained by subtracting from percent time spent freezing during contextual fear retrieval the percent time freezing in the same context before conditioning (see Figure 2). Mice for each condition were as follows: wt, low = 7; wt, high = 11; wt, high flx = 10; MeCP2-308, low = 6; MeCP2-308, high = 7; MeCP2-308, high flx = 4. *Horizontal axis*: days from shock delivery. ▬: full-dose flx treatment (20 mg/Kg/day); ▪▪▪ :gradual flx dose tapering (10, 5, 2 mg/Kg/day). Data are mean ± SEM. *: $p < 0.05$; **: $p < 0.01$.

To evaluate the persistence of the fear response, the experimental mice were re-exposed to the conditioning context 9, 32 or 70 days after conditioning (Figure 1). Moreover, 2 subgroups of both wt and MeCP2-308 mice receiving the high shock were subjected to a chronic treatment with flx, starting from 2 days after shock delivery and lasting 1 month, and their behavioral response was compared to that presented by mice receiving tap water, to verify whether flx can reverse the fear response of trauma-exposed MeCP2-308 mice to normal. We found that, even though less marked, the hyperresponsiveness of untreated high-shocked MeCP2-308 mice was still evident 1 week after

trauma exposure and persisted until the last monitoring, which was conducted 2 months after the shock delivery [Figure 3B; day*genotype*manipulation interaction: $F_{(4,78)} = 1.529$, $p = 0.202$; *post hoc*: $p < 0.05$ (MeCP2-308, high vs wt, low)]. Treatment with flx had the overall effect of reducing context-related freezing of high-shocked MeCP2-308 mice, restoring levels of fear response to the conditioning context comparable to those of mice receiving the low shocks [genotype*manipulation interaction: $F_{(2,39)} = 2.421$, $p = 0.102$; *post hoc*: $p < 0.01$ (MeCP2-308, high vs MeCP2-308, high flx); Figure 3B, inset]. Significant effects of flx treatment were indeed found as early as after 1 week from the beginning of treatment, i.e. 9 days from shock delivery, and lasted until its end (Figure 3B; day*genotype*manipulation interaction; *post hoc*: $p < 0.01$ (MeCP2-308, high vs MeCP2-308, high flx)].

Increased long-lasting fear response to salient non-predicting cues in trauma-exposed MeCP2-308 mice is partially normalized by fluoxetine treatment

The exposure to the tone in a novel context 24 hours after shock delivery did not elicit fear responses in wt mice, thus confirming that this salient cue was not predictive of the trauma (Δ *freezing*: wt = 0.147 ± 1.551 %). A freezing response was instead observed in high-shocked MeCP2-308 mice, who performed more freezing than all the other groups in response to the tone [Figure 4A; $H_{(3)} = 26.282$, $p < 0.001$; *post hoc*: U = 4, $p < 0.05$ (MeCP2-308, high vs MeCP2-308, low); U = 4, $p < 0.01$ (MeCP2-308, high vs wt, low) and U = 8, $p < 0.001$ (MeCP2-308, high vs wt, high)]. Importantly, increased tone-related freezing in high-shocked MeCP2-308 mice compared to high-shocked wt mice was persistent, as it lasted for 1 month after conditioning (Figure 4B; day*genotype*manipulation interaction: $F_{(4,78)} = 3.096$; $p = 0.020$; *post hoc*: $p < 0.01$).

Contrary to our expectation, we found that even MeCP2-308 mice that received mild footshocks presented higher levels of freezing compared to high-shocked wt mice when exposed to the tone (genotype*manipulation interaction: $F_{(2,39)} = 3.057$, $p = 0.058$; *post hoc*: $p < 0.01$). This abnormal response was not due to low shocks being sufficient to induce fear memory of the non-predicting cue in MeCP2-308 mice, since we found that MeCP2-308 mice froze more than wt in response to auditory cue even in the absence of any conditioning (Figure 4C; genotype: $t_{(29)} = -3.717$, $p < 0.001$). These data suggest that hyperarousal may account for the increased freezing response in low-shocked MeCP2-308 mice, and confirm that long-lasting fear memory alterations, a key feature of PTSD symptomatology, selectively occur in trauma-exposed MeCP2-308 mice.

Flx treatment led to mild beneficial effects on the aberrant fear response to the non-predicting cue in high-shocked MeCP2-308 mice, halving the levels of tone-related freezing presented by

untreated traumatized MeCP2-308 mice after 4 weeks of treatment (32 days after the shock delivery; Figure 4B).

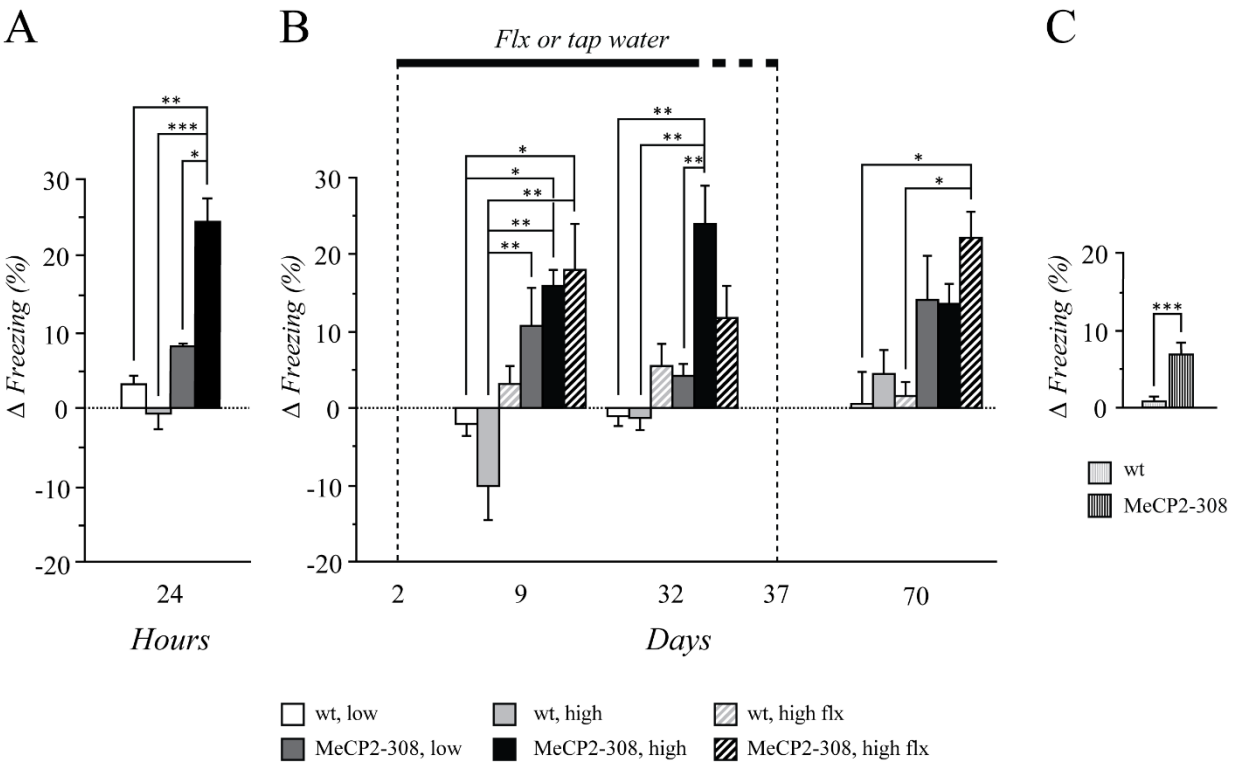

Figure 4 – ***Increased long-lasting fear response to salient non-predicting cues in trauma-exposed MeCP2-308 mice is partially normalized by fluoxetine treatment. A)*** *Auditory fear recall in a non-conditioned context 24 hours after the shock delivery*. Exposure to the tone in a novel context did not elicit a fear response in wild type (wt) mice. Trauma-exposed MeCP2-308 mice (MeCP2-308, high) performed more freezing when exposed to the tone than wt littermates and MeCP2-308 mice subjected to low shocks (MeCP2-308, low), thus demonstrating an incapacity to restrict fear to the correct predictors of the threat. Mice for each condition were as follows: wt, low = 6; wt, high = 21; MeCP2-308, low = 4; MeCP2-308, high = 14. **B)** *Long-lasting auditory fear recall and effects of fluoxetine (flx)*. Increased tone-related freezing in trauma-exposed MeCP2-308 compared to trauma-exposed wt mice (wt, high) was still present at 32 days after shock delivery and disappeared after 70 days. Flx partially reduced tone-elicited freezing after 1 month of treatment (32 days after shock delivery). Mice for each condition were as follows: wt, low = 7; wt, high = 11; wt, high flx = 10; MeCP2-308, low = 6; MeCP2-308, high = 7; MeCP2-308, highflx = 4. *Horizontal axis*: days from shock delivery. ▬: full-dose flx treatment (20 mg/Kg/day); ▪ ▪ ▪ : gradual flx dose tapering (10, 5, 2 mg/Kg/day). **C)** *Auditory fear response in non-conditioned mice.* MeCP2-308 mice froze significantly more than wt in response to the auditory cue in the absence of any conditioning. Mice for each condition were as follows: wt = 16; MeCP2-308 = 15. *Δ freezing* was calculated by subtracting the percent time freezing displayed during the tone delivery from that displayed during the baseline in the non-conditioned context (see Figure 2). Data are mean ± SEM. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

<u>Exaggerated long-lasting avoidance of trauma-related cues in MeCP2-308 high-shocked mice is dampened by fluoxetine treatment</u>

High-shocked MeCP2-308 mice spent significantly less time in the aversive compartment compared to low-shocked mice from both genotypes [Figure 5A; genotype*manipulation interaction: $F_{(2,38)} = 5.663$; $p = 0.007$; *post hoc*: $p < 0.05$ (MeCP2-308, high vs wt, low); $p < 0.01$ (MeCP2-308, high vs MeCP2-308, low)], demonstrating active avoidance of the conditioning chamber scent when this was presented in a different context. Furthermore, the occurrence of the risk assessment behavior head out was selectively increased in MeCP2-308 mice receiving the high shock [Figure 5B;

genotype*manipulation interaction: $F_{(2,39)} = 1.722$; $p = 0.192$; *post hoc*: $p < 0.05$ (MeCP2-308, high vs MeCP2-308, low and MeCP2-308, high vs wt, high); $p < 0.01$ (MeCP2-308, high vs wt, low)]. Long-lasting effects of the flx treatment were observed. Flx in fact increased the time spent in the aversive compartment of the apparatus and decreased the frequency of head out in high-shocked MeCP2-308 mice, thus restoring wt-like levels [*head out*: genotype*manipulation interaction; *post hoc*: $p < 0.05$ (MeCP2-308, high vs MeCP2-308, high flx)].

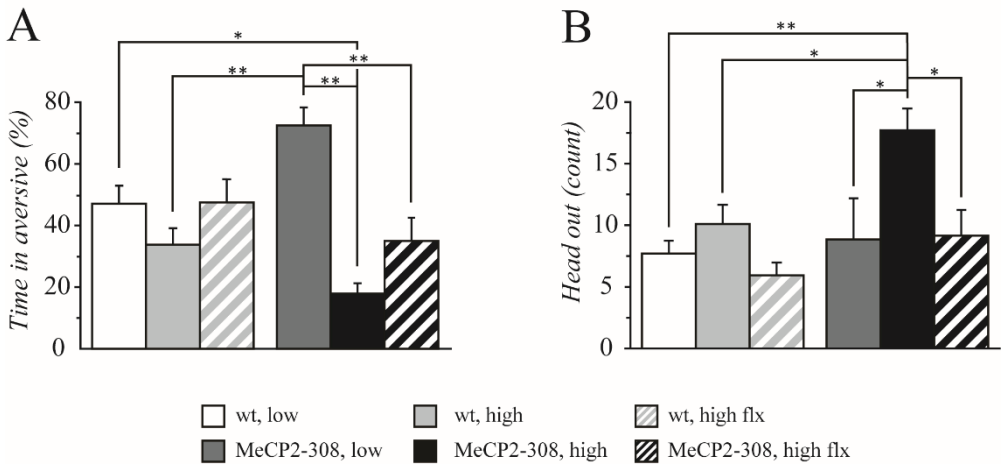

Figure 5 - ***Exaggerated long-lasting avoidance of trauma-related cues in MeCP2-308 high-shocked mice is dampened by fluoxetine treatment***. Mice were shocked in an acetic acid scented compartment (conditioning context) and tested 53 days later in the conditioned odor active avoidance task. The apparatus consisted of 2 chambers, scented with acetic acid (starting chamber, aversive) or ethanol (neutral). **A)** *Percent time spent in the aversive compartment*. Trauma-exposed MeCP2-308 mice (MeCP2-308, high) spent significantly less time in the aversive compartment compared to low-shocked groups (wt, low and MeCP2-308, low). Fluoxetine (flx) increased the percent time spent in the aversive compartment by MeCP2-308, high mice, thus restoring wt-like levels. Mice for each condition were as follows: wt, low = 7; wt, high = 11; wt, high flx = 10; MeCP2-308, low = 5; MeCP2-308, high = 7; MeCP2-308, high flx = 4. **B)** *Number of time (counts) performing head out risk assessment behavior towards the aversive compartment*. Trauma-exposed MeCP2-308 mice performed significantly more frequently head out risk-assessment behavior towards the aversive compartment compared to all the other groups. This abnormal behavioral response was normalized by flx. Mice for each condition were as follows: wt, low = 7; wt, high = 11; wt, high flx = 9; MeCP2-308, low = 6; MeCP2-308, high = 7; MeCP2-308, high flx = 5. Data are mean ± SEM. *: $p < 0.05$; **: $p < 0.01$.

<u>The hypoactive profile of MeCP2-308 mice was not affected by either shock intensity or fluoxetine treatment</u>

As expected, MeCP2-308 mice were significantly less active than wt mice in the home cages (genotype: $F_{(1,39)} = 17.579$; $p < 0.001$), especially during the dark/active phase of the L/D cycle ($p < 0.01$ after *post hoc* comparison between dark, wt and dark, MeCP2-308 on the phase*genotype interaction: $F_{(1,39)} = 25.807$; $p < 0.001$). A main effect of manipulation was found (manipulation: $F_{(2,39)} = 6.496$; $p = 0.004$), related to a strong sedative effect of fluoxetine on wt mice during the first assessment ($p < 0.01$ after *post hoc* comparisons on the day*phase*genotype*manipulation interaction: $F_{(2,39)} = 0.855$; $p = 0.433$; Figure S2). No significant effect of manipulation on MeCP2-308 mice or of day of assessment were detected.

Shock-induced plasma levels of corticosterone are increased in trauma-exposed MeCP2-308 mice

We found that high-shocked MeCP2-308 mice showed higher CORT plasma levels 15 minutes after the delivery of the footshocks than all the other groups (Figure 6; genotype*shock interaction: $F_{(1,29)} = 10.128$, $p = 0.004$; *post hoc*: $p < 0.01$).

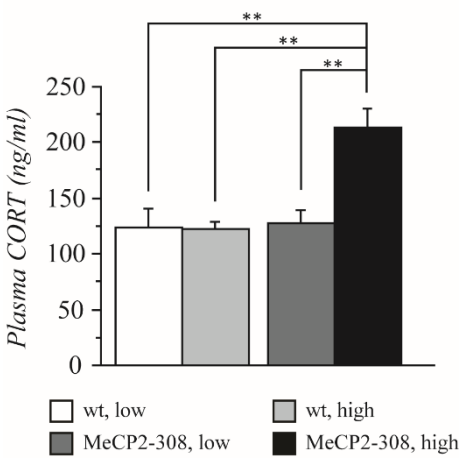

Figure 6 – *Shock-induced plasma levels of corticosterone are increased in trauma-exposed MeCP2-308 mice.* Blood samples were collected 15 minutes after the last shock delivery during conditioning on the first day of the schedule to evaluate corticosterone (CORT) plasma levels. Trauma-exposed MeCP2-308 mice (MeCP2-308, high) showed significantly increased CORT plasma levels compared to all the other groups. Mice for each condition were as follows: wt, low = 7; wt, high = 10; MeCP2-308, low = 7; MeCP2-308, high = 9. Data are mean ± SEM. **: $p < 0.01$.

High-shocked MeCP2-308 show altered peripheral gene expression of PTSD-related proteins

We found that *Sgk1*, *Fkbp5* and *Tspan32* were significantly under-expressed (respectively, genotype: $t_{(10)} = 4.471$, $p = 0.001$; genotype: $t_{(10)} = 4.318$, $p = 0.002$; genotype: $t_{(10)} = 2.401$, $p = 0.037$), and *Creb1* significantly over-expressed (genotype: $t_{(10)} = -5.322$, $p < 0.001$) in the blood of high-shocked MeCP2-308 mice compared to high-shocked wt controls (Figure 7). *Avp* mRNA showed only a trend to be increased in MeCP2-308 compared to wt (MeCP2-308, high = 1.324 ± 0.162 fold change vs wt, high).

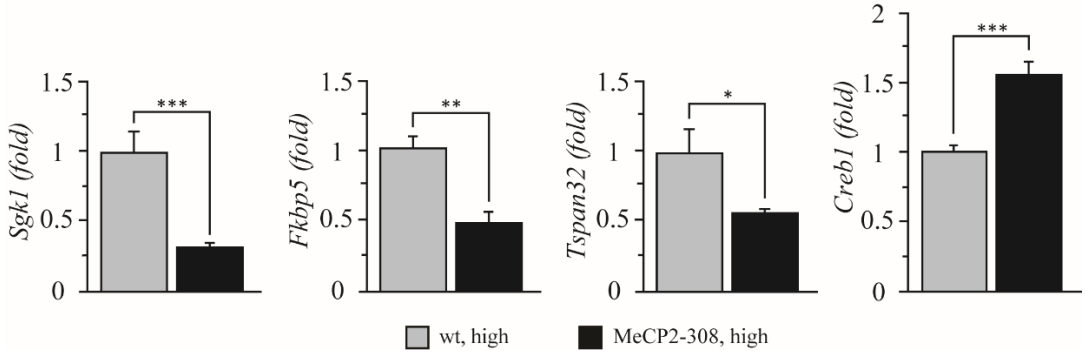

Figure 7 – *Trauma-exposed MeCP2-308 mice mirror the alterations in peripheral gene expression found in patients*. Real-time PCR analyses were performed on whole blood collected 9 days after trauma exposure. *Sgk1*, *Fkbp5* and *Tspan32* were under-expressed in trauma-exposed MeCP2-308 mice (MeCP2-308, high) in comparison with trauma-exposed wild type group (wt, high). *Creb1* was found to be over-expressed in blood of MeCP2-308, high mice compared to wt, high mice. Measures are fold change vs wt, high group and normalized to *βactin*. N=6 for each condition. Data are mean ± SEM. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Discussion

The present work provides evidence that male mice carrying a truncated form of MeCP2 protein develop PTSD-like symptoms in response to traumatic events occurring at adulthood. This study identifies a new possible factor involved in PTSD etiology and stresses the importance of environment-driven epigenetic changes in defining the boundaries of susceptibility to this disorder of multifactorial polygenic etiology. Furthermore, results presented here reveal that MeCP2-308 male mice are a powerful tool to investigate the biological underpinnings of interindividual susceptibility to develop PTSD.

Differential epigenetic states are increasingly recognized as critical contributors to risk and resilience to develop PTSD, with experience-dependent methylation changes representing the most intriguing epigenetic marks of increased susceptibility. Distinctive DNA methylation profiles have been in fact highlighted in the blood of PTSD patients compared to healthy people exposed to trauma (23, 51-53). Moreover, the methylation machinery itself undergoes diverse regulation in resilient and vulnerable people, at both pre- and post-trauma timepoints (51). The present study demonstrates that MeCP2, the founding member of a family of proteins recognizing and binding methylated DNA, is involved in shaping vulnerability to develop PTSD-like symptoms in the aftermath of traumatic events.

Indeed, the exposure to a traumatic event induced PTSD-like memory impairments and exaggerated avoidance behavior, key features of PTSD symptomatology, selectively in MeCP2-308 mice. In particular, a substantial fear response to salient cues not predicting the threat was found in trauma-exposed MeCP2-308 mice. The traumatic event indeed triggered an incapacity to restrict fear to the correct predictors of the threat, suggesting an over-representation of amygdala-dependent emotional memory of the aversive experience leading to a generalization of fear to non-predictive, highly arousing stimuli in MeCP2-308 mice. (34). Moreover, traumatized MeCP2-308 mice tend to show an increased, exaggerated hippocampal-dependent fear recall when re-exposed to the context, the correct predictor of the threat. Such persistent behavioral distress in response to external reminders of the threat is a core symptom of PTSD (11). These findings clearly evidence the onset of PTSD-like memory impairments as a consequence of exposure to an intense stressor specifically in MeCP2-308 mice. In fact, the use of a mild stressor as a control allows us to unequivocally confirm that MeCP2-308 mice require a threatening event (i.e. going above a certain threshold of elicited distress) to develop PTSD-like features, thus matching the first, fundamental, criterion of PTSD diagnosis (11, 54). Moreover, both auditory and contextual exaggerated fear responses exhibited by trauma-exposed

MeCP2-308 mice are persistent and protracted over time (49). The long-lasting aberrant fear memory profile is indeed modeling a fundamental diagnostic criterion of PTSD requiring the symptoms to last at least 1 month (1), thus further corroborating the disease-specificity of such alterations. Indeed, repeated exposures to the conditioning context seem to have a fear sensitization effect in transgenic mice, triggering recurrent recalls and reconsolidation of traumatic memories, rather than progressive habituation and extinction, leading to the increase or even persistence of fear responses over time (45).

The peculiar behavioral consequences triggered by the trauma in MeCP2-308 mice also include exaggerated long-lasting avoidance of trauma-related cues, a particularly reliable behavioral marker for PTSD (11, 42). Although assessed with more than 1 month distance from shock delivery, avoidance behavior was still strongly pronounced in trauma-exposed MeCP2-308 mice. This symptom is often modeled using approach-avoidance conflict tests, which evaluate generalized anxiety-like behavior instead of the trauma-specific avoidance typical of PTSD condition (54). Our work shows a strong and persistent avoidance of stimuli specifically related to the aversive event, hence further increasing the relevance for PTSD condition of the trauma-induced phenotype in MeCP2-308 mice.

An additional important point in favor of the similarity between the outlined aberrancies and PTSD symptomatology is that the behavioral syndrome induced in MeCP2-308 mice by exposure to high-intensity footshocks is relieved by chronic treatment with flx, the most widely used drug for PTSD in the clinical setting (28). Specifically, chronic flx administration exerted some beneficial effects on fear responses and normalized the avoidance of trauma-related cues.

Further similarity between the proposed model's phenotypic alterations and PTSD symptomatology is supported by our finding of decreased activity during the dark, active phase of the day, suggesting alterations in the circadian rhythm in transgenic mice. It is indeed known that MeCP2-308 mice show decreased locomotion during the dark and increased fine movements during the light phase (59). The reduced ambulatory activity during the active phase has been suggested to stand for increased sleepiness, although specific measurements of sleep have not been performed until now, as far as we know. However the exposure to a trauma did not further disrupt circadian activity in MeCP2-308 mice, suggesting that this alteration may not be selectively related to PTSD in this mouse model.

The abnormal memory recall profile in trauma-exposed MeCP2-308 mice was also associated with an altered plasma CORT secretion 15 minutes after the shock delivery, confirming that aberrant

trauma-related physiological reactions occur, ultimately influencing traumatic memory consolidation. Such evidence is in accordance with findings that PTSD-like memory impairment is triggered by elevated levels of glucocorticoids during conditioning with an intense threat (34, 55). However, the literature on this topic is inconsistent, probably due to the presence of biologically distinct subtypes of PTSD patients displaying different physiological stress reactivity and comorbidity (36, 56-58).

The finding that trauma-exposed MeCP2-308 mice showed an abnormal peripheral gene expression profile, specifically mirroring that found in PTSD patients, confirms the presence of disease-specific phenotypical aberrancies at the molecular level. Although literature on human research is not uniform in identifying peripheral biomarkers of the disease, arguably on account of the high clinical variability of the PTSD syndrome (56), our results broadly match findings from patients with multiple backgrounds. In fact, MeCP2-308 threatened mice displayed the same expression profile of a subset of genes (namely *Sgk1*, *Fkbp*5, *Tspan32*, *Creb1* and *Avp*) that have been found to be deregulated in blood samples of various groups of PTSD patients (23, 43, 44), highlighting the strength of these molecular alterations as potential biomarkers. Interestingly, some of these genes have been reported to be MeCP2 targets (*Sgk1*, *Fkbp5*, *Creb1*, *Avp* (5, 60, 61), providing further support the involvement of MeCP2 in shaping vulnerability to traumatic events. Present results do not provide any clue about the molecular alterations at the central level (62). Further analyses on brain tissue are needed to understand the mechanism underlying the behavioral and physiological alterations here highlighted. Given the finding of important alterations in the methylome of PTSD patients (19, 23), the relation between DNA methylation and the observed gene expression alterations may be assessed in the future (63-65).

Taken together, present results provide convincing evidence that dysfunctions in MeCP2 result in increased susceptibility to develop psychopathology in response to a trauma in male mice. Even though it is not possible to distinguish between a direct involvement of the truncated MeCP2 protein and the deregulation of its downstream signaling pathways based on data presented here, present findings support the hypothesis that DNA methylation plays a major role in mediating vulnerability to life adversities (19, 23, 51, 52) and suggest a novel signaling network involved in PTSD etiology. Moreover, an innovative powerful tool to investigate brain changes that result from a PTSD-inducing traumatic experience and to identify innovative biomarkers for risk and resilience to PTSD is provided. Indeed, the strong genetic-based variability we highlighted in the outcome of threatening stimuli, with the discrimination of susceptible (MeCP2-308) from resilient (wt) populations, opens a

completely new window of opportunity for a mechanistic understanding of the biological underpinnings of PTSD vulnerability.

Accumulating evidence outline the presence of *MECP2* gene variants or expression alterations in a number of diseases besides Rett syndrome (28), ranging from *lupus erythematosus* to schizophrenia (7, 8, 29, 30, 66). Present results broaden the spectrum of MECP2-associated disorders, suggesting that the emerging role of MeCP2 as a master regulator of experience-dependent epigenetic programming (18) may be critical in mediating those gene × environment interactions that underlie PTSD vulnerability (21, 23, 24). Further studies are currently in progress on human cohorts that will clarify the relative involvement of MECP2 in the etiology of PTSD and the possibility of pinpointing specific MECP2-related innovative markers of disease susceptibility.

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Disclosures

None of the authors declare financial interests or potential conflict of interests.

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Supplementary methods

Animals

Experimental animals were obtained by mating MeCP2-308 heterozygous females (+/-) with wt males (+/Y). Upon weaning at postnatal day 25 mice were housed according to sex in groups of 2-3 in polycarbonate transparent cages (33(d)×13(w)×14(h)cm) with sawdust bedding and kept on a 12h light-dark schedule, with lights off at 7am (winter time) or 8am (summer time). Temperature was maintained at 21±1°C and relative humidity at 60±10%. Animals were provided *ad libitum* with tap water and a complete pellet diet (Altromin, 1324 - 10mm pellets, Germany). Experimental mice were isolated at least 1 week before the beginning of the testing protocol. Behavioral testing was performed by experimenters blind to mouse genotype and treatment during the dark/active phase, starting at least 1 hour after lights off.

Genotyping

DNA was prepared from a small tail-tip biopsy taken at weaning, and at sacrifice as previously described (1). The MeCP2 alleles were identified by PCR using 2 sets of primers. Primer set 1 (5′ primer: 5′-AAC GGG GTA GAA AGC CTG-3′ and 3′ primer: 5′- ATG CTC CAG ACT GCC TTG -3′) yields a product of 396 bp identifying the wt allele. Primer set 2 (5′ primer: same as for primer set 1 and 3′ primer: 5′- TGA TGG GGT CCT CAG AGC -3′) yields a product of 318 bp identifying the null allele (2). The cycling conditions used were 3’ at 94 °C, 38x(45’’ at 94 °C, 45’’ at 62 °C, 45’’ at 72 °C), and 7’ at 72 °C.

PCR products were electrophoresed through a 2% NuSieve 3:1 agarose gel (Cambrex Bio Science, Rockland, ME, USA) containing 0.1 µl/ml GelRed™ and examined under UV light.

Fluoxetine Treatment

Fluoxetine (flx) (fluoxetine HCl, SantaCruz, USA) was dissolved in water and delivered *ad libitum* for 35 days via light-proof drinking bottles (the only source of drinking water) and changed weekly. Although less precise in daily dose assumption, we preferred an indirect administration method to direct ones, such as oral gavage or intraperitoneal injection, to avoid the stress deriving from handling procedures (3, 4). The solutions were prepared in order to provide an average daily intake of 20 mg/kg for 30 days of treatment, 10 and 5 mg/kg for the following 4 days (2 and 2, respectively) and 2.5 mg/kg for the last day of treatment. This gradual reduction of dosage was preferred to a sudden interruption of treatment to avoid the occurrence of something comparable to the discontinuation syndrome seen in some people taking antidepressant drugs. The dosage of 20

mg/kg was chosen according to literature (5-7) and the choice to progressively halve the dosage was supported by the protocol used in (5).

Drug concentration necessary to achieve the desired dose of 20 mg/kg/day was determined for each genotype from average body weight and average daily water consumption, measured from bottle weights, corrected for evaporation and spillage (8), on the first week of isolation, before the beginning of the testing protocol, resulting in 88 mg/l for both wt and MeCP2-308 mice. The same procedure was used to calculate flx and water consumption during the testing protocol, to control for effective drug dose assumption.

Fear conditioning

Conditioning procedure. A previously published protocol of fear conditioning was applied with slight modifications (9). We used an automated fear conditioning system consisting in a computer-controlled operant chamber (conditioning chamber), comprising a Plexiglas box (25(d)x30(w)x30(h)cm) with electrified grid floor (27 stainless-steel rods (4 mm diameter), spaced 7 mm apart, connected to a shock generator), and a removable speaker, all inside a soundproof cubicle (Coulbourn Instruments, Allentown, PA, USA) (10). Fear conditioning was performed on the first day of the experimental schedule. Mice were placed in the conditioning chamber (context A), in a brightness of 4 lux, for 256 s during which they were exposed to 2 tone cues (65 dB, 1 kHz, 15 s of length) and 2 footshocks (0.3 or 0.8 mA, 50 Hz, 3 s of length) combined in a mixed backward trace conditioning/trace conditioning schedule (cue-shock unpairing procedure). Specifically, 120 s after being placed into the chamber for animal acclimatization [baseline (BL-A)], animals received a shock that was followed, after a 20 s delay, by a tone; after a 30 s delay, the same tone and the same shock spaced by a 30 s interval were again presented (Figure 2). A trace interval between CS and US was used to weaken the association to the salient cue proportionally to the time distance of delivery, while reducing the relevance of the footshock grid as a tonic contextual element (9, 11). Wt and MeCP2-308 mice were assigned to receive a footshock of 0.8 mA (high) or 0.3 mA (low) intensity, as control, balancing for general health score and age (4 or 5 months) (i.e. comparable means between groups of animals with the same genotype).

Plasma corticosterone levels after shock. Plasma levels of CORT (the primary glucocorticoid produced by rodents) were evaluated from blood samples collected on the first day of the schedule, 15 minutes after the last footshock delivery. Samples were collected into potassium ethylenediaminetetraacetic acid (EDTA)-coated tubes (1.6 mg EDTA/ml blood, Sarstedt, Germany), cool centrifuged at 4°C and the plasma stored at -80°C until assayed. We collected approximate volumes of 0.1 ml of plasma. CORT was measured in 5 µl plasma sample using a commercial

radioimmunoassay (RIA) kit (ImmunChemTM 125I Corticosterone RIA, MP Biomedicals, Orangeburg, NY) with 200 µl trace and 200 µl antibody as previously described (12). The sensitivity of the assay was 7.7 ng/ml.

Fear memory retrieval. Mice were tested for both auditory and contextual fear memory retrieval by the means of the following protocols:

Auditory fear memory retrieval: mice were placed in a context consisting of a clear Plexiglas cylinder (20 cm in diameter) with sawdust bedding (context B), placed inside a grey PVC open field apparatus (40(d)x40(w)x35(h) cm), to prevent access to the visuospatial cues of the experimental room. The speaker was moved from the fear conditioning chamber to the top of the cylinder. Animals were given 120 s of acclimatization (BL-B) after which they were presented with the cue (65 dB, 1 kHz) for 120 s (cue test) and then returned to the home cages (Figure 2). Brightness was set at 15 lux. The cylinder was cleaned with 70% ethanol and the sawdust bedding was changed before each trial. As an index of auditory fear memory, the Δ *freezing* was calculated by subtracting the percent time freezing performed in BL-B from percent time spent freezing during cue test.

Contextual fear memory retrieval: two hours after auditory fear retrieval, mice were placed in the conditioning chamber (context A) and left there for 120 s (context test), after which they were returned to the home cages (Figure 2). Brightness was the same as during conditioning (4 lux). Before each trial the box was cleaned with 4% acetic acid. As an index of contextual fear memory, the Δ *freezing* was calculated by subtracting the percent time freezing in BL-A from percent time spent freezing during context test.

Auditory and contextual fear recalls were assessed either 24 hours and 9, 32 and 70 days after shock delivery, to assess for both recent and remote fear memory. The 9 day distance from conditioning was chosen based on previous studies claiming that behavioral changes observed 1 week following stress exposure in rodents are stable over the next month (13, 14). Moreover, choosing this timepoint allowed us to evaluate early effects of fluoxetine treatment, namely after 1 week of administration (15). Effects of the 1 month chronic treatment on remote memory retrieval were also assessed on the last day of whole dose (20mg/Kg/day) administration, i.e. 32 days after conditioning, to avoid acute effects of dose tapering (5). Far remote fear retrieval was assessed up to 70 days after conditioning to look for long-lasting effects of trauma and treatment (after 1 month of wash out). Each trial was videotaped and freezing behavior, a species-specific response to fear defined as the absence of any movement except for respiratory-related ones (16), was scored by a trained observer, blind to mouse genotype and treatment using dedicated software (The Observer v10.5, Noldus Information Technology, Wageningen, the Netherlands).

Pain sensitivity

To control for genotype-driven differences in pain sensitivity, the hot plate test was carried out on a different cohort of experimentally naïve mice (N=16; wt=9, MeCP2=7) as previously described (17). The hot plate apparatus (Socrel model-DS 37) was maintained at 50 ± 0.1 °C. Mice were placed into a clear Plexiglas cylinder (20 cm in diameter) on the heated surface and the time between placement and licking/lifting hindpaws/forepaws or jumping (whichever occurred first) was recorded and considered as latency to exhibiting pain behaviors. As soon as one of these symptoms occurred, the animals returned to their home cages. To prevent tissue damage, a 60s cut-off was used, at which animals not showing reactions were taken from the hot plate and given the latency score of 60s (18). The hot plate was cleaned with 70% ethanol before testing the next animal.

Conditioned odor active avoidance

The conditioned odor active avoidance test was applied 53 days after fear conditioning to assess avoidance symptoms and their persistence, as in (19). The experimental apparatus consisted of an opaque Plexiglas rectangular box (40(d)×14(w)×30(h) cm) with smooth walls, subdivided into 2 compartments of equal size, connected by an open door. Both compartments had grey walls, except for the external short-side walls, which were black. Brightness was maintained at 1 lux. The day before the test, thus 52 days after shock delivery, mice were individually placed in the starting chamber (the same for all the animals) and allowed to freely explore the apparatus for 10 minutes, for habituation. Prior to each animal habituation the apparatus was cleaned with 70% ethanol solution. Twenty-four hours later, mice were placed in the starting chamber and given again 10 minutes of free exploration. This time, however, each chamber was previously cleaned with 2 differently scented solutions: the starting chamber (aversive) with 4% acetic acid (the same as for the conditioning chamber) while 70% ethanol was used for the opposite chamber (neutral). The test assesses active avoidance, where mice are placed first in the chamber with the aversive cue (the same scent as the one to which they were exposed while receiving the footshocks), from which they have the possibility to escape. Both habituation and test were videotaped and scored by a trained observer blind to the genotype and treatment of mice, using dedicated software (The Observer v10.5, Noldus Information Technology, Wageningen, the Netherlands). *Percent time spent in the aversive compartment* and counts of *head out* behavior towards the aversive compartment, defined as the animal protruding his head in the aversive compartment while maintaining the whole body in the neutral one, were considered as an index of active avoidance of the aversive odor.

Odor preference

To control for genotype-driven differences in odor preference, the conditioned odor active avoidance protocol was applied using an additional cohort of experimentally naïve MeCP2–308 and wt mice that were not conditioned. (N=16; wt=9, MeCP2=7).

General health status and spontaneous locomotor activity

To control for the effects of the manipulations (shock intensity and drug treatment) on well-established phenotypic features of the transgenic mice under investigation (20, 21), general health status and locomotor activity in the home cage were repeatedly evaluated throughout the experimental schedule, respectively at 3, 35 and 71 days after conditioning for general health, and at 21 and 65 days distance from the shock for locomotor activity.

General health assessment. The general health of the experimental mice was qualitatively evaluated by a trained observer blind to the genotype and treatment of the experimental mice as previously described (21, 22), with little modifications. Briefly, mice received a score (ranging from 0 - normal appearance to 4 - highly compromised) for each of the following symptoms: gait, mobility, breathing, kyphosis, fur, hindlimb clasping, tremors, seizures and general condition. The individual scores for each category were subsequently summed up to obtain a semi-quantitative measure of symptom status, called throughout the text the *general health score* (GH). Body weight and rectal temperature of the animals were also recorded at each scoring session.

Home cage spontaneous locomotor activity. Spontaneous locomotor activity in the home cages was monitored continuously for 24 hours by means of an automatic device using small passive infrared sensors positioned on the top of each cage (ACTIVISCOPE system, NewBehaviour Inc., Zurich, Switzerland, website: www.newbehaviour.com). The sensors detected any movement of mice with a frequency of 20 events per second (20 Hz). Data were recorded by an IBM computer with dedicated software. No movements were detected by the sensors when mice were sleeping, inactive, or performed moderate self-grooming. Scores were obtained during 1 hour intervals and expressed as counts per minute (cpm). The position of cages in the rack was such that mice of each group were equally distributed in rows and columns. The access of the authorized personnel to the animal room was not restricted and followed the routine schedule.

Gene expression in the blood

Blood collection and RNA extraction. Retro-orbital sampling was performed 10 minutes after ocular anesthetic (0.4% oxybuprocaine hydrochloride eye drops, Novesin, Novartis, Switzerland) topical administration (23) by penetrating the retro-orbital sinus with a Pasteur pipette wetted with

EDTA 0.5 M. Around 500 µl of blood were collected and immediately transferred in a 4 µl-EDTA 0.5M-containing Eppendorf, gently shaken to prevent coagulation at 4°C. RNA was extracted from whole blood with the Quick-RNA MiniPrep Plus kit (Zymoresearch, Irvine, CA, U.S.A) according to manufacturer's instructions. Briefly, 200 µl of blood were lysed in an equal volume of lysis buffer contains proteinase K then added to a filter column and centrifuged. The eluate was mixed to 1 volume of ethanol and centrifuged in a second filter column where a DNAse1 treatment was performed. Then the RNA was washed and eluted by subsequent centrifugation with the given buffers.

Gene expression analysis. One µg of total RNA was used for cDNA synthesis, with the iScript cDNA Synthesis Kit (Biorad, Milan, Italy), as indicated by the manufacturer. One µg of total cDNA was used for each real-time reaction; analyses were performed in triplicate for each sample. cDNA was amplified for each gene using a mix of specific primers and FAM labelled TaqMan Gene Expression Assays probes (Applied Biosystems, CA, U.S.A.) and the 2× FluoCycleII MasterMix for Probe (Euroclone, Milan, Italy) to a final reaction volume of 20 µl. HE-labeled TaqMan primers and probes mix were used to amplify *β-actin* and *GAPDH* genes used as housekeeping genes in duplex Real-Time PCR reactions. Reactions were performed on an Opticon2 DNA Engine (MJ Research) with a hot start step of 95 °C for 5 min followed by 40 amplification cycles (95 °C, 10 s; 62 °C, 10 s; 72 °C, 20 s). Amplification efficiency for each primer and probe set was determined by amplification of a linear standard curve (from 16 ng to 100 ng) of total cDNA as assessed by A_{260}/A_{280}spectrophotometry. All standard curves displayed good linearity and amplification efficiency (90–99%). As negative controls, each sample was previously run with *β-actin* primers without reverse transcription to detect genomic DNA contamination; moreover, blank controls were assayed in each reaction and for each primer/probe mix to detect DNA contamination of the reagents. cDNA levels were standardized by normalizing them to the *β-actin* and to the *GAPDH* controls. In figure 7 the data related to the normalization versus *β-actin* are presented since the two housekeeping genes returned almost completely matching results. Histograms represent the fold increase (ratio of the experimental gene value/*β-actin* gene value) over the control sample.

Statistical analyses

Shapiro-Wilks and Levene tests were applied to confirm that data were normally distributed and that variances did not differ between group. Mauchly's test was employed to confirm data sphericity in case of repeated measurements. Statistical analyses were performed using Statview (SAS institute inc. Vers. 5) and SPSS (IBM statistics, vers. 20).

Supplementary results

Wt and MeCP2-308 mice consumed the same amount of fluoxetine across the 4 weeks of treatment

An evaluation of the average daily dose of drug consumption for each of the 4 weeks of the treatment showed no significant differences between wt and MeCP2-308 animals (wt = 14.341 ± 0.280 mg/Kg/day; MeCP2-308 = 14.846 ± 0.367 mg/Kg/day).

MeCP2-308 mice displayed comparable pain sensitivity to wt

The assessment of pain sensitivity in the hot plate test in naïve MeCP2-308 mice that had received no footshock at all showed no genotype difference in latency to exhibiting pain behaviors (genotype: $t_{(14)} = 1.805$, $p = 0.093$; wt = 24.444 ± 1.966 s; MeCP2-308 = 20.000 ± 1.155 s).

MeCP2-308 mice did not show any preference between the odors used in the behavioral tests

To exclude that the presence of odor preferences between ethanol and acetic acid scent in MeCP2-308 mice might have biased the results, an additional cohort of naïve mice was tested in the odor active avoidance test in the absence of any conditioning, and percent time spent in the acetic acid compartment was recorded. We did not detect any preference in wt neither in MeCP2-308 mice when the olfactory cues under investigation were not associated with exposure to any footshock (wt = 49.593 ± 3.316 %; MeCP2-308 = 42.492 ± 10.432 %).

MeCP2-308 mice displayed worse health conditions and a hypoactive profile, which were not affected by either shock intensity or fluoxetine treatment

General health assessment. As expected, the evaluation of the general health status of the experimental mice confirmed that MeCP2-308 mice displayed worse general health conditions than wt animals (genotype: $F_{(1,39)} = 144.970$; $p < 0.001$). This profile was confirmed across all the 3 assessments. No significant effect of manipulation was found. Moreover, the general health status of all mice significantly worsened across the 3 assessments (day: $F_{(2,39)} = 14.103$; $p < 0.001$), but no genotype or manipulation interaction with the day of testing was observed. Figure S1A represents day*genotype*manipulation interaction: $F_{(4,78)} = 0.657$; $p = 0.623$.

Body weight was found to be significantly lower in MeCP2-308 compared to wt mice (genotype: $F_{(1,39)} = 20.130$; $p < 0.001$). No significant effect of manipulation was found. Conversely, a main effect of day of assessment was observed, together with a significant day*genotype interaction (day: $F_{(2,78)} = 11.310$; $p < 0.001$; day*genotype: $F_{(2,78)} = 3.570$; $p = 0.033$), with wt mice increasing body weight over time while MeCP2-308 maintained quite constant values ($p < 0.01$ after *post hoc*

comparisons for wt, day3 vs wt, day71 on day*genotype interaction). Moreover, a significant day*manipulation interaction was found, with fluoxetine determining an increase in body weight after 1 month of treatment (day35) ($p < 0.01$ after *post hoc* comparisons for day3, high flx vs day35, high flx and for day35, high flx vs day35, high flx on the day*manipulation interaction: $F_{(4,78)} = 7.232$; $p < 0.001$). Figure S1B represents the day*genotype*manipulation interaction: $F_{(4,78)} = 0.906$; $p = 0.465$).

No significant differences in rectal temperature were found among groups.

Home cage spontaneous locomotor activity. As expected, MeCP2-308 mice were significantly less active than wt mice in the home cages (genotype: $F_{(1,39)} = 17.579$; $p < 0.001$), especially during the dark/active phase of the L/D cycle ($p < 0.01$ after *post hoc* comparison between dark, wt and dark, MeCP2-308 on the phase*genotype interaction: $F_{(1,39)} = 25.807$; $p < 0.001$). A main effect of manipulation was found (manipulation: $F_{(2,39)} = 6.496$; $p = 0.004$), related to a strong sedative effect of fluoxetine on wt mice during the first assessment ($p < 0.01$ after *post hoc* comparisons on the day*phase*genotype*manipulation interaction: $F_{(2,39)} = 0.855$; $p = 0.433$; Figure S2). No significant effect of day of assessment was detected.

Supplementary figures

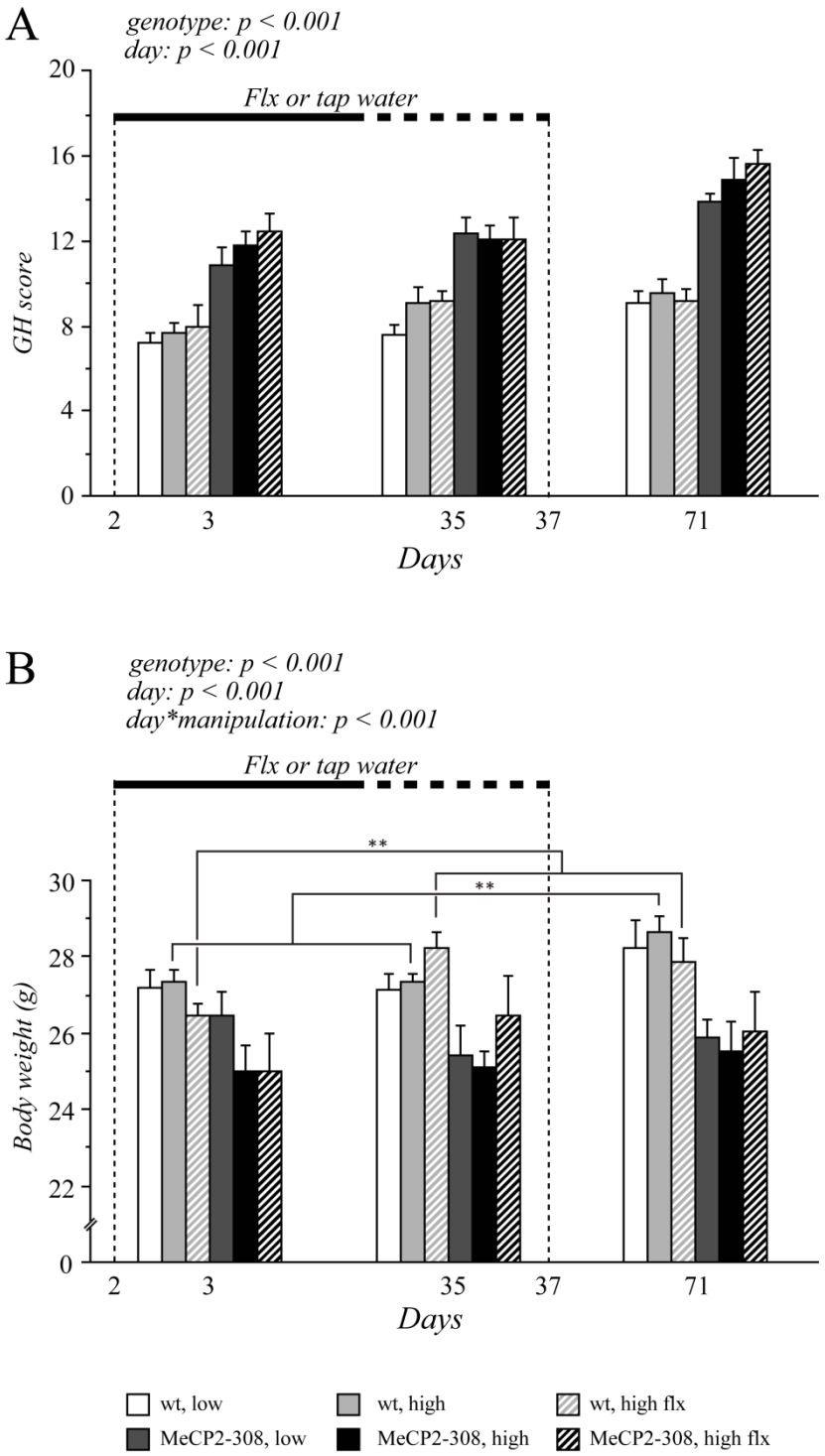

Figure S1 – MeCP2-308 mice depicted worse health conditions, which were not affected by either shock intensity or fluoxetine treatment. A) *Mice's general health status, assessed throughout the experimental schedule (3, 35 and 71 days after conditioning).* The general health score (GH) was obtained by summing up the individual scores (0, normal appearance – 4, highly compromised) of 9 parameters (see supplementary methods). MeCP2-308 mice displayed worse general health conditions than wild type (wt) animals across all the three assessments. No significant effect of manipulation (shock + drug treatment) was found. GH worsened with increasing distance from the shock delivery in all groups. **B)** *Body weight, measured at each general health scoring session.* MeCP2-308 mice weighted significantly less than wt animals throughout the three assessments, with wt mice increasing body weight over time. Fluoxetine (flx) selectively increased body weight of wt mice after 1 month of treatment. *Horizontal axis*: days from shock delivery. ▬: full-dose flx treatment (20 mg/Kg/day); ■ ■ ■: gradual flx dose tapering (10, 5, 2 mg/Kg/day). Mice for each condition were as follows: wt, low = 7; wt, high = 11; wt, high flx = 10; MeCP2-308, low = 6; MeCP2-308, high = 7; MeCP2-308, high flx = 4. Data are mean ± SEM. **: $p < 0.01$.

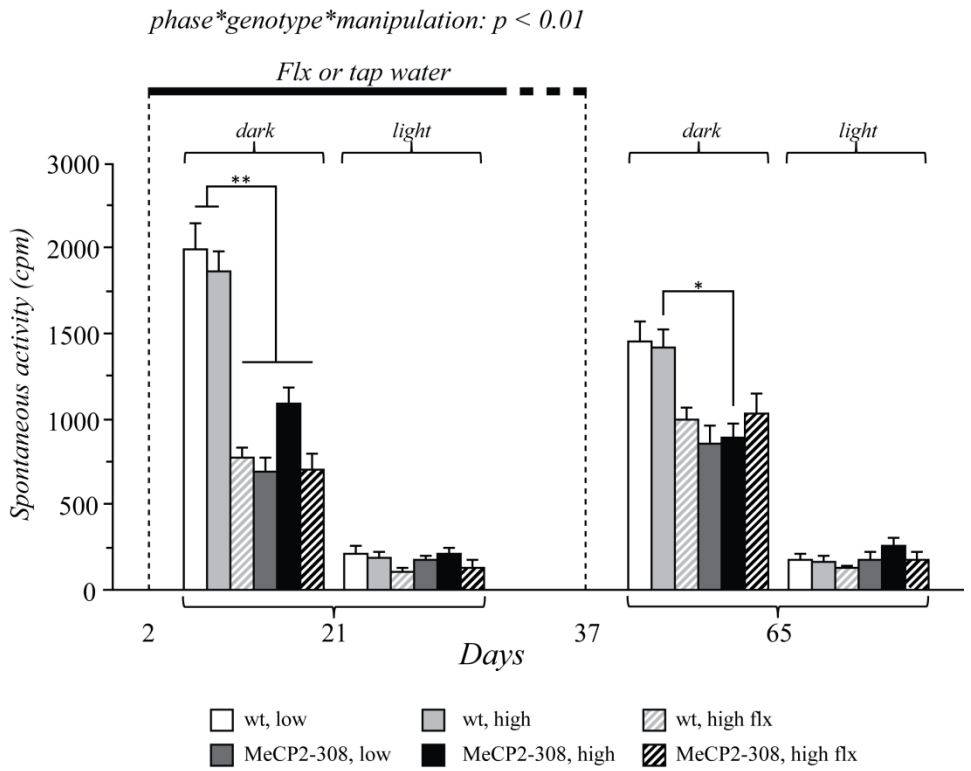

Figure S2 –The hypoactive profile shown by MeCP2-308 mice was not affected by either shock intensity or fluoxetine treatment. Spontaneous locomotor activity of mice was measured continuously for 24 hours at 21 and 65 days after the shock delivery by means of infrared sensors positioned on the top of each home cage. The sensors detected any movement with a frequency of 20 events per second and the scores, obtained during 1 hour intervals, are expressed as counts per minute (cpm). The 24h profile of daily activity was obtained by averaging the 12h lights off (dark, D) and on (light, L) of continuous registration for each day of assessment. MeCP2-308 mice were significantly less active than wild type (wt) mice in the home cage, especially during the dark/active phase of the L/D cycle. A main effect of manipulation was found, given by a strong sedative effect of fluoxetine (flx) selectively on wt mice at 21 days after the conditioning. *Horizontal axis*: days from shock delivery. ▬: full-dose flx treatment (20 mg/Kg/day); ■ ■ ■ : gradual flx dose tapering (10, 5, 2 mg/Kg/day). Mice for each condition were as follows: wt, low = 7; wt, high = 11; wt, high flx = 10; MeCP2-308, low = 6; MeCP2-308, high = 7; MeCP2-308, high flx = 4. Data are mean ± SEM. *: $p < 0.01$; **: $p < 0.01$.

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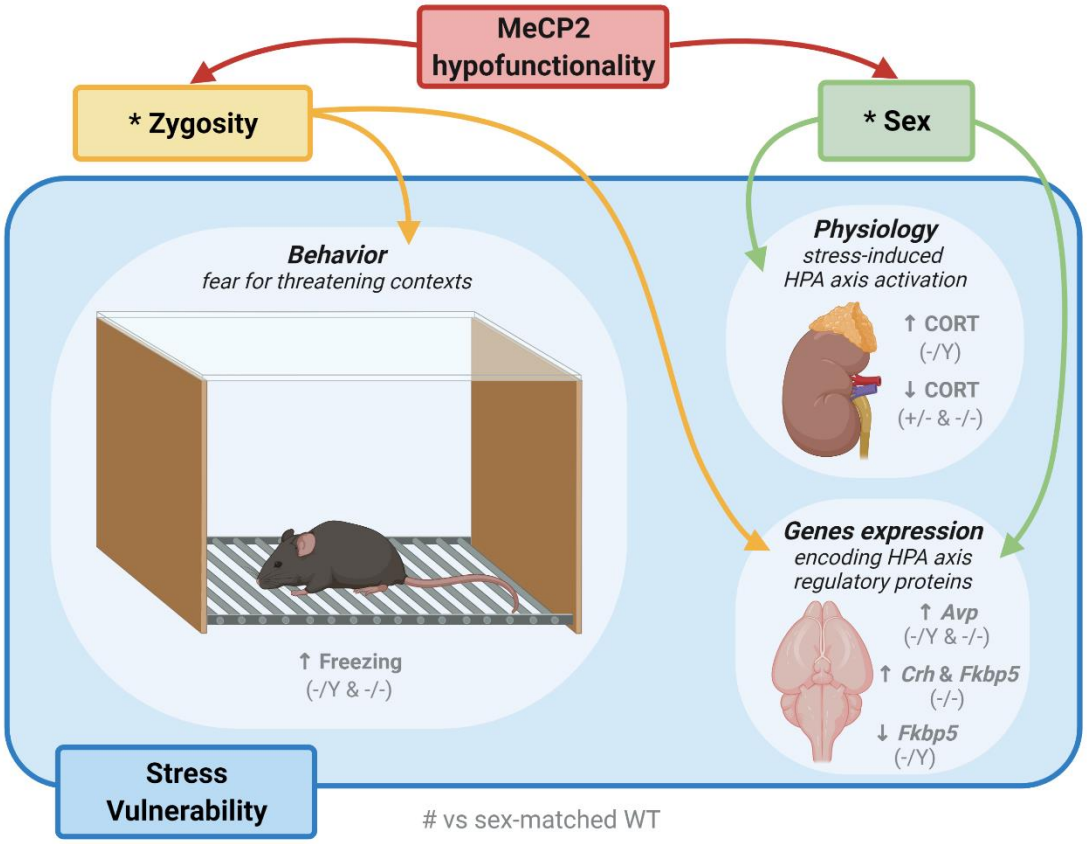
2.2 Study 2: Methyl-CpG binding protein 2 dysfunction provides stress vulnerability with sex- and zygosity-dependent outcomes[1]

[1]Publication: Cosentino L, Bellia F, Pavoncello N, Vigli D, D'Addario C, De Filippis B. *Methyl-CpG binding protein 2 dysfunction provides stress vulnerability with sex- and zygosity-dependent outcomes*. European Journal of Neuropharmacology. 2021. doi: 10.1111/ejn.15165 (online ahead of print).

Graphical abstract



Zygosity for a truncating mutation at the X-linked MeCP2 gene and sex differentially contribute to the onset of stress vulnerability in transgenic mice. Zygosity is crucial in rising contextual fear and hypotalamic HPA axis-regulatory genes expression (*Crh* and *Avp*) compared to wt, while sex primarily modulates the detrimental consequences of MeCP2 hypofunctionality on HPA axis reactivity. Sex and zygosity together influence cortical expression of the HPA axis negative feedback regulator *Fkbp5*.

Abstract

Stress vulnerability is a critical factor for the development of trauma-related disorders, however its biological underpinnings are not clear. We demonstrated that dysfunctions in the X-linked epigenetic factor methyl-CpG binding protein 2 (MeCP2) provide trauma vulnerability in male mice. Given the prominent role of sex in stress outcomes, we explored the effects of MeCP2 hypofunctionality in females. Female mice carrying truncated MeCP2 (heterozygous and homozygous) and wild type controls (wt) were tested for fear memory. Stress-induced corticosterone release and brain expression of hypothalamic-pituitary-adrenal (HPA) axis regulatory genes were also evaluated in wt and mutant mice of both sexes. While heterozygous females displayed a normal stress-related behavioral profile, homozygous mice showed enhanced memory recall for the threatening context compared to wt, thus recapitulating the phenotype previously evidenced in hemizygous males. Interestingly, MeCP2 truncation abolished the sex differences in stress-induced corticosterone release, which was found increased in mutant males, while blunted in mutant females in a zygosity-independent manner. While heterozygous mice did not differ from controls, homozygous females and hemizygous males showed increased hypotalamic *Crh* and *Avp* mRNAs and a differentially altered expression of *Fkbp5* in cortical areas. Present results demonstrate that in female mice carrying truncated MeCP2 altered stress responsivity is driven by homozygosity, while heterozygosity does not lead to maladaptive stress outcomes. MeCP2 dysfunctions thus provide stress vulnerability in mice with sex- and zygosity-dependent outcomes.

Keywords: Epigenetics, Susceptibility, PTSD, HPA axis, Sex differences.

Introduction

Methyl-CpG binding protein 2 (MeCP2) is an epigenetic factor that acts as a reader of methylated loci in the DNA. Upon DNA binding, MeCP2 recruits chromatin remodeling complexes, ultimately modulating gene expression (Lyst & Bird, 2015). Besides its ubiquitous expression throughout the body, MeCP2 is particularly abundant in the brain (Guy et al., 2011). The gene encoding MeCP2 is located in the X-chromosome, and undergoes random X-inactivation, a process that leads, in females, to the mosaic expression of single alleles throughout the body (Ribeiro & MacDonald, 2020). In keeping with the leading role of MeCP2 protein in the central nervous system, mutations in its gene cause a severe intellectual disability disorder, called Rett syndrome (RTT), in heterozygous females (+/-) and neurological conditions ranging from mental retardation to neonatal encephalopathy in hemizygous males (-/Y) (Amir et al., 1999; Villard, 2007).

Beyond its widely acknowledged role in learning and memory, MeCP2 controls the expression of a number of genes regulating the activity of the hypothalamic-pituitary-adrenal (HPA) axis, whose activation is required in response to stressors (McGill et al., 2006; Nuber et al., 2005). In line with this and its crucial role during critical periods of development (Picard & Fagiolini, 2019), it has been demonstrated that MeCP2 exerts key functions in the long-lasting modulation of the stress response following aversive experiences early in life. Indeed, maternal separation-triggered temporary phosphorylation of MeCP2 was found to induce persistent up-regulation of HPA axis regulatory proteins in male mice (Murgatroyd et al., 2009; Zimmermann et al., 2015).

Since an adequate neurophysiological response to stress is needed to adaptively face aversive situations (Walker et al., 2017), we previously hypothesized that an aberrant functionality of MeCP2 protein could prime individuals to increased stress vulnerability. We took advantage of a transgenic mouse line carrying an hypofunctional form of the protein, that lacks the C-terminal domain. Similar mutations are found in 10% of girls diagnosed with RTT, and associate with a mildly compromised neurobehavioral profile in both mouse models and patients (Shahbazian et al., 2002). We demonstrated that male mice carrying the truncated MeCP2 protein are more likely to develop post-traumatic stress disorder-like symptomatology in the aftermath of an intense stressor, including a persistent increased memory recall of the threatening context and increased stress-induced corticosterone (CORT) release (Cosentino et al., 2019), thus suggesting MeCP2 to be a promising marker of susceptibility to stressor-related disorders.

Of note, vulnerability to stress is strongly biased between sexes, and males and females are increasingly sensitive to stressors in different periods of development. Perinatal stress is thought to exert most serious proximal effects on males, while females are more severely affected throughout

puberty and adulthood (Hodes & Epperson, 2019). Stress outcomes also appear to be sex-dependent. As a result of stressful experiences, males are more likely to develop autism spectrum or psychotic disorders, and to make greater use of substances of abuse, while females are more prone to contract mood and anxiety disorders (Wittchen et al., 2011). Interestingly, MeCP2 is emerging as a factor involved in the establishment of sex differences throughout life. It has been suggested to exert different roles in male versus female developing brains, ultimately leading to the organization of molecular and behavioral divergences (Forbes-Lorman et al., 2012, 2014; Kurian et al., 2008). Importantly, MeCP2 sexually-dimorphic functions do not seem to be limited to the developmental period. Indeed, MeCP2 expression was persistently downregulated in early-life stressed male, but not female rats (Blaze & Roth, 2013). Moreover, MeCP2 downregulation in adult neurons was shown to differently influence male and female behaviors related to stress (Philippe et al., 2018).

Drawing on these findings, in the present work we sought to verify whether MeCP2 dysfunction provides stress vulnerability in female mice. To this aim, we explored the effects of stress exposure in female mice carrying truncated MeCP2 (heterozygous: +/- and homozygous: -/-) and wild type (wt: +/+) littermates as a control. Heterozygous females were included in the experimental design as we reasoned that studying their stress-related profile may be interesting from a clinical perspective as they reproduce both the genetic and hormonal milieu of RTT patients (Bianca De Filippis et al., 2015; Katz et al., 2012). In fact, although homozygous females may be more informative from a mechanistic point of view, homozygous expression of a mutated MeCP2 gene has rarely been described in humans, apparently linked to cases of parental disomy (Bhanushali et al., 2016; Karall et al., 2007). To assess vulnerability to stress, female mice were subjected to a fear conditioning task and their memory for the fearful context was evaluated 24 h later. Present results were compared with those previously obtained on hemizygous males (-/Y) (Cosentino et al., 2019) to assess whether sex might modulate the stress-related behavioral profile of mutant mice. To test whether MeCP2 dysfunction associates with distinct biological profiles of stress vulnerability in males and females, basal and stress-induced plasma levels of CORT were assessed, as a measure of HPA axis activation. In order to verify whether alterations in the expression of important stress regulatory genes, which are known to be targeted by MeCP2 (McGill et al., 2006; Nuber et al., 2005), might contribute to the stress-related phenotype of male and female mutant mice, corticotropine releasing hormone (Crh), arginine-vasopressine (Avp), and FK501 binding protein (Fkbp5) mRNA levels were also evaluated in hypothalamic and cortical tissues of wt and mutant mice of both sexes. The expression of the gene coding for Glucocorticoid Receptor protein (Nuclear Receptor Subfamily 3 Group C Member 1, Nr3c1) was also addressed to better characterize the HPA axis activation profile.

Materials and methods

Animals

Experimental animals were 8-12 month old MeCP2-308 transgenic mice of both sexes, carrying a form of the protein truncated at the aminoacid (aa) 308 (+/-, -/-, -/Y), and wt (+/+, +/Y) littermates (B6.129S-MeCP2tm1Hzo/J, stock number: 005439, backcrossed to C57BL/6J mice for at least 12 generations (De Filippis et al., 2010)). Protein truncation was obtained through insertion of a premature STOP codon after aa 308, thus eliminating the C-terminal domain of the protein, while retaining the methyl-CpG binding and the transcriptional repression domains (Shahbazian et al., 2002). Experimental subjects were obtained by mating MeCP2-308 heterozygous females with wt males, to obtain wt and heterozygous females, or hemizygous males, to obtain homozygous females, wt and hemizygous males. Upon weaning at postnatal day 25 mice were housed according to sex in groups of 2-3 in polycarbonate transparent cages (33×13×14 cm) with sawdust bedding, and kept on a 12h light-dark schedule, with lights off at 7:00 am (winter time). Temperature was maintained at 21±1°C and relative humidity at 60±10%. Animals were provided ad libitum with tap water and a complete pellet diet (Altromin, 1324 - 10mm pellets, Germany). Behavioral testing was performed by experimenters blind to mouse genotype during the dark/active phase, between 2 and 4 pm. All procedures were carried out in accordance with the European Communities Council Directive (10/63/EU) of the European Parliamenat and the Council of 22 September 2010 on the protection of animals used for scientific purposes as well as the Italian law (26/2014). The study was approved by the National Center for Animal Research and Welfare of the Istituto Superiore di Sanità and authorized by the Italian Ministry of Health (Project D9997.91, research protocol number 93/2019-PR).

Genotyping

DNA was extracted from a small tail-tip biopsy taken at weaning, and the MeCP2 alleles were identified by PCR, as previously described (Vigli et al., 2018). PCR products were electrophoresed through a 2% NuSieve 3:1 agarose gel (Cambrex Bio Science, Rockland, ME, USA) containing 0.5µg/mL ethidium bromide, and examined under UV light.

Experimental design

On the first experimental day, female mice were exposed to a novel context in which after 3 min they received a 1 min long series of mild foot shocks (*training*). Memory consolidation for the context in which mice received the foot shocks was evaluated 24 h later (*test*). After one week, CORT release

in the blood of female mice was measured before and after 30 min of restraint stress. A separate cohort of male mice, previously subjected to a comparable battery of behavioural tests, underwent restraint stress with the aim of collecting blood for stress-induced CORT release evaluation.

Fear conditioning test

Training: experimental subjects were exposed for 4 min to a computer-controlled operant chamber (conditioning chamber), comprising a Plexiglas box (25x30x30 cm) with electrified grid floor (27 stainless-steel rods (4 mm diameter), spaced 7 mm apart, connected to a shock generator) inside an opaque soundproof cubicle (Coulbourn Instruments, Allentown, PA, USA) (Cosentino et al., 2019). After 3 min of familiarization with the conditioning chamber (baseline), *mice* received a 1 min long series of mild foot shocks (0.4 mA, 1s, spaced 25 s each). The apparatus was cleaned with 70% ethanol solution between trainings.

Test: 24 h after fear conditioning training, mice were put back in the conditioning chamber for 4 min, to test their memory for the context where they were shocked. The apparatus was cleaned with 70% ethanol solution between testings.

Training and test were video-recorded and animal behavior was scored by a trained observer blind to mouse genotype, using dedicated software (The Observer XT, Noldus Information Technology, Wageningen, the Netherlands). Two perpendicular lines were traced to divide the conditioning chamber area in 4 quadrants of equal size (12x15 cm) and the number of times mice crossed the lines with all 4 paws was recorded and used as a measure of general activity. The time animals spent immobile, defined as the absence of body motions without considering head shifts or spontaneous respiratory movements, was also scored, and considered an index of memory retention.

Stress-induced plasma CORT release

Evaluation of plasma CORT release was done as previously described (De Filippis et al., 2013). Briefly, blood was obtained through tail incision within 2 min from entering the surgery room, to analyze basal levels of CORT (t0). Mice were then introduced in a Plexiglas restraint of 2.8 cm diameter. After 30 min mice were removed from the restraint, bled a second time to measure stress-induced levels of CORT (t30), and put back to their home cages. Sixty min later, a third blood sample was obtained from an additional tail incision to evaluate recovery from the stressor (t90). Blood sampling was performed between 10:30 and 12:30 h. Samples of approximately 100 µl volume were individually collected in potassium EDTA coated tubes (1.6 mg EDTA/ml blood, Sarstedt, Germany), cool centrifuged (4°C) and plasma was stored at 80°C until assayed. CORT was measured using a

commercial radioimmunoassay (RIA) kit (ICN Biomedicals, Costa Mesa, CA) with 0.125 mg/dl sensitivity.

Stress axis gene expression

For the evaluation of gene expression, experimental animals were sacrificed between 10 and 12 am. Hypothalami and cortices were dissected, and immediately frozen in dry ice and stored at -80°C until use. Total RNA was isolated using TRIzol Reagent (Thermo Scientific, Waltham, MA, USA), quantified by spectrophotometry, and the integrity was checked by gel electrophoresis (Arosio et al., 2012). A total of 1 µg of total RNA was converted to cDNA using the RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Scientific, Waltham, MA, USA). The abundance of each studied genes mRNA was assessed by real-time RT-qPCR using SensiFASTTM SYBR ® Lo-ROX Mix (Bioline Reagents, London, UK) on a DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA). Primer sequences are reported in Table S1. The specificity of the amplification products after PCR, was evaluated through a dissociation curve (melting curve) constructed in the range of 60 to 95°C (Lyon, 2001). All data were normalized to the endogenous reference genes beta-actin (B-Act), glyceraldehyde-3-phosphate dehydrogenase (Gapdh), and 18s ribosomal RNA (18s). The relative expression of each gene was calculated by the Delta-Delta threshold cycles (ΔΔCt) method and converted to relative expression ratio ($2^{-\Delta\Delta Ct}$) for statistical analysis (Livak & Schmittgen, 2001). A comparison between male and female wt mice was made to control for the presence of basal sex differences in gene expression. All samples have been run in duplicate, and subjects were excluded when doubles differed more than 1.5 Ct. Male and female samples were collected concomitantly, processed and tested for gene expression in the same run.

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics for Windows (IBM Corp. Version 25.0. Armonk, NY, USA). Non directional Student's T-test or One-way ANOVA was used for the analysis of continuous variables, using genotype as a 2-level or 3-level independent factor. Two-way mixed ANOVA was used in case of repeated measurements. Normality, homoschedasticity and sphericity of residuals were controlled with Shapiro-Wilks, Breush-Pagan and Mauchly's tests, respectively. *Post hoc* comparisons were performed using Tukey's HSD test. When assumption of normality was not met, Kruskal-Wallis test was performed, using Mann Whitney test with Bonferroni corrections for *post hoc* comparisons. Outliers were identified by the use of Grubb's test (Table S3). Type I error probability was set at $\alpha=0.05$.

Results

Homozygous females display increased fear when re-exposed to the conditioning context

To assess if the increased emotional response to aversive contexts shown by male mice carrying truncated MeCP2 (Cosentino et al., 2019) was present also in mutant females, heterozygous and

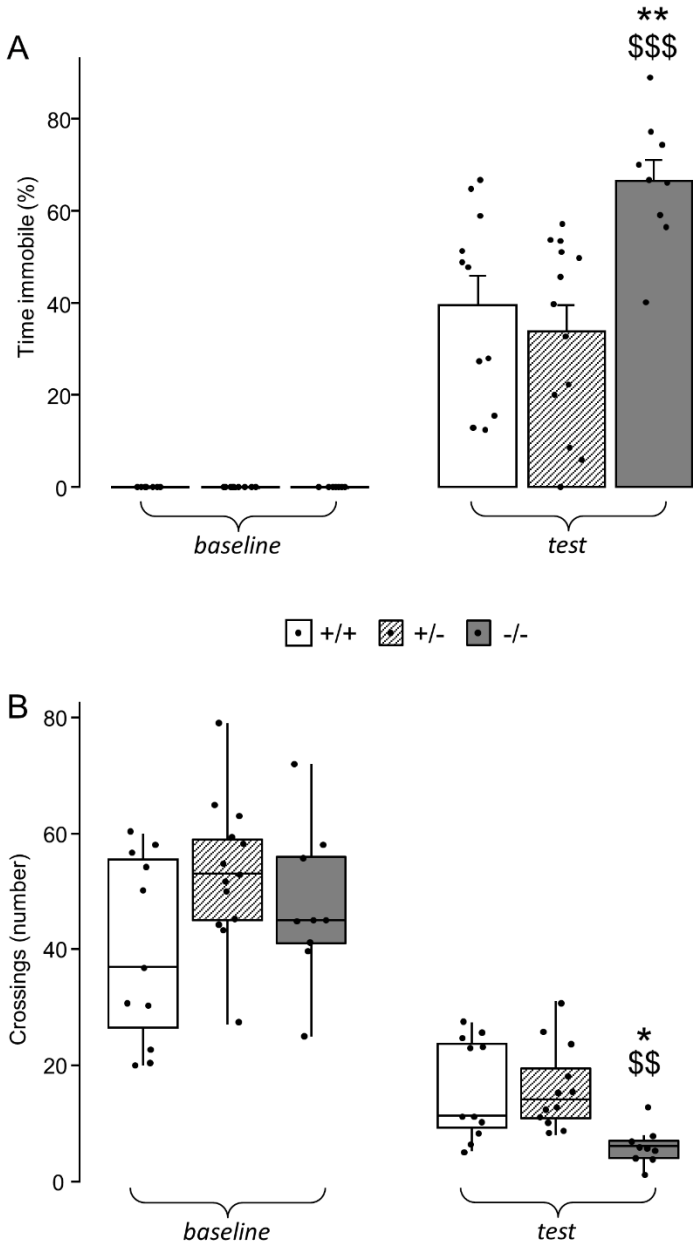


Figure 1 - Homozygous females display increased fear for an aversive context. Homozygous females (-/-) spend more time immobile, an index of fear memory **(A)**, and perform a lower number of crossings, a measure of locomotor activity **(B)**, after re-exposure to a threatening context compared to both heterozygous females (+/-) and wild type controls (+/+), suggesting increased emotionality when recalling aversive situations. Two-way mixed ANOVA with Tukey's *post hoc* tests (A) and Kruskal Wallis test and Mann Whitney *post hoc* tests with Bonferroni's corrections (B) were used for statistical analyses. *, **: $p<0.05$, $p<0.01$ (-/- versus +/+); $$, $$$: $p<0.01$, $p<0.001$ (-/- versus +/-). Boxplots represent interquartile range (IQR) ± 1.5*IQR; bar plots represent mean ± standard error of the mean (SEM).

homozygous female mice were subjected to a contextual fear conditioning protocol, and their performance was compared to that of wt littermates of the same sex. A significant effect of genotype

was found for the time spent immobile as well as for the number of crossings performed during conditioning context re-exposure (immobility – genotype*phase: $F_{2,30}$=8.512, p=0.001; crossings – genotype: H_2=12.574, p=0.002; Figure 1A,B). Specifically, homozygous females showed increased fear responses, as demonstrated by the longer time they spent immobile (p<0.01 and p<0.001 respectively after *post hoc* comparisons on the genotype*phase interaction; Figure 1A) and the lower number of crossings (p<0.05 and p<0.01 respectively after *post hoc* comparisons on the main effect of genotype; Figure 1B) they performed during the *testing* phase compared to wt and heterozygous female mice, thus recapitulating the phenotype shown by hemizygous males (Cosentino et al., 2019). Such effect was likely related to conditioning, since no differences were found in mice's activity (crossings - genotype: H_2=3.698, p=0.157; Figure 1B), and no experimental animal showed immobility behavior during *baseline* (Figure 1A), suggesting that the heterozygous condition may protect from increased fear memory retrieval.

MeCP2 truncation induces sex-dependent alterations in CORT release after stress

To assess whether fear memory alterations in homozygous females and hemizygous males (Cosentino et al., 2019) are paralleled by aberrant HPA axis activation in response to stress, we evaluated CORT release in the blood of male and female mice carrying truncated MeCP2, and of sex-matched wt controls, before and after restraint. As expected, stress induced a significant increase in CORT release in both male and female mice (males – time points: $F_{2,26}$=64.170, p<0.001; females - time points: $F_{2,38}$=165.072, p<0.001, Figure 2). Moreover, wt females displayed a significantly increased rise of CORT after stress compared to wt males (t30 and t90 - p<0.001 after *post hoc* comparisons on sex*time points interaction: $F_{2,24}$=10.562, p<0.001; Figure 2).

Interestingly, genotype significantly influenced the amount of CORT released over time in both sexes, as specified by the significant genotype*time points interaction (males - $F_{2,26}$=5.166, p=0.013; females - $F_{2,38}$=2.667, p=0.047, Figure 2). As expected, while basal CORT (t0) did not differ among wt and mutants, restraint stress induced a significant increase in CORT release in hemizygous males compared to sex-matched controls (t30 - p<0.001 after *post hoc* comparisons on genotype*time points interaction; Figure 2, left). Such difference disappeared at 1 h from the stress (t90) since CORT levels in the blood of mutant mice underwent a significant reduction (p<0.01 for t30 vs t90 after *post hoc* comparisons on genotype*time points interaction; Figure 2, left). Contrary to our expectation, instead, we found that both homozygous and heterozygous females released significantly lower levels of CORT compared to wt at the end of restraint stress (t30 - p<0.01 after *post hoc* comparisons on genotype*time points interaction; Figure 2, right). Such difference was maintained up to 1 h after the end of restraint (t90 - p<0.01 and p<0.1 for heterozygous and homozygous females respectively vs

wt sex-matched controls after *post hoc* comparisons on genotype*time points interaction; Figure 2, right). At this final time point, however, CORT levels did not return to baseline in any experimental group (p<0.001 for t0 vs t90 after *post hoc* comparisons on genotype*time points interactions; Figure 2).

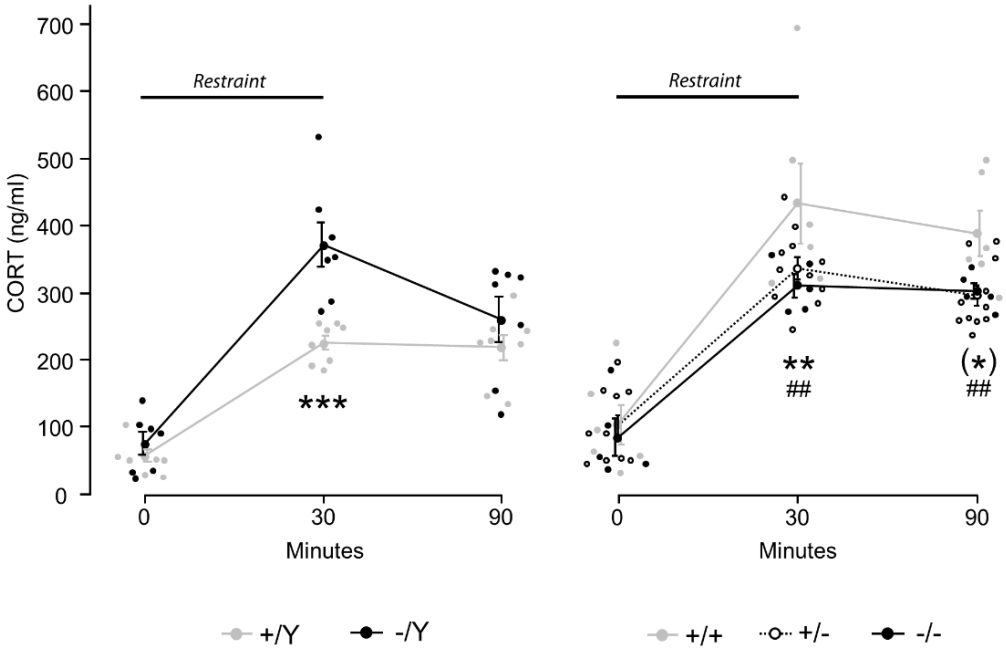


Figure 2 – MeCP2 truncation induces sex-dependent alterations in CORT release after stress. Stress-induced release of the glucocorticoid hormone corticosterone (CORT) in the blood is increased in hemyzigous males (-/Y; left), while is blunted in homozygous and heterozygous females (-/-, +/-; right), compared to sex-matched wild type controls (+/+, +/Y). Two-way mixed ANOVA and Tukey's *post hoc* tests were used for statistical analyses. (*), **, ***: p<0.1, p<0.01, p<0.001 (-/- versus +/+ and -/Y versus +/Y); ##: p<0.01 (+/- versus +/+). Line plots represent mean ± standard error of the mean (SEM).

<u>The expression of stress axis regulatory genes is altered in mice hemizygous and homozygous for MeCP2 truncating mutation</u>

The observation that mutant males and females show opposite aberrant profiles of stress-induced CORT release prompted us to explore the expression of stress axis regulatory genes in the *hypothalamus* and in the *cortex* of MeCP2-308 male and female mice, and sex-matched controls.

Hypotalamic expression of *Avp* was increased in hemizygous versus wt males (t_{11}=-2.248, p=0.046; Figure 3A, left, and Table S2). Although apparently higher compared to wt (Figure 3B, left, and Table S2), the high variability of hypothalamic *Crh* expression among hemizygous males prevented us from drawing conclusions regarding the statistical significance of such comparison. No differences between genotypes was detected for *Fkbp5* expression in the hypothalamus of male mice, instead (Figure 3C, left, and Table S2). Similarly, in the *hypothalamus* of homozygous females, the *Avp*, *Crh* and *Fkbp5* mRNAs were overexpressed compared to both wt and heterozygous female mice (p<0.05 after *post hoc* comparisons on the main effect of genotype: *Avp* - $F_{2,11}$=5.753, p=0.019; *Crh* - $F_{2,9}$=7.065, p=0.014; *Fkbp5* - $F_{2,9}$=4.241, p=0.050; Figure 3A,B,C, right, and Table S2).

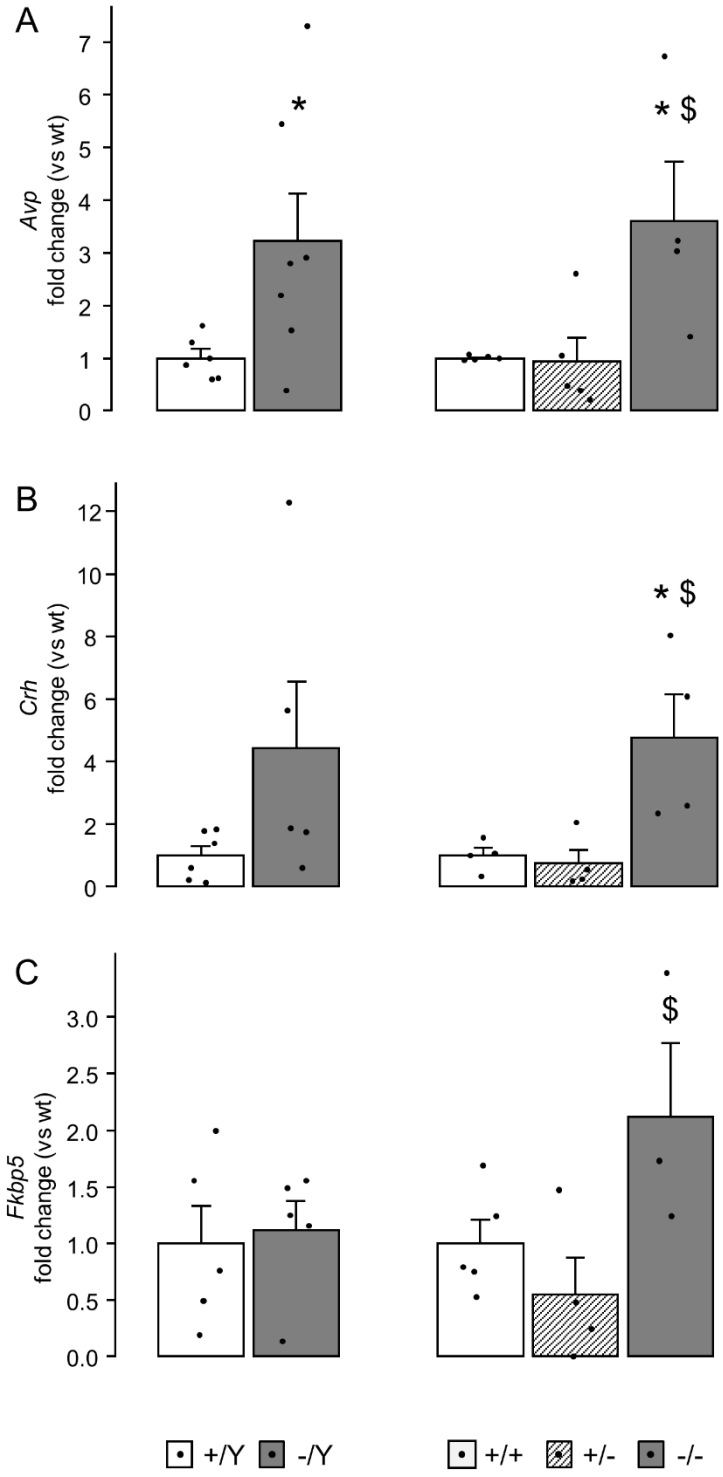


Figure 3 – Genes encoding stress axis regulatory proteins are overexpressed in the hypothalamus of homozygous females and hemizygous males. (A) *Arginine-vasopressine* (*Avp*) the prime-mover of HPA axis stress response together with *corticotropine releasing hormone* (*Crh*), is overexpressed in the *hypothalamus* of hemizygous males (-/Y) compared to wild type sex-matched controls (+/Y; left) and of homozygous females (-/-) compared to both wild type and heterozygous mice (+/+ and +/-; right). **(B)** Although apparently increased compared to wild type controls, the high variability of hypothalamic *Crh* expression among hemizygous males prevented us from drawing conclusions regarding the significance of such comparison (left); *Crh* is significantly overexpressed in homozygous females compared to both wild type and heterozygous mice (right). **(C)** Hypotalamic expression of *Fk501 binding protein* (*Fkbp5*) a major modulator of glucocorticoid-mediated negative feedback, is not altered in hemizygous males relative to sex-matched wild type (left), while increased in homozygous females compared to heterozygous mice and wild type controls (right). One-way ANOVA with Tukey's *post hoc* tests and non-directional Student's T test were used for statistical analyses. *: $p<0.05$ (-/- versus +/+ and -/Y versus +/Y); $: $p<0.05$ (-/- versus +/-). Data are mean ± standard error of the mean (SEM).

A different profile was found in the *cortex*, where *Avp* and *Crh* mRNA levels did not differ between genotypes in mice of both sexes (Table S2). In the *cortex*, however, hemizygous males showed lower levels of *Fkbp5* mRNA compared to wt controls (t_8=2.835, p=0.022; Figure 4, left, and Table S2). Interestingly, female mice displayed an opposite profile of *Fkbp5* expression, which was higher in homozygous females cortices compared to heterozygous and wt sex-matched controls (p<0.05 after *post hoc* comparisons on the main effect of genotype: $F_{2,11}$=6.298, p=0.015; Figure 4, right, and Table S2).

Nr3C1 mRNA was instead unaltered in both brain areas of mutant mice (Table S2).

No differences in gene expression were found in the selected areas between male and female wt mice, with the exception of a significant overexpression of hypothalamic *Avp* mRNA in females compared to males (sex: t_9=-3.480, p=0.007; Table S2).

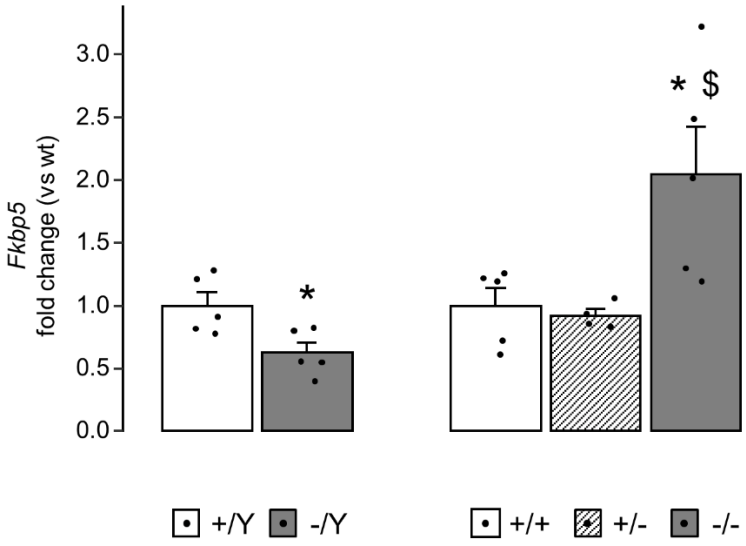


Figure 4 – *Fkbp5* expression is altered in the cortex of MeCP2-308 mice in a sex- and zygosity- dependent manner. The glucocorticoid receptor inhibitor *Fk501 binding protein* (*Fkbp5*) is downregulated in the *cortex* of hemizygous males compared to wild type males (+/Y; left); Higher *Fkbp5* mRNA expression was found in homozygous females (-/-) compared to both wild type (+/+) and heterozygous (+/-) female mice cortices (right). One-way ANOVA with Tukey's *post hoc* tests and non-directional Student's T test were used for statistical analyses. *: p<0.05 (-/- versus +/+ and -/Y versus +/Y); $: p<0.05 (-/- versus +/-). Data are mean ± standard error of the mean (SEM).

Discussion

Alterations in the behavioral response to stress and HPA axis activation in either normal or aversive conditions are key indicators of a vulnerable phenotype (Ebner & Singewald, 2017; Russell et al., 2018). Further characterization of the biology underneath such phenotype is required to develop a discriminative index of vulnerability, and epigenetic markers have been suggested to play a crucial role in the risk of contracting stressor-related disorders (Rakesh et al., 2019).

We recently demonstrated that an aberrant functionality of the epigenetic modulator MeCP2 deeply influences the behavioral and physiological response to high intensity stressors in transgenic adult male mice (Cosentino et al., 2019). We here provide evidence that reduced MeCP2 functionality associates with an increased fear after re-exposure to the conditioning context also in middle-aged females homozygous for the same truncating mutation, confirming the fundamental role of MeCP2 in the consolidation of emotional memories (Finsterwald et al., 2015; Gupta et al., 2010).

In this line, MeCP2 truncation also appears to disrupt the proper physiological response to stress, with significant deviations from control-levels of plasma CORT being evident in MeCP2-mutated mice from both sexes. Interestingly, however, the direction of such altered stress response appears strongly influenced by sex. Indeed, hemizygous males display an increase in stress-induced CORT release compared to wt male mice, as already demonstrated in previous works (Cosentino et al., 2019; De Filippis et al., 2013). By contrast, homozygous and heterozygous females show decreased levels of the glucocorticoid hormone after stress relative to sex-matched controls. It is important to underline that stress-induced glucocorticoids release is sexually-dimorphic, with females usually displaying higher levels of CORT compared to males (Bangasser & Valentino, 2014). The opposite tendency to decreased and increased CORT release in mutant males and females may then suggest that reduced functionality of MeCP2 protein could mitigate such diversity between sexes, in line with previous findings supporting a role for MeCP2 in the organization of sex differences during development (Forbes-Lorman et al., 2012).

Also, a region-specific pattern of stress-related gene expression alterations was found in mice lacking fully functional MeCP2. In the hypothalamus both hemizygous males and homozygous females display an increased expression of Crh and Avp, the prime-movers of HPA axis stress response, mirroring the increased context-related fear outlined in either groups. Of note, there is a general agreement that CRH is increased in the cerebrospinal fluid of people suffering from PTSD, which could suggest this to be a feature underlying vulnerability to stress and alterations in fear-related circuitries (Raglan et al., 2017). However, hypothalamic Crh and Avp expression cannot explain the alterations in stress-induced peripheral CORT levels we outlined in mutant mice. Given

the sex-dependent outcomes triggered by MeCP2 truncation over CORT release, it is likely that sex-specific mechanisms acting downstream Crh and Avp within the HPA axis may combine with MeCP2 dysfunctions to determine opposite phenotypes in males and females. This would not be surprising given that Crh receptors sensitivity as well as Crh binding protein expression, for instance, have been previously described as sexually dimorphic (Bangasser & Valentino, 2012, 2014; Wiersielis et al., 2019). MeCP2 dysfunctions might then have contributed to reducing the developmental establishment of such differences between sexes (Forbes-Lorman et al., 2012), although dedicated studies would be needed to clarify whether the disruption of activational, rather than organizational, effects of sex might have actually played a role (Romano et al., 2016).

Similarly, the gene expression profile of mutant mice appears to be influenced by sex in cortical tissue. Of particular interest is the opposite expression profile of Fkbp5, a major modulator of glucocorticoid-mediated negative feedback, which is increased in homozygous female and decreased in hemizygous male cortices. In fact, such oppositely altered patterns of Fkbp5 expression in both hemizygous males and homozygous females are accompanied by an increased behavioral sensitivity to stress, as demonstrated by their increased fear for aversive contexts. This is apparently consistent with other studies demonstrating that multiple single nucleotide polimorphisms (SNPs) in FKBP5 gene provide increased risk for PTSD, although they are associated with opposite variations in protein levels (Hawn et al., 2019; Mehta et al., 2011). Furthermore, some PTSD patients have been reported to display lower, while others higher levels of FKBP5 mRNA, according to their genetic variation (Klengel et al., 2013), suggesting that distinct biological profiles may account for stress vulnerability in MeCP2-mutated hemizygous males and homozygous females.

It is interesting to note that brain *Fkbp5* levels also, as *Crh* and *Avp*, do not help explaining the sex-dependent alterations in stress-induced CORT evidenced in mutant mice, suggesting that the modulation of HPA axis negative feedback might not be the primary cause underlying these physiological disruptions.

An interesting aspect highlighted by our results is that MeCP2 mutation, when present in heterozygosity, does not lead to any behavioral effect attributable to stress vulnerability. Indeed, heterozygous females, the condition that more closely recapitulates the genetic and hormonal profile of RTT patients, do not display significant alterations in conditioned fear. Further studies will have to verify whether the lack of the stress vulnerability phenotype in heterozygous females might be attributable to the presence of at least one copy of wt MeCP2. The mosaicism due to the X inactivation phenomenon might in fact confer different cellular properties, possibly leading to a rearrangement of relevant networks during development. We cannot however rule out the possibility that a skewed X inactivation may have played a role in softening the phenotype of heterozygous females, as might

also happen in patients with RTT (Huppke et al., 2006; Ribeiro & MacDonald, 2020). Noteworthy, however, present results are in line with current knowledge regarding RTT symptomatology, where stress-dependent phenotypes are usually not described.

Conclusion

Overall, present results reaffirm the need for functional MeCP2 protein to properly regulate stress-related behavior and physiology, by extending our preceding results to the feminine hormonal and genetic background (Table 1). Via highlighting that heterozygosity does not lead to maladaptive stress outcomes, the present work demonstrate that altered stress responsivity is driven by homozygosity in female mice carrying truncated MeCP2. This further substantiates the need of recurring to heterozygous females for studying potential RTT therapeutics, while heterozygous and homozygous mice appear valuable models of stress-related disorders. MeCP2 dysfunctions thus provide stress vulnerability in mice with sex- and zygosity-dependent consequences. Further studies are needed to dissect the mechanisms underneath such different outcomes, which may provide insight into biologically distinct profiles of stress vulnerability.

Table 1 - MeCP2 dysfunction provides stress vulnerability with sex- and zygosity-dependent outcomes

Stress-related measures		**vs +/+**		**vs +/Y**	
		+/-	**-/-**	**+/+**	**-/Y**
Behavior	*Contextual Fear*	↔	↑	NA	↑[a]
Physiology	*Stress-induced CORT*	↓	↓	↑	↑[a]
Gene expression	*Avp hypothalamus*	↔	↑	↑	↑
	Crh hypothalamus	↔	↑	↔	↔
	Fkbp5 hypothalamus	↔	↑	↔	↔
	Avp cortex	↔	↔	↔	↔
	Crh cortex	↔	↔	↔	↔
	Fkbp5 cortex	↔	↑	↔	↓

Abbreviations: NA - Not applicable; CORT - corticosterone; *Avp* - arginine-vasopressine; *Crh* -corticotropine releasing hormone; *Fkbp5* -Fk501 binding protein 51; **Symbols**: ↑ - Increased; ↔ - Unchanged; ↓ - Decreased.

[a] Cosentino et al. 2019. Neuropharmacology 160, 107664. https://doi.org/10.1016/j.neuropharm.2019.06.003

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Conflicts of Interest

The authors declare no conflict of interest.

Author's contributions

LC: Data curation, Formal analysis, Investigation, Software, Visualization; Writing - original draft, Writing - review & editing; FB, DV and NP: Investigation, Writing - review & editing; CDA: Methodology, Resources, Supervision, Validation, Writing - review & editing; BDF: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

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Supplementary methods

Table S1 - Primer sequences used for gene expression analyses

Gene	Gene ID	Sequence
18S	19791	Fwd: 5'-CGCCGCTAGAGGTGAAATTCT-3'
		Rev: 5'-CGAACCTCCGACTTTCGTTCT-3'
B-Act	11461	Fwd: 5'-TGTTACCAACTGGGACGA-3'
		Rev: 5'-GTCTCAAACATGATCTGGGTC-3'
Gapdh	14433	Fwd: 5'-AACGGGAAGCTCACTGGCAT-3'
		Rev: 5'-GCTTCACCACCTTCTTGATG-3'
Avp	11998	Fwd: 5'-ATCTGCTGCAGCGACGAGAG-3'
		Rev: 5'-TGTACCAGCCTTAGCAGCAG-3'
Crh	12918	Fwd: 5'-ACCTTCTGCGGGAAGTCTTG-3'
		Rev: 5'-TTTTGGCCAAGCGCAACATT-3'
Fkbp5	14229	Fwd: 5'-TGGTGTTCGTTGTTGGGGAA-3'
		Rev: 5'-CCAAAACCATAGCGTGGTCC-3'
Nr3c1	14815	Fwd: 5'-TCAAGCCCTGGAATGAGACC-3'
		Rev: 5'-ATTGTGCTGTCCTTCCACTG-3'

Abbreviations: *18S - 18S ribosomal RNA*; *B-Act - Beta Actin*; *Gapdh - Glyceraldehyde 3-phosphate dehydrogenase*; *Avp - Arginine Vasopressin*; *Crh - Corticotropin Releasing Hormone*; *Fkbp5 - Fkbp Prolyl Isomerase 5*; *Nr3c1 - Nuclear receptor subfamily 3, group C, member 1*.

Supplementary results

Table S2 – ΔCt values of the analyzed target genes

	+/+	+/-	-/-	+/y	-/y
			Hypothalamus		
Avp	6.157 ± 0.060	6.824 ± 1.430	4.521 ± 0.924	6.958 ± 0.571	5.624 ± 1.393
Crh	13.764 ± 0.924	14.626 ± 1.534	11.517 ± 0.878	13.803 ± 1.631	10.593 ± 2.434
Fkbp5	9.075 ± 0.660	11.428 ± 3.433	7.998 ± 0.738	9.007 ± 1.344	8.805 ± 1.488
Nr3c1	5.371 ± 0.071	7,536 ± 1.662	5.480 ± 0.867	5.585 ± 1.026	4.672 ± 1.763
			Cortex		
Avp	11.206 ± 1.105	10.603 ± 1.227	9.925 ± 0.747	11.783 ± 1.033	10.857 ± 0.874
Crh	11.921 ± 1.024	11.808 ± 0.853	11.276 ± 0.382	11.617 ± 0.696	12.061 ± 0.890
Fkbp5	9.570 ± 0.492	9.633 ± 0.160	8.577 ± 0.611	9.031 ± 0.333	9.728 ± 0.433
Nr3c1	6.056 ± 0.636	5.786 ± 0.532	5.833 ± 0.735	5.715 ± 0.371	6.017 ± 0.649

Data are expressed as mean ± standard deviation. Abbreviations: *Avp*: *-Arginine Vasopressin*; *Crh - Corticotropin Releasing Hormone*; *Fkbp5 - Fkbp Prolyl Isomerase 5*; *Nr3c1 - Nuclear receptor subfamily 3, group C, member 1*.

Table S3 – Number of observations included in the statistical analyses for each group

Parameter	**Figure**	**+/+**	**+/-**	**-/-**	**+/Y**	**-/Y**
Crossing	1B	*11*	*12 (1)*	*9*	NA	NA
Immobility	1A	*11*	*13*	*9*	NA	NA
CORT	2	*6*	*11*	*5 (2)*	*8*	*7*
Avp hypothalamus	3A	*5 (1)*	*5 (1)[1]*	*4 [1]*	*6 (2)*	*7*
Crh hypothalamus	3B	*4 (1)[1]*	*4 (2)[1]*	*4 (1)*	*6 (2)*	*5 (2)*
Fkbp5 hypothalamus	3C	*5*	*4 (1)[1]*	*3 [2]*	*5 (1)[1]*	*5 (2)*
Nr3c1 hypothalamus	NA	*3 (1)[1]*	*3 [2]*	*5*	*4 (1)*	*6*
Avp cortex	NA	*4 (1)*	*5*	*5*	*4 (1)*	*5*
Crh cortex	NA	*4 (1)*	*5*	*5*	*5*	*5*
Fkbp5 cortex	4	*5*	*4 (1)*	*5*	*5*	*5*
Nr3c1 cortex	NA	*5*	*5*	*5*	*5*	*5*

The number of outlying observations ($p<0.05$ after Grubb's test) which were excluded from the analyses is indicated between round brackets; the number of samples excluded from statistical analyses because duplicates differed more than 1.5 Ct is indicated between square brackets. Abbreviations: NA - not applicable; CORT - corticosterone; *Avp - Arginine Vasopressin*; *Crh - Corticotropin Releasing Hormone*; *Fkbp5 - Fkbp Prolyl Isomerase 5*; *Nr3c1 - Nuclear receptor subfamily 3, group C, member 1*.

2.3 Study 3: Stress in early life mediates the association between *Methyl-CpG binding protein 2* expression and symptoms of stress-related disorders in a gender-dependent manner[1]

[1]Manuscript in preparation: Cosentino L, Zidda F, Dukal H, Witt SH, De Filippis B[§], FlorH[§]. *Stress in early life mediates the association between Methyl-CpG binding protein 2 expression and symptoms of stress-related disorders in a gender-dependent manner*. [§]equally contribution authors.

Abstract

Numerous mental illnesses arise following stressful events in vulnerable individuals, with females being generally more affected than males. Adverse childhood experiences are known to increase the risk of developing psychopathologies, and DNA methylation was demonstrated to drive the long-lasting effects of early life stress and promote stress susceptibility. *Methyl-CpG binding protein 2* (*MECP2*), an X-linked reader of the DNA methylome, is altered in numerous mental disorders of stress origin, suggesting *MECP2* as a marker of stress susceptibility. The present work explored whether peripheral *MECP2* expression marks increased vulnerability to stress-related disorders in healthy men and women. To this aim, *MECP2* mRNA levels were analyzed in blood samples from 63 people with no history of mental health disorders. Childhood adversities and present symptomatology pertaining to depressive and anxiety symptom clusters were evaluated through self-report. Structural Equation Modeling was used to test the hypothesis that *MECP2* expression associates to stress-related symptomatology via the mediation of childhood adversities. We found that gender moderates *MECP2* association to depressive and anxiety symptoms via the mediation of childhood adverse experiences. Indeed, although *MECP2* levels were overall increased in women compared to men, decreased *MECP2* expression was associated with more severe childhood experiences, increased trait anxiety and depressed mood, especially in females. These results suggest a gender-specific modulation of *MECP2* expression upon childhood adversities that supports vulnerability to suffer from symptoms of stress-related disorders, and shed new light on the complex biology underlying gender bias in stress vulnerability.

Introduction

Trauma- and stress-related disorders such as post-traumatic stress disorder (PTSD), are a serious public health issue with highly debilitating outcomes that have a significant impact on both affected individuals and society [1–3]. The global burden associated with mental illnesses of stress origin is considerable, with an estimated 10% of the population suffering from them [4,5]. Moreover, treatment efficacy remains poor, with a high degree of resistance or relapse [6–9].

Although more than two thirds of the general population experience at least one event that can be defined as traumatic throughout life [10], the outcomes of experiencing adverse events and the probability to develop disorders of stress origin rely on individual vulnerability [11]. Although the factors underlying interindividual differences in stress susceptibility have not yet been completely clarified, the overall disease risk profile appears to depend on the interaction among genes and environmental factors [12–14], which eventually shapes the way individuals respond to stress [11]. The inability to adaptively face stressors in fact clearly determines an higher incidence of stress-related disorders such as PTSD [15–17].

Epigenetic regulation of gene transcription is a mechanism by which the gene × environment interaction occurs. Epigenetics in fact provides a mechanism to translate environmental exposures into the modulation of gene expression, thus altering stress adaptability and influencing the probability that an individual will display susceptibility or resilience to future stressors [11].

Among the many possible epigenetic modifications, DNA methylation has been largely studied due to its extreme responsiveness to environmental stimuli. Indeed, the methylation status of specific loci appears to quantify the amount of stressors experienced throughout life [18–20]. Such a dynamic nature makes it a promising mediator of behavioral adaptations to environmental challenges, suggesting that it can prompt individuals to risk or resilience beyond the variability attributable to genetic factors alone. Of note, risk and resilience have been proposed to associate with an opposite methylation profile on similar genes, consistent with the idea that a disrupted balance between activation and repression of gene expression may interfere with the ability to adaptively respond to stress [20,21].

The X-linked Methyl-CpG binding protein 2 (MECP2) is a reader of the DNA methylome and plays a major role in the regulation of gene expression in the brain [22]. MECP2 activity, which is regulated by post-translational modifications of the protein, is modulated by neuronal firing, thus strongly contributing to the adaptability of neurons to a dynamic environment [23]. Of note, stressful events early in infancy, which are recognized to be a major cause of increased vulnerability to future challenges [24,25], were found to affect MECP2 functionality, thus allowing an enduring

reorganization of methylation and expression of stress-related genes [26], suggesting a potential involvement of this protein in shaping vulnerability to develop stress-related psychopathology [27]. In line with this, *MECP2* was found to be mutated or differentially expressed in a number of mental disorders whose onset can be triggered by stress such as schizophrenia, bipolar disorder and depression [28–30]. Moreover, regulation of MECP2 functionality has been suggested to contribute to both depressive-like symptoms and their mitigation by selective serotonin reuptake inhibitors, as well as to drug craving in substance abuse disorders [31–33].

In spite of the increasing evidence of MECP2 involvement in stress-related psychopathology, its role in prompting disease vulnerability has so far received little attention. We have shown that a hypomorphic mutation in the *MeCP2* gene provides vulnerability to develop behavioral and molecular features of PTSD in trauma-exposed mice, suggesting that *MECP2* could represent a marker of stress susceptibility [34,35].

Based on this evidence, this study explored whether *MECP2* expression levels may be associated with the severity of traits and symptom clusters that could confer vulnerability to PTSD in healthy humans. To this aim we examined *MECP2* expression levels in blood samples from healthy volunteers and assessed *MECP2* interaction with depressive and anxiety symptom clusters. Previous studies have in fact demonstrated that individuals suffering from trauma- and stress-related disorders receive higher scores in the selected measures compared to non-clinical population [36,37], suggesting that healthy people rating higher in the above dimensions might be vulnerable to stressful events. Given the reported tight connection between MECP2 function and stress early in life [27], and the widely recognized influence that the latter exerts in determining the outcome of traumatic experiences, we tested the possibility that childhood adversities might mediate the association between blood *MECP2* levels and stress-related symptomatology. In particular, we hypothesized that reduced *MECP2* expression associates with increased severity of stress-related disorders, especially in people who experienced adversities during infancy or adolescence. Since women are known to be more prone to develop trauma- and stress-related disorders following abuse or neglect in childhood, and MECP2 function has been linked to the establishment of developmental sex differences in mouse models [38–40], we assumed that women might be more affected by a putative reduction of *MECP2* expression following childhood adverse experiences, and thus examined the role of gender in this correlational model. Present findings underline the role of MECP2 in the risk of developing stress-related disorders and strengthen our knowledge on the gender-specific biology beneath stress vulnerability.

Materials and methods

Study participants

Sixty-three healthy persons of Caucasian ethnicity (23 females; mean age 36.79, standard deviation 15.78, range: 18-69 years of age) participated in the study. The sample considered in the present study partly overlaps with that of previous works [41,42]. Individuals with current or lifetime mental disorders such as major depressive disorder, current or chronic substance abuse, schizophrenia or borderline personality disorder, as assessed with the Structured Clinical Interview for the Diagnostic and Statistical Manual of Mental Disorders-IV [43,44], were excluded. Written informed consent was obtained before the experiment, which was approved by the Ethics Committee of the Medical Faculty Mannheim, Heidelberg University. The study conformed to the Code of Ethics of the World Medical Association (Declaration of Helsinki, 6th revision, 2008).

MECP2 expression

Whole blood was collected in PAXgene Blood RNA (PreAnalytiX) or Tempus Blood RNA (Applied Biosystems) Tubes (N=16 and 47, respectively). Total RNA including miRNA was extracted using the PAXgene Blood miRNA Kit (Qiagen), or the Tempus™ Spin RNA Isolation Kit (Applied Biosystems), respectively, following the manufacturer´s protocols. The concentration of the RNA samples and the sample purity was assessed with NanoDrop 1000 Spectralphotometer (Thermo Scientific). The RNA Integrity was determined with the Agilent 2100 Bioanalyzer System (Agilent Technologies). The cDNA was synthesized by a reversed transcription reaction using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed on the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems by Life Technologies) by using TaqMan Fast Advanced Mastermix (Applied Biosystems), and the *MECP2* TaqMan Gene Expression Assay Hs00172845_m1 (Applied Biosystems). The *Actin Beta* (*ACTB*) TaqMan Gene Expression Assay Hs01060665_g1 (Applied Biosystems) was used as an internal standard. Results were calculated with the QuantStudio Real-Time PCR Software v1.3 (Applied Biosystems by Thermo Fisher Scientific) by the comparative $2^{-\Delta\Delta Ct}$ method and normalized to ACTB. Analyses were carried out in triplicates.

Unpaired t test revealed that blood tubes did not significantly influence *MECP2* levels (t_{61}=-0.534, p=0.595).

Psychometric measures

Depression and anxiety measures have been selected in representation of stress-related symptom clusters. Trait anxiety was measured with the German version of the State-Trait Anxiety Inventory (STAI-T; [45]), and depressive symptoms were assessed by administering the German version of the Center for Epidemiological studies Depression Scale (CES-D; [46]). The anxiety and depression scales of the revised version of the Symptom Checklist-90 (SCL-90-R [47]), a self-report instrument evaluating a broad range of psychological problems, were also used for validation. Importantly, these scales are all psychometrically validated (see supplementary methods) and designed to be subjected to clinical and non-clinical (healthy) populations, mainly for research purposes or as a screening tool to identify people who may be diagnosed with a mental disorder [48,49].

Participants' recall of adverse experiences during childhood was assessed using the Childhood Trauma Questionnaire (CTQ; [50]), a broadly used retrospective self-report instrument aimed at quantifying the severity of emotional/physical abuse and neglect, and sexual abuse experienced up to 18 years of age. Current exposure to chronic stress was evaluated with the Trier Inventory for Chronic Stress (TICS; [51]), aimed at measuring the presence of chronic stressors in everyday life in terms of their intensity, duration and frequency (see supplementary methods for further details).

Statistical analyses

All statistical analyses were conducted using SPSS 20.0 and AMOS 20.0 (IBM Statistics).

Unpaired t tests and Pearson correlations were used to evaluate whether *MECP2* expression differed between genders or changed with age. Multiple regression analyses were used to evaluate the main and interaction effects of *MECP2* expression and gender (females=1, males=2) on the severity of childhood adversities and of stress-related symptomatology (depressive and anxiety symptoms). In addition, regression analyses were used to evaluate the direct relationships between childhood adversities and the symptomatology of stress-related disorders.

Structural Equation Modeling (SEM) with maximum likelihood estimation was then employed to test the prediction that gender moderates the association between reduced *MECP2* expression and increased childhood adversities, thus contributing to the appearance of symptomatology typical of stress-related disorders. Since the parameters included in the model were measured at the same time, the tested hypothesis is purely correlational.

Exclusion criteria for the model were: failure to converge after 240 iterations, the presence of squared multiple correlation values greater than 1 ($R^2>1$) and poor fit, estimated via the following goodness-of-fit measures: the χ^2 statistic (with a good fit indicated by χ^2/degrees of freedom (df) $\leq$ 3), the root mean square error of approximation (RMSEA with a good fit indicated by an index <

0.08) and the comparative fit index (CFI with a good fit indicated by an index > 0.95) [52]. When the overall model fit was poor, model respecifications were made by removing nonsignificant directed arcs ($p > 0.05$) and adding correlated paths as indicated by modification indices that were consistent with the hypothesis. The statistically significant improvements between hierarchical nested models could be assessed using the likelihood ratio (calculated as the difference in χ^2 and df between the models of interest [53]). To establish mediation, indirect paths were tested for significance using a Bias-Corrected (BC) Bootstrapping method (95% confidence intervals; 2000 resamples [54]).

To examine whether the final model was specific for early life stressful experiences, it was retested with a measure of current perceived chronic stress replacing the childhood trauma scale. Also, we checked whether the final model predicted equally well anxiety and depressive symptoms when using different symptoms scales established in the literature. For each of the analyses the alpha level was set to 0.05 [53,55]. See supplementary methods, and Table S2 for more details regarding data preparation..

Results

MECP2 is overexpressed in females compared to males

We tested whether gender influenced the expression levels of *MECP2*, an X-linked gene, in the blood of participants. Interestingly, we found that *MECP2* mRNA was significantly higher in women compared to men (t_{61}=2.689, p=0.009; Figure 1A). *MECP2* expression did not significantly correlate with participants' age (r_{61}= -0.155, p = 0.224).

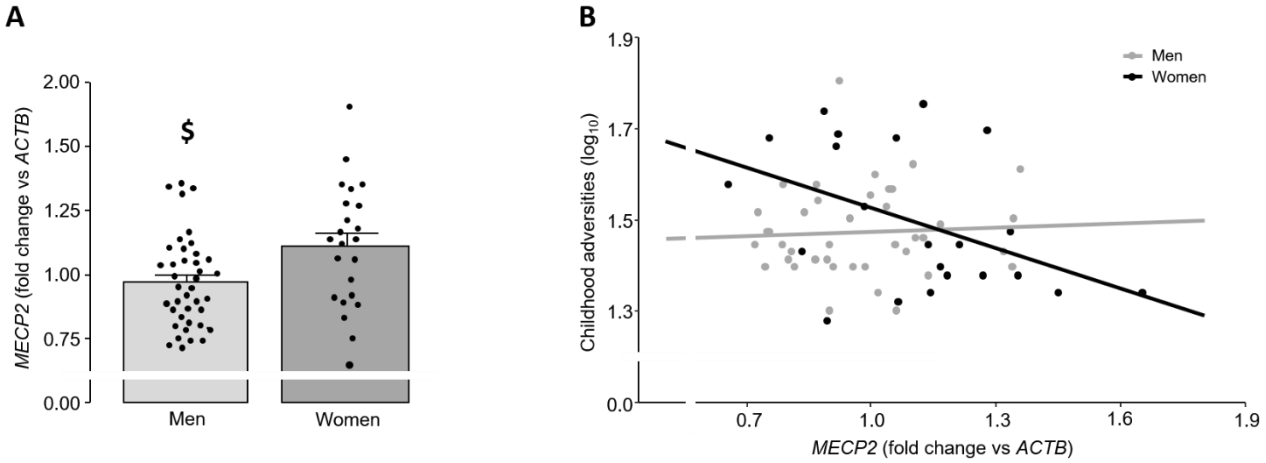

Figure 1 – *MECP2* is overexpressed in women compared to men and gender moderates *MECP2* association with childhood adversities. A) Blood mRNA levels of *methyl-CpG binding protein 2* (*MECP2*) are increased in women compared to men. Statistical significance was calculated by the means of non-directional Student's t test. $: p<0.05. Data are Mean±standard error of the mean (SE). **B)** Decreased expression of *MECP2* is associated to more severe childhood adverse experiences, particularly in women. Statistical significance was calculated by the means of multiple linear regression. R^2: 0.206 (Women); 0.003 (Men).

Low *MECP2* levels are associated with severe childhood adversities especially in females

To test the hypothesis that *MECP2* expression might be directly associated with stress-related measures in a gender-dependent manner, multiple regression analyses were performed. Gender moderated *MECP2* association with childhood adversities (*MECP2**gender - β=0.285, p=0.030). In particular, lower peripheral *MECP2* expression levels were related to higher childhood adversity scores especially in female participants (Figure 1B). Of note, neither *MECP2* nor gender alone were directly associated with the severity of childhood aversive experiences and no significant main or interaction effects of *MECP2* and gender were found for the association with depressive or anxiety symptoms (Table 1). Regression analyses were then performed to test whether the severity of adverse childhood experiences might be directly associated with stress-related symptomatology. Consistent with previous findings, growing up in an adverse environment was significantly related to higher levels of depressive symptoms (β=0.271, p=0.032) and anxiety (β=0.373, p=0.003). See Table 1 for further details.

Table 1 - Results of multiple regression analyses

Regression models	b±SE	β	t	p-value
***MECP2+gender+MECP2*gender*→childhood adversities**				
MECP2	-0.019±0.016	-0.153	-1.144	0.257
gender	-0.021±0.016	-0.175	-1.356	0.180
*MECP2**gender	0.033±0.015	0.285	2.217	0.030
***MECP2+gender+MECP2*gender* →depressive symptoms**				
MECP2	-0.036±0.045	-0.114	-0.805	0.424
gender	-0.030±0.043	-0.094	-0.692	0.492
*MECP2**gender	0.031±0.042	0.101	0.742	0.461
***MECP2*+gender+*MECP2*gender* →anxiety symptoms**				
MECP2	0.001±0.016	0.006	0.046	0.964
gender	-0.014±0.016	-0.124	-0.912	0.365
*MECP2*gender*	0.016±0.015	0.145	1.071	0.288
***Childhood trauma*→depressive symptoms**	0.712±0.324	0.271	2.196	0.032
***Childhood trauma*→anxiety symptoms**	0.355±0.113	0.373	3.142	0.003

Abbreviations: *MECP2* - *methyl-CpG binding protein 2*; b - unstandardized coefficient; .SE - standard error; β - standardized coefficient; t - Student's t statistic. Symbols: underlined - significant results.

MECP2 association with stress-related symptomatology is mediated by exposure to childhood adversities

The SEM analysis focused on the hypothesis that decreased *MECP2* expression would associate with more severe stress-related symptomatology via the mediation of childhood experiences, in a gender-dependent manner. In particular, in the initial base model (Table 2, 1i and Figure S1A), we tested the hypothesis that gender moderates *MECP2* prediction of stress-related symptoms via childhood adversities. The second model (Table 2, 1ii and Figure S1B) used modification indices to test the addition of four paths to the initial model. Three of these paths included correlations: one between the symptoms of stress-related disorders (anxiety and depression) and two between the input variables (*MECP2* and gender with the interaction term *MECP2**gender), while the fourth path was a direct arc from gender to *MECP2*. The addition of these four paths significantly improved the model fit (significant likelihood ratio, $p<0.001$). In the final model (Table 2, 1iii and Figure 2) remaining non-significant paths were removed, and the model fit was further, although not significantly, improved. The overall model fit of this final model was satisfactory (Table 2).

In terms of variance accounted for (R2), the final model accounted for 13.9% of the variance in anxiety symptoms and 7.3% of depressive symptoms, and these symptoms were predicted by the effects of the *MECP2**gender interaction on childhood adversities (9.2%).

In the final model, gender was found to moderate the association between *MECP2* expression and increased severity of childhood adversities (*MECP2**gender - β=0.303, p=0.012). Worse experiences during childhood were associated with greater depressed mood (β=0.271, p=0.027) and increased anxiety (β=0.373, p=0.002). Depression and anxiety were positively correlated (r=0.459, p<0.001). See Table S3 for further details.

Bootstrapping confirmed a significant indirect pathway from *MECP2**gender down to symptom outcomes, indicating that childhood adversities significantly mediated the association between *MECP2* expression and symptomatology typical of stress-related disorders in a gender-dependent manner (see Table 3).

Table 2 - Goodness of fit indices

Model	N	χ^2	df	χ^2/df	$\Delta\chi^2$	Δdf	RMSEA	CFI
(1) Hypothesized *MECP2* model								
(i) *Base model*	63	30.108***	10	3.101			0.180	0.465
(ii) *Four paths added*	63	1.559	6	0.260	28,549***	4	0	1
(iii) *Final model: nonsignificant paths removed*	63	0.114	2	0.057	1,445	4	0	1
(2) Confirmatory models								
(iv) *Chronic stress model*	62	0.669	2	0.335	-	-	0	1
(v) *Depression/anxiety scales substitution model*	63	0.674	2	0.337	-	-	0	1

Abbreviations: *MECP2 - methyl-CpG binding protein 2*; N – sample size; χ^2 - chi square statistic; df - degrees of freedom; $\Delta\chi^2$- nested chi square difference; RMSEA - root mean square error of approximation; CFI - comparative fit index. Symbols: *p<0.05; ***p<0.001.

Current stress load does not mediate gender-dependent *MECP2* association with stress-related symptomatology

To examine whether *MECP2* association with symptoms of depression and anxiety was specifically mediated by stress experienced during childhood, the final model was retested with current chronic stress load (TICS) replacing childhood adversities (Chronic stress model). The goodness of fit (GOF) indices of this confirmatory model matched the criteria for model acceptance (see Table 2, 2v), thus allowing the interpretation of model paths. As expected, chronic stress was significantly associated with stress-related symptomatology (depression - β=0.426, p<0.001; anxiety - β=0.588, p<0.001), leading to a high percent of variance explained for both depression and anxiety symptoms (18.1% and 34.6%, respectively). However, current stress load was not significantly predicted by the *MECP2**gender interaction (Table S3). This resulted in a lack of significance of the indirect effects of *MECP2**gender on symptom outcomes (Table 3), suggesting that childhood, but

not current stressful experiences, selectively mediate the gender-moderated *MECP2* association with stress-related symptomatology.

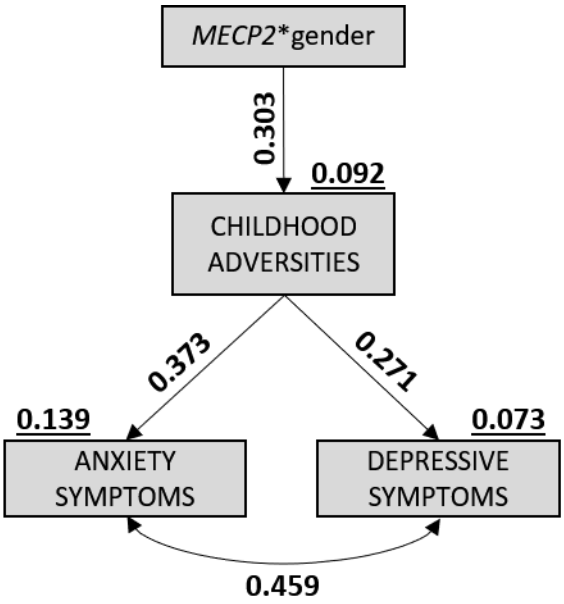

Figure 2 – Childhood adversities mediate the gender-dependent association of *MECP2* with stress-related symptomatology. Expression of *methyl-CpG binding protein 2* (*MECP2*) interacts with gender in associating with the severity of childhood adversities. This, in turn, mediates an indirect relationship with depressed mood and trait anxiety. Symbols: → directed arcs ($p<0.05$); ↔ correlations ($p<0.05$); **black numbers** - standardized coefficients; black underlined numbers - R^2 values.

Table 3 - Indirect effects in the final and confirmatory models

Total indirect pathway	b (SE)	β	p-value
***MECP2*gender* → depressive symptoms**			
Final model	0.025 (0.015)	0.113	0.029
Chronic stress model	0.014 (0.018)	0.044	0.315
Depression/anxiety scales substitution model	0.011 (0.007)	0.091	0.017
***MECP2*gender* → anxiety symptoms**			
Final model	0.013 (0.007)	0.082	0.012
Chronic stress model	0.007 (0.009)	0.061	0.368
Depression/anxiety scales substitution model	0.005 (0.004)	0.064	0.043

Abbreviations: *MECP2 - methyl-CpG binding protein 2*; b - unstandardized coefficient; SE - standard error; β - standardized coefficient. Symbols: underlined – significant results.

The gender-dependent indirect association between *MECP2* and stress-related symptomatology is confirmed using different scales of depression and anxiety

A third confirmatory model aimed at validating the strength of the final model by retesting the same path with the use of different scales measuring the severity of depression and anxiety symptoms. The GOF indices were acceptable (see Table 2, 2vi). In terms of variance accounted for (R^2) the model was able to explain 4.4% of the variance for anxiety and 9% for depressive symptoms.

As expected, gender moderated the association between *MECP2* expression and childhood adversities (β=0.303, p=0.012). Worse childhood experiences, in turn, were associated with more severe stress-related symptomatology, although the association with anxiety missed significance (depression - β=0.300, p=0.013; anxiety - β=0.210, p=0.090). Importantly, the indirect effect of the *MECP2**gender interaction on symptoms of anxiety and depression remained significant (see Table 3), confirming the gender-dependent mediating effect of childhood adversities on the association between *MECP2* expression and stress-related symptoms over different psychometric scales..

Discussion

The translation of stressful experiences into mental disorders appears to be highly dependent on each subject's vulnerability. There is a strong interest in determining the biological underpinnings of such interindividual differences in stress responses and outcomes, which would be helpful for identifying measurable markers of stress vulnerability and targets to increase resilience. The present study provides novel evidence that in healthy humans peripheral expression of *MECP2* is associated with the severity of adverse experiences at childhood, a major factor increasing the risk of developing stress-related disorders. *MECP2* is also indirectly associated with stress-related disorder symptom clusters via the mediation of stress load specifically during childhood, further corroborating the link between *MECP2* levels and stress susceptibility. Importantly, these effects were all moderated by gender, suggesting that the role of MECP2 in the regulation of stress vulnerability might differ between men and women.

The present results provide evidence that MECP2 is involved in providing vulnerability to trauma- and stressor-related disorders. This is supported by multiple evidence of an association between MECP2 alterations and several mental disorders, including depression, bipolar disorder, schizophrenia and substance abuse [28,29,32,56], many of which also represent significant PTSD comorbidities [57]. The data on MECP2 involvement in such a large spectrum of disorders, whose major common factor is the etiological component of stress, led us to postulate a role for MECP2 in shaping vulnerability to traumatic events. In line with this, we recently provided evidence that hypomorphic mutations in *MeCP2* increase the likelihood of developing PTSD-like symptomatology in traumatized mice [34,35]. The present study extends the connection between stress and MECP2 to healthy conditions in humans and provides evidence that reduced *MECP2* expression is associated with higher depressed mood and trait anxiety, especially in females.

Of major importance, this association is mediated by exposure to adversities during childhood. Indeed, in accordance with previous findings [58–60] stressful events experienced before 18 years of age were found to positively correlate with higher depressed mood and trait anxiety. Increased stress exposure during early life was also significantly linked to reductions in blood *MECP2* mRNA levels. Previous studies have demonstrated that an interplay between MECP2 functionality and stress exposure during infancy occurs in rodents [26,61,62]. Early life stressors were in fact found to induce a transient detachment of MECP2 from the DNA, with lasting consequences for the expression of its target genes. These findings acknowledge the role of MECP2 as crucial for the long-lasting programming of stress responsivity after adverse experiences at infancy [27]. The notion of a tight interaction between MECP2 and early life stress was further strengthened by research reporting a

persistent modulation of *MECP2* expression in the brain of rodents experiencing different kinds of perinatal stressors [63,64]. The present results, by evidencing an association between childhood stress and *MECP2* levels, confirm and expand previous findings to humans. Although the mechanisms underneath the outlined connection between reduced *MECP2* and increased childhood adversities need to be further explored, a possibility is that early life stressors trigger lasting epigenetic changes at the *MECP2 locus*, leading to a persisting downregulation of *MECP2* expression. Indeed, the lasting reduction of *MECP2* mRNA following early life challenges has previously been associated with stable changes in *MECP2* promoter methylation in rodents [65]. We cannot however rule out that other mechanisms could explain the downregulation of *MECP2* we observed, including a putative persistent alteration at the charges of *MECP2* transcriptional regulators following childhood adverse experiences [66].

Interestingly, gender turned out to play a major role in this scenario. Indeed, *MECP2* association with childhood adversities as well as its indirect link with stress-related symptoms, were moderated by gender. In particular, decreased *MECP2* expression was associated with more severe childhood experiences especially in females, suggesting a gender-specific involvement of MECP2 in supporting stress vulnerability. In this line, gender was previously found to moderate the detrimental consequences of childhood traumas, with girls reporting histories of abuse or neglect in adolescence displaying more severe depressive symptoms than boys [67,68]. The gender-dependent modulation of *MECP2* hereby evidenced might then provide a biological framework for the differential vulnerability to childhood traumas outlined in females and males. Importantly, sex differences in the alteration of *MECP2* expression upon early stress have already been shown in rodent studies with inconsistent findings, however, with respect to the relative direction of mRNA or protein modulation [69–71]. Different type and timing of the stressors taken into account might have played a role in the lack of uniformity across studies, and further research is needed to clarify and dissect the moderating role of gender in the lasting modulation of *MECP2* expression by early life stress.

It is important to note that we did find significant differences in *MECP2* mRNA levels in the blood of male and female participants, with women overexpressing *MECP2* compared to men. Although *MECP2* is not expected to escape X inactivation outside embryonic development [72,73], multiple mechanisms, involving hormonal or genetic factors in interaction with age and tissue specificity, have been proposed to account for differences in the expression of X-linked genes between males and females [74] and may be involved in the gender-dependent regulation of blood *MECP2* mRNA outlined in the present work [75]. Importantly, perinatal differences in *MECP2* expression between male and female rodent brains were previously described to depend on the specific region investigated [75,76], corroborating the relevance of a tissue-dependent regulation. A

gender-specific modulation of *MECP2* expression in the postnatal brain is also sustained by the sex-dependent effects of MECP2-associated neurological disorders, with MECP2 loss of function (Rett syndrome) and duplication syndromes affecting primarily girls and boys, respectively [77]. Consistently, we here report that women are particularly sensitive to *MECP2* downregulation upon experiencing adversities during childhood, which, in turn, mediates the association between reduced *MECP2* levels and increased depressive and anxiety symptoms. These findings are also in line with the fact that girls are known to be more affected than boys by the detrimental effects of stress exposure throughout puberty, leading to a higher risk of developing mood- and stress-related disorders [78].

It is important to consider that the present findings have been obtained in healthy participants. This condition allowed us to assess significant associations between *MECP2* expression and symptom clusters known to be related to an increased risk of contracting PTSD following traumatic experience. Although the present results are limited by their correlational nature, which does not allow us to derive conclusions on the predictive role of decreased *MECP2* expression with respect to disease development after traumatization, they may help uncovering an innovative marker of vulnerability. Highly discriminative measures able to characterize vulnerable individuals are in fact still missing, and the multiplicity of factors involved in the outcomes of traumatic events challenges the finding of a common framework, which is needed to develop a predictive susceptibility index [79]. So far the most promising attempts leverage merging candidate biological markers of stress susceptibility to construct integrative vulnerability measures [80]. Further research aimed at outlining novel promising markers, including epigenetic modulators, is thus encouraged [79].

As a whole, in line with recent evidence acknowledging the contribution of X-linked genes to the existing gender bias in PTSD [81], the present results suggest an involvement of MECP2 in providing vulnerability to trauma- and stress-related disorders, especially in females. Due to the small sample size, however, the results have to be interpreted with caution and need to be replicated in larger groups. Also, to verify whether such associations can be predictive of the disorder itself, future large-scale longitudinal studies will have to be performed, that may help assessing whether decreased *MECP2* expression in healthy individuals actually predicts disease onset in the aftermath of a traumatic event longitudinal evaluations might also strengthen the interpretation of the role played by childhood adversities, since, as a retrospective evaluation, it might be influenced by vulnerability to stressors.

Overall, present results may suggest a gender-specific involvement of low peripheral *MECP2* levels in supporting vulnerability to PTSD when facing adversities during childhood. Further studies are necessary to longitudinally confirm the proposed association, by monitoring if *MECP2* levels in health actually predict the likelihood of disease onset, thus representing a measurable marker of

increased susceptibility for developing stress-related disorders. The present findings shed new light on the complex biology underlying stress vulnerability and provide a novel promising candidate vulnerability marker to be further explored.

Conflicts of interest

The authors declare no competing interests.

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Authors contributions

Conceptualization: De Filippis Bianca, Herta Flor. Methodology: Cosentino Livia, Zidda Francesca, Dukal Helene, Witt Stephanie. Software: Cosentino Livia, Zidda Francesca. Validation: Witt Stephanie, De Filippis Bianca, Flor Herta. Formal analysis: Cosentino Livia, Dukal Helene. Investigation: Zidda Francesca, Dukal Helene. Resources: De Filippis Bianca, Flor Herta, Witt Stephanie. Data curation: Cosentino Livia, Zidda Francesca, Dukal Helene. Writing – original draft: Cosentino Livia. Writing – Review & Editing: all authors. Visualization: Cosentino Livia, De Filippis Bianca. Supervision: De Filippis Bianca, Flor Herta. Project administration: De Filippis Bianca, Flor Herta. Funding acquisition: De Filippis Bianca, Flor Herta.

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Supplementary methods

Psychometric measures

The trait scale of the German version of the State-Trait Anxiety Inventory (STAI-T) is composed of 20 questions rated on a 4-point Likert scale ranging from 1 - nearly never to 4 - nearly always. Scores range from 20 to 80, with higher scores indicating greater trait anxiety. STAI-T has excellent internal reliability, good test-retest reliability, and high convergent validity in terms of correlations with parallel anxiety tests [1–3].

The German version of the Center for Epidemiological Studies Depression Scale (CES-D; German Allgemeine Depressions Skala, ADS) is a self-report instrument assessing the frequency of 20 depressive symptoms in the last week using a 4-point scale ranging from 0 - rare to 3 - mostly. The score ranges from 0 to 60. The ADS shows good internal and test-retest reliability, and high convergent validity [4,5].

The German version of the Symptom Checklist-90-R (SCL-90-R) consists of 90 items rated on a 5-point Likert scale ranging from 0 - not at all to 4 – very strong. The depression and anxiety subscales scores are calculated as the mean score and range from 0 to 4, and the scale shows high internal consistency and good convergent and divergent validity [6–8].

The Childhood Trauma Questionnaire (CTQ) is composed by 34 items rated on a 5-point Likert scale (1 = never true - 5 = very often true) that assesses the severity of adverse childhood experiences. The 5 subscales (emotional abuse, physical abuse, sexual abuse, emotional neglect, and physical neglect) have good test-retest reliability, and high internal consistency [9,10]. The CTQ also contains a denial scale that evaluates the likelihood of underreporting traumatic experiences. In the present study the sum score of the 5 subscales, not including the denial scale, has been used, that ranges from 5 to 125. We used tha validated German version of the scale [11,12].

The Trier Inventory for Chronic Stress (TICS) is composed by 57 items assessing 9 chronic stressors: work and social overload, pressure to perform, work discontent, excessive demand at work, lack of social recognition, social tensions or isolation and chronic worrying. Each item is rated on a 5-point Likert scale (from 0 - never to 4 - very often) in respect to how often the subject had experienced a certain situation within the last 3 months. The internal consistency and test-retest reliability for this scale are good to very good [13,14].

Statistical Analyses

In case of violation of normality, assessed by the means of Shapiro Wilk’s test, a logarithmic transformation was performed (Table S1).

Supplementary results

Table S1 - Data transformation

	Non-transformed			Log_{10} transformed		
	Mean±SD	**Skewedness**	**Curtosis**	**Mean±SD**	**Skewedness**	**Curtosis**
Childhood adversities	31.635±9.759	1.334	1.506	1.483±0.121	0.725	-0.020
Depressive symptoms (CES-D)	9.365±6.361	1.293	1.569	0.870±0.318	-0.541	0.475
Anxiety symptoms (STAI-T)	32.984±9.454	1.157	0.963	1.503±0.115	0.574	-0.149
Depressive symptoms (SCL-90-R)	0.319±0.514	2.697	8.282	0.098±0.129	1.762	3.072
Anxiety symptoms (SCL-90-R)	0.205±0.281	2.431	6.815	0.072±0.085	1.738	3.410

Logarithmic transformation was performed to reduce the skewedness and curtosis of childhood adversities, dissociative and anxiety symptoms measures distributions. Abbreviations: SD - standard deviation; CES-D - center for epidemiological studies depression scale; STAI-T – trait scale of the state and trait anxiety inventory; SCL-90-R – Symptoms Checklist-90-R.

Table S2 - Direct effects and correlation coefficients of the final and confirmatory models

Direct effects / Correlation coefficients	b/s (SE)	β/r	p-value
Final Model			
*MECP2**gender → childhood adversities	0.036 (0.014)	0.303	0.012
childhood adversities → depressive symptoms	0.712 (0.321)	0.271	0.027
childhood adversities → anxiety symptoms	0.355 (0.112)	0.373	0.002
depressive symptoms ↔ anxiety symptoms	0.015 (0.004)	0.459	<0.001
Chronic stress model			
*MECP2**gender → chronic stress	0.101 (0.124)	0.104	0.412
chronic stress → depressive symptoms	0.136 (0.037)	0.426	<0.001
chronic stress → anxiety symptoms	0.068 (0.012)	0.588	<0.001
depressive symptoms ↔ anxiety symptoms	0.01 (0.004)	0.373	0.006
Depression/anxiety scales subtitution model			
*MECP2**gender → childhood adversities	0.036 (0.014)	0.303	0.012
childhood adversities → depressive symptoms	0.148 (0.087)	0.300	0.013
childhood adversities → anxiety symptoms	0.32 (0.129)	0.210	0.090
depressive symptoms ↔ anxiety symptoms	0.008 (0.002)	0.825	<0.001

Abbreviations: *MECP2 - methyl-CpG binding protein 2*; b - unstandardized coefficient; s - covariance estimate; SE - standard error; β - standardized coefficient; r - Pearson's correlation coefficient. Symbols: underlined - significant results.

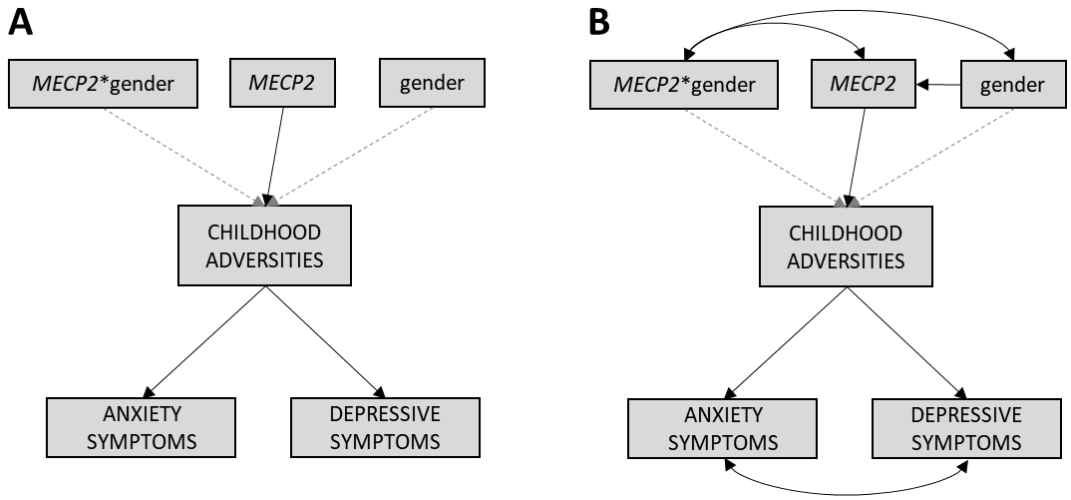

Figure S1 – Initial hypothesized models. A) The initial model tested the hypothesis that *methyl-CpG binsing protein 2 expression* (*MECP2*) and gender predicted alone and in interaction the severity of anxiety and depressive symptoms through the mediation of childhood adversities. **B)** Model respecifications were made by removing nonsignificant directed arcs and adding correlated paths as indicated by modification indices that were consistent with the hypothesis. Symbols: ⇢ directed arcs (nonsignificant); → directed arcs ($p<0.05$); ↔ correlations ($p<0.05$).

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2.4 Study 4: *Methyl-CpG binding protein 2* is modulated by stressors and associates with post-traumatic stress disorder in a gender-dependent manner[1]

[1]Manuscript in preparation: Cosentino L, Witt SH, Dukal H, Zidda F, Siehl S, Flor H[§], De Filippis B[§]. *Methyl-CpG binding protein 2 is modulated by stressors and associates with post-traumatic stress disorder in a gender-dependent manner*. [§]equally contributing authors.

Abstract

Background: Post-traumatic stress disorder (PTSD) is a chronic and severely debilitating mental disorder that affects twice as many women as men. Disease pathophysiology is still unclear, and reliable markers are currently lacking. Altered DNA methylation profiles in patients suggest an important role for epigenetic mechanisms in the pathogenesis of PTSD. Specifically, a mutation at the level of the X-linked Methyl-CpG binding protein 2 (MeCP2), an important reader of the DNA methylome, was demonstrated to provide susceptibility to develop PTSD-like alterations in traumatized mice. The present study used a human cohort to examine whether peripheral MECP2 expression may relate to PTSD symptom severity in traumatized men and women and their history of stress exposure.

Methods: *MECP2* mRNA levels were analyzed in the blood of 132 subjects (58 women). Participants were interviewed by expert clinicians to assess trauma exposure and PTSD symptomatology, and were required to retrospectively report the severity of adverse experiences during childhood.

Results: *MECP2* expression was persistently altered by stressful events in a gender- and time window-dependent manner. Women displayed *MECP2* underexpression when exposed to childhood adversities, while men traumatized in adulthood overexpressed *MECP2* in the blood. Most importantly, among trauma-exposed participants, reduced *MECP2* levels were indirectly associated with increased severity of PTSD symptoms via the mediation of adverse experiences at childhood, and this association was stronger in women than in men.

Conclusions: *MECP2* expression emerges as a potential contributor to post-trauma pathophysiology suggesting novel studies on the molecular mechanisms underlying its potential gender-dependent role in PTSD onset and progression.

Keywords: human cohorts, MECP2, PTSD, gender, childhood trauma, structural equation modeling

Introduction

Post-traumatic stress disorder is a chronically debilitating mental disorder that affects in average 5.6% of individuals exposed to traumatic events and is more prevalent in women than men (1). The distressing symptoms arising in the aftermath of traumas are long-lasting (2) and can be grouped in three main categories: i) re-experiencing, since affected people suffer from intrusive thoughts, nightmares and flashbacks; ii) hyperarousal, including manifestations as attentional threat bias, sleep problems and enhanced startle reactivity; iii) avoidance of trauma reminders, that often entails patients' inability to recall important aspects of the traumatic event (3). In the last update of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (4) a further cluster of symptoms related to negative alterations in cognition and mood has been added, and PTSD has been reallocated to the novel category of "trauma and stressor-related disorders" in recognition of the fact that the development of the disorder is specifically ascribed to the presence of a known stressor. Following traumatization, the diagnosis of PTSD is currently based on the patients' symptomatology. However, disease biomarkers based on molecular mechanisms could increase diagnostic accuracy and ultimately contribute to the development of more effective interventions (5,6).

In recent years we have gained a better understanding of the neurobiological basis of PTSD (7), and a growing body of evidence has underscored the fundamental contribution of epigenetic mechanisms to PTSD pathogenesis and symptom presentation in the aftermath of traumas (8). Among the multiple epigenetic signatures, DNA methylation (DNAm) has been thoroughly studied, and numerous findings of altered levels of methylated DNA have been associated with PTSD (6,9).

The potential involvement of the X-linked epigenetic reader methyl-CpG binding protein 2 (MECP2) in PTSD pathophysiology is so far not known. MECP2 appears to be an excellent candidate for mediating post-trauma epigenetic rearrangements, since it serves as a scaffold protein for the recruitment of chromatin remodeling complexes (10–12) and DNA methyltransferases (13,14) on methylated DNA *loci*. Also, MeCP2 is extremely responsive to the surrounding environment, and early life, as well as adult, stressors have been described to alter its activity and expression (15–17). Besides, MeCP2 is known to control the transcription of genes involved in the regulation of the stress response (18,19), whose expression has been consistently found altered in people diagnosed with PTSD (20–23). These characteristics make of MECP2 a promising mediator of the lasting epigenetic adjustments taking place following trauma exposure, that could direct towards vulnerability or resilience (24). Based on this evidence, we recently addressed the potential involvement of altered MeCP2 functionality in the onset of PTSD-like pathophysiology, using transgenic mice carrying a hypofunctional form of MeCP2 (25). We demonstrated that *MeCP2*-mutated mice display an

increased propensity to develop enduring neurobehavioral alterations, comparable to those observed in PTSD patients, in the aftermath of intense, acute stressors (26).

Notably, male and female carriers of the *MeCP2* hypomorphic mutation, while being both behaviorally sensitive to stressors, exhibited an opposite modulation of stress markers at the molecular level (27). This finding opens to the intriguing possibility that MeCP2 protein may be involved in the regulation of gender-dependent pathways of disease vulnerability. In this line, it is noteworthy that MeCP2 has been proposed to participate in the establishment of sex differences in the developing brain (28,29), suggesting that it might play a role in setting the basis for the existing gender bias in PTSD vulnerability (30,31). Also, a sex-specific modulation of MeCP2 has been previously shown to be triggered by stress exposure early in life (32,33), which is a widely recognized vulnerability factor for PTSD onset upon further trauma exposure (34).

Based on this body of evidence, we hypothesized that *MECP2* levels may be altered within a traumatized population, with its underexpression possibly representing a disease marker for PTSD. We also reasoned that *MECP2* might be differentially regulated in men and women following exposure to stressful events experienced during childhood or at adulthood, in line with the existing gender bias in PTSD vulnerability. To test our assumptions we evaluated *MECP2* mRNA levels in the blood of a total of 132 male and female traumatized and non-traumatized participants with and without PTSD and assessed whether early stress or adult traumas altered *MECP2* expression. To examine the hypothesized contribution of MECP2 functions to PTSD psychopathology, we tested the possibility that reduced *MECP2* blood levels may be associated to increased PTSD symptom severity. Furthermore, since early stress is known to increase the likelihood of maladaptive trauma outcomes, we assessed the mediating role of childhood adversities in this association.

Materials and methods

Study participants

The subjects included in the present study are a joint sample of the participants recruited between 2010 and 2018 to take part in multiple studies on learning and memory alterations in people suffering from PTSD. A total of 132 subjects (58 women, mean age 41.718±13.911 years) were included in the present study, 85 (40 women) of whom experienced at least one traumatic event, as assessed by the means of the Posttraumatic Diagnostic Scale (35,36) (see Table 1 and supplementary methods for detailed demographic and clinical information). PTSD diagnosis and possible comorbidities were evaluated by the Structured Clinical Interviews for DSMIV-TR (3) I and II (SCID) (37,38) (see supplementary methods). Participants were excluded in case of severe traumatic experiences before 18 years of age, comorbid psychotic symptoms, borderline personality disorder, alcohol/drug dependence or abuse, and cardiovascular or neurological disorders. The study conformed to the Code of Ethics of the World Medical Association (Declaration of Helsinki, 6th revision, 2008) and was approved by the Ethics Committee of the Medical Faculty Mannheim, Heidelberg University. All participants gave written informed consent.

MECP2 expression

Whole blood was collected in PAXgene Blood RNA Tubes (PreAnalytiX) and stored until analysis at -80°C (39). A PAXgene Blood miRNA Kit (Qiagen) was used to extract total RNA, following the manufacturer´s instructions. RNA concentration and sample purity were assessed with a NanoDrop 1000 Spectralphotometer (Thermo Scientific), and RNA integrity was determined with the Agilent 2100 Bioanalyzer System (Agilent Technologies). The cDNA was synthesized by a reversed transcription reaction using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed on the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems by Life Technologies) by using TaqMan Universal PCR Master Mix, no AmpErase UNG (Applied Biosystems), and the *MECP2* TaqMan Gene Expression Assay Hs00172845_m1 (Applied Biosystems). The *Actin Beta* (*ACTB*) TaqMan Gene Expression Assay Hs01060665_g1 (Applied Biosystems) was used as an internal standard. Results were calculated with the QuantStudio Real-Time PCR Software v1.3 (Applied Biosystems by Thermo Fisher Scientific). Analyses were carried out in triplicates. All data were normalized to the endogenous reference gene ACTB. For statistical analyses, the relative expression with respect to non-traumatized participants, not exposed to adversities during childhood (controls) was calculated by the Delta-Delta threshold

cycles (ΔΔCt) method, and converted to the relative expression ratio ($2^{-\Delta\Delta Ct}$), separately for men and women (40)

Psychometric measures

Childhood Trauma Questionnaire. Participants completed the childhood trauma questionnaire (CTQ) (41) in order to retrospectively evaluate the severity of adverse childhood experiences (≤ 18 years of age), including emotional and physical abuse/neglect and sexual abuse. CTQ is a psychometrically validated self-report inventory composed by 28 items each rated on a 5-point Likert scale (1, never true – 5, very often true). The total CTQ score ranges from 5 to 125. To include childhood adversities as a 2-levels independent factor in analysis of variance (ANOVA) tests, we transformed the continuous total CTQ scores in a dichotomous variable: 1, none to minimal (CTQ ≤ 36) and 2, minimal to extreme (CTQ > 36) (42), based on the classification included in the CTQ manual (41).

Trier Inventory for Chronic Stress. The load of current chronic stress was assessed by the means of the Trier Inventory for Chronic Stress (TICS) (43), a self-assessment instrument composed by 57 items evaluating 9 chronic stressors: work and social overload, pressure to perform, work discontent, excessive demand at work, lack of social recognition, social tensions or isolation and chronic worrying. Each item is rated on a 5-point Likert scale indicating how often the subject had experienced a certain situation within the last 3 months (0, never – 4, very often).

Clinician-Administered PTSD Scale. PTSD symptomatology was assessed by the means of the Clinician-Administered PTSD Scale interview (CAPS) (44,45), a 30-item structured interview that corresponds to the DSMIV criteria for PTSD (3). Frequency and severity of each item are rated on a 5-point Likert scale ranging from 0, never/not affected to 4, every day/extremely affected. Three subscales measuring re-experiencing, avoidance and arousal symptom clusters were then calculated as the mean frequency and severity values of the relative items (5 for re-experiencing and arousal and 7 for avoidance symptoms). The total CAPS score was calculated as the overall sum of the ratings, ranging from 0 to 68.

Center for Epidemiological Studies Depression Scale and State-Trait Anxiety Inventory: see supplemetary methods.

Statistical analyses

All statistical analyses were conducted using SPSS 20.0 and AMOS 20.0 (IBM Statistics).

A logarithmic transformation was performed to reduce skewedness and kurtosis of non normally distributed variables. Outliers, defined as observations lying outside the 3 standard deviations from the mean cutoff, were excluded.

Three-way ANOVA was performed to evaluate the relative role of gender (men & women), childhood adversities (none to minimal & minimal to extreme) and trauma exposure at adulthood (non traumatized & traumatized) in the modulation of peripheral *MECP2* expression. Normality and homoscedasticity of residuals were assessed by the means of Shapiro Wilk, Levene and Breush Pagan tests. Post hoc comparisons were performed by Tukey's test.

Structural Equation Modeling (SEM) with maximum likelihood estimation was used to test the hypothesis that, in traumatized participants, reduced *MECP2* expression would be associated with increased PTSD symptom severity via the mediation of childhood adversities. Gender was also included in the model (women=1, men=2). Exclusion criteria for the model were: failure to converge after 240 iterations, the presence of squared multiple correlation values greater than 1 ($R^2>1$) and poor fit, estimated via the following goodness-of-fit (GOF) measures: the χ^2 statistic (with a good fit indicated by χ^2/degrees of freedom (df) ≤ 3), the root mean square error of approximation (RMSEA with a good fit indicated by an index < 0.08) and the comparative fit index (CFI with a good fit indicated by an index > 0.95) (46). To examine whether the final model was specific for early life stressful experiences, it was retested with a measure of current perceived chronic stress replacing the Childhood Trauma Questionnaire. Moreover, the model was separately re-specified on male and female subsamples to further dissect the moderating role of gender. For each of the analyses the alpha level was set to 0.05.

Table 1 - Demographic and clinical informations

Variable			Group						Group statistic
			HC (N=47)		TC (N=48)		PTSD (N=37)		
			Men (N=29)	Women (N=18)	Men (N=25)	Women (N=23)	Men (N=20)	Women (N=17)	
Age (in years)		Mean (SD)	40.069 (14.155)	45.529 (15.961)	42.040 (14.383)	43.130 (16.347)	39.700 (9.879)	40.706 (11.994)	Diagnosis: $F_{2,125}$=0.411, p=0.664 Gender: $F_{1,125}$=1.012, p=0.316
Education	≤ 12 years	N	0	3	2	1	4	4	χ^2_{15}=18.377, p=0.243
	> 12 years	N	28	14	20	20	13	13	
	Other	N	1	1	1	0	1	0	
	Missing	N	0	0	2	2	2	0	
Ancestry	European	N	26	17	22	21	14	14	χ^2_{10}=11.060, p=0.353
	Other[a]	N	3	1	2	0	3	2	
	Missing	N	0	0	1	2	3	1	
CES-D		Mean (SD)	7.207 (4.221)	7.500 (5.834)	10.040 (6.985)	10.696 (8.110)	31.100 (9.159)	29.059 (10.028)	Diagnosis: $F_{2,126}$=109.448, p<0.001 (PTSD > HC & TC) Gender: $F_{1,126}$=0.077, p=0.782
STAI-T		Mean (SD)	32.241 (9.376)	31.611 (6.464)	35.833 (8.245)	35.909 (11.690)	58.579 (9.794)	52.706 (11.526)	Diagnosis: $F_{2,123}$=66.878, p<0.001 (PTSD > HC & TC) Gender: $F_{1,123}$=1.537, p=0.217
Medication	None	N	13	8	16	6	2	6	χ^2_{15}=41.095, p<0.001
	Psychopharmacological[b]	N	11	6	6	12	4	4	
	Non-psychopharmacological[c]	N	2	2	2	2	7	7	
	Missing	N	3	2	1	3	7	0	
CTQ		Mean (SD)	31.318 (8.519)	32.611 (12.821)	33.120 (10.035)	35.870 (17.057)	36.650 (13.240)	48.177 (22.565)	Diagnosis: $F_{2,126}$=5.877, p=0.004 (PTSD > HC & TC) Gender: $F_{1,126}$=4.310, p=0.040 (F>M)
TICS		Mean (SD)	2.067 (0.564)	2.039 (0.468)	2.335 (0.776)	2.190 (0.652)	3.042 (0.580)	2.811 (0.594)	Diagnosis: $F_{2,120}$=20.383, p<0.001 (PTSD > HC & TC) Gender: $F_{1,120}$=1.434, p=0.233
Time since trauma (in years)		Mean (SD)	-	-	9.227 (8.512)	11.796 (12.414)	10.375 (9.091)	8.906 (11.055)	Diagnosis: $F_{1,72}$=0.129, p=0.720 Gender: $F_{1,72}$=0.051, p=0.821
Index trauma	Caused voluntarily[d]	N	-	-	11	6	12	12	χ^2_6=11.067, p=0.086
	Caused involuntarily[e]	N	-	-	14	16	7	5	
	Missing	N	-	-	0	1	1	0	

Variable		Group						Group statistic
		HC (N=47)		TC (N=48)		PTSD (N=37)		
		Men (N=29)	Women (N=18)	Men (N=25)	Women (N=23)	Men (N=20)	Women (N=17)	
CAPS	Mean (SD)	-	-	7.739 (8.835)	9.368 (11.398)	67.184 (23.415)	65.912 (20.531)	Diagnosis: $F_{1,74}$=235.441, p<0.001 (PTSD > TC) Gender: $F_{1,74}$=0.002, p=0.962
PDS	Mean (SD)	-	-	8.042 (7.166)	9.000 (10.872)	36.353 (7.770)	32.125 (8.928)	Diagnosis: $F_{1,75}$=173.435, p<0.001 (PTSD > TC) Gender: $F_{1,75}$=0.660, p=0.419

Abbreviations: SD – standard deviation; N – sample size; HC – non-traumatized healthy controls; TC – trauma-exposed healthy participants; PTSD – patients diagnosed with Post-Traumatic Stress Disorder; χ^2 - chi square statistic; F – F statistic; p value of the chi square or F statistics; CES-D –Centre for Epidemiological Studies Depression Scale; STAI-T - State-Trait Anxiety Inventory – Trait Anxiety; CTQ – Childhood Trauma Questionnaire; TICS – Trier Inventory for Chronic Stress; CAPS – Clinician Administered PTSD Scale; PDS – Post-traumatic Diagnostic Scale; [a]: Mixed, Turkish, Columbian, Pakistani, Palestinian; [b]Aripiprazole, Pregabalin, Methylphenidate, Mirtazapine, Quetiapine, Sertraline, Trimipramine, Venlafaxine; [c]Etoricoxib, Bisoprolol, Beta-Blocker, Ibuprofen, Metamizole, Levothyroxine; [d] Imprisonment, torture, physical violence, sexual abuse/rape, wartime experience, witness of sudden death/serious injury of someone, other experience; [e]fire or explosion, accident, sudden death of someone, other experiences.

Results

Stress exposure in different periods of life differentially alters *MECP2* expression in men and women

To assess the relative contribution of stressors experienced in different periods of life on the modulation of *MECP2* expression, and the moderating effects of gender, we tested an ANOVA model with gender, trauma exposure at adulthood and childhood adversities as independent factors. In spite of an unsignificant three-way interaction (gender*childhood adversities*adult trauma: $F_{1,124}$=2.319, p=0.130), gender turned out to have a major influence on *MECP2* expression levels, with men displaying significantly higher blood *MECP2* mRNA compared to women (main effect of gender: $F_{1,124}$=37.890, p<0.001). This was mainly driven by the effect of trauma exposure in adulthood, which was related to selectively increased *MECP2* in males (p<0.01 for non-traumatized vs traumatized men after post hoc comparisons on gender*trauma interaction: $F_{1,124}$=3.792, p=0.054; Figure 1A). Conversely, childhood adversities significantly decreased peripheral *MECP2* expression (main effect of childhood adversities: $F_{1,124}$=7.458, p=0.007) with females being most affected (p<0.01 for women with none to minimal vs minimal to extreme childhood adversities after post hoc comparisons on gender*childhood adversities interaction: $F_{1,124}$=7.211, p=0.008; Figure 1B).

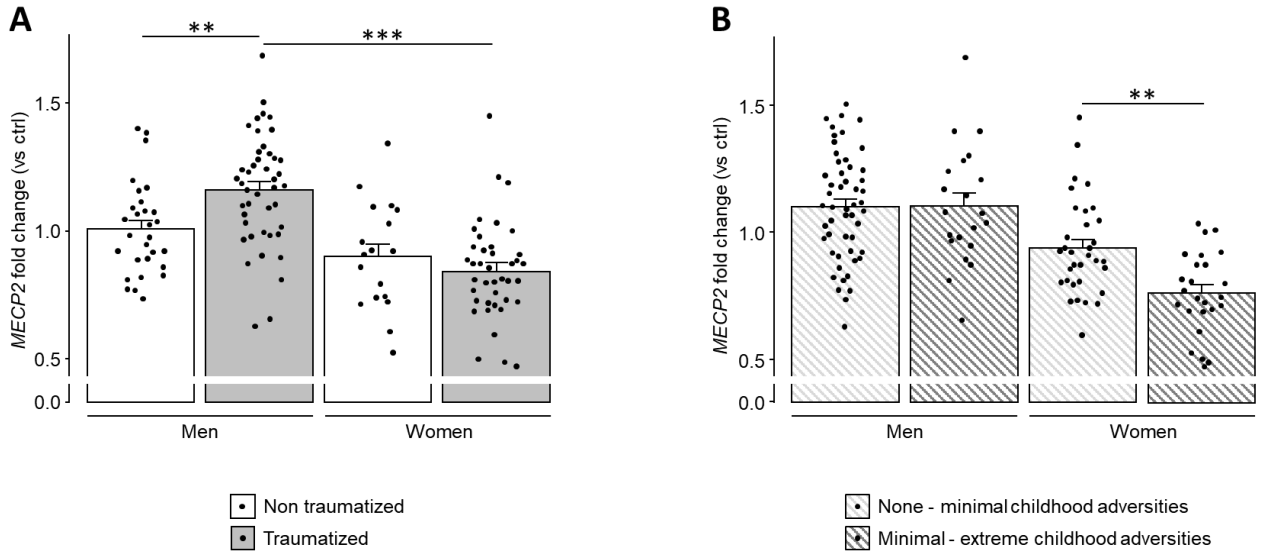

Figure 1 – *MECP2* is overexpressed in traumatized men and underexpressed in women exposed to childhood adverse experiences. A) Blood mRNA levels of *methyl-CpG binding protein 2* (*MECP2*) are increased in traumatized men, compared to non-traumatized men and women. **B)** *MECP2* is underexpressed in the blood of women exposed to adverse experiences during childhood compared to non-stressed women and men. *MECP2* levels were normalized to total actin beta (ACTB) contents and expressed as a proportion of those of non-traumatized participants, not exposed to adversities during childhood (ctrl), separately for men and women. Statistical significance was calculated by the means of three-way ANOVA, and Tukey's post hoc tests. **: p<0.01; ***: p<0.001. Data are mean±standard error of the mean.

Childhood adversities mediate the association between reduced *MECP2* expression and PTSD symptomatology

Given that *MECP2* expression is modulated by the exposure to both childhood stress and adult traumas, and that early adverse experiences are known to increase the risk of PTSD onset in the aftermath of traumatic events, we next tested the hypothesis that decreased *MECP2* expression in trauma-exposed participants would associate with more severe PTSD symptomatology (CAPS scores) via the mediation of childhood adversities using structural equation modeling (Figure 2). Overall PTSD symptomatology was represented as a single latent factor in the present model, based on the a priori assumption that the three PTSD symptoms subscales may be all be associated within the same latent construct (47,48).

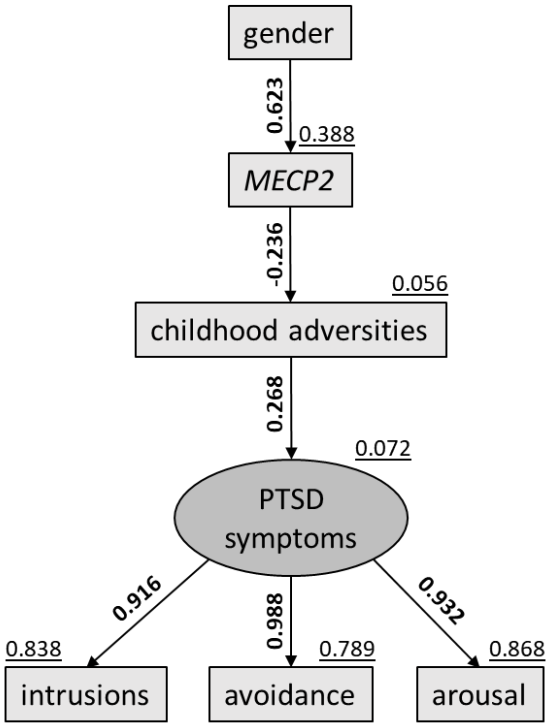

Figure 2 – Childhood adversities mediate the association of *MECP2* with PTSD symptomatology. Expression of *methyl-CpG binding protein 2* (*MECP2*) is indirectly associated with post-traumatic stress disorder (PTSD) symptomatology via the mediation of childhood adverse experiences. Symbols: → directed arcs ($p<0.05$); **black numbers** - standardized coefficients; black underlined numbers – variance explained (R^2).

Table 2 - Goodness of fit indices

Model	N	χ^2	df	χ^2/df	RMSEA	CFI
(1) Hypothesized model	75	5.335	9	0.593	0	1
(2) Confirmatory models						
(vi) *Chronic stress model*	74	11.827	9	1.314	0.066	0.988
(vii) *Men*	40	1.415	5	0.282	0	1
(viii) *Women*	35	5.594	5	1.119	0.059	0.993

Abbreviations: N – sample size; χ^2 - chi square statistic; df - degrees of freedom; RMSEA - root mean square error of approximation; CFI - comparative fit index.

The model fit was satisfactory (Table 2, 1), suggesting that the hypothesized path (Figure 2) describes the data well, thus allowing further interpretations of the results obtained. A significant effect of gender on *MECP2* mRNA levels was hereby confirmed, with traumatized men overexpressing *MECP2* compared to trauma-exposed women (β=0.623, p<0.001), explaining up to 38.8% of the variance in *MECP2* expression. Of note, *MECP2* expression was inversely proportional to the severity of childhood adversities (R^2=5.6%), suggesting that decreased *MECP2* levels were associated with more adverse childhood experience (β=-0.236, p=0.037). In turn, as expected, higher childhood stress scores were associated with increased frequency and severity of PTSD symptomatology among traumatized individuals (β=0.268, p=0.022). Most importantly, *MECP2* expression turned out to be indirectly associated with PTSD symptoms, with childhood adversities mediating the association between lower levels of *MECP2* and higher CAPS scores (β=-0.063, p=0.029), suggesting that *MECP2* underexpression is associated to increased vulnerability to PTSD, particularly when experiencing more childhood adversities (see Table 3 and Supplementary Table S1 for further details on direct and indirect effects in the hypothesized model).

Overall, the hypothesized model explained 7.2% of variance for the latent construct representing PTSD symptomatology and up to 83.8, 78.9 and 86.8% for re-experiencing, avoidance and arousal symptoms, respectively.

Chronic stress exposure does not mediate *MECP2* association with PTSD symptomatology

To examine the specificity of the observed effects for stress experienced during childhood, we assessed whether the hypothesized path was still valid when replacing the childhood trauma score with a measure of chronic stress load.

The model had acceptable fit indices (Table 2, 2i), and explained a relatively high proportion of variance for the PTSD symptomatology latent construct (R^2=26%). This was entirely due to the highly significant association between chronic stress load and PTSD symptoms (β=0.510, p<0.001) (see Supplementary Table S2 for further information on direct and indirect effects). Indeed, *MECP2* was not significantly associated with chronic stress and failed to have significant indirect effects on PTSD symptomatology in the present model (see Table 3), suggesting that the association between *MECP2* expression and PTSD symptomatology is mediated specifically by stressors experienced during childhood.

Childhood adversities mediate the association between *MECP2* expression and PTSD symptomatology selectively in women

In order to confirm and dissect the significant effects of gender outlined in the correlational model under investigation, we explored the validity of the selected path on two different subsamples, composed of men or women only (see Figure 3). The GOF indices for both subsamples were acceptable (see Table 2, 2ii and 2iii). In terms of R2 the model explained up to 27.8% of PTSD symptom variance in the female subsample, but failed to significantly explain PTSD symptom variance in the male subsample. Of note, the R2 values for the single symptom subscales were similarly high in both samples (88.6, 76, and 95.8% in men, and 77, 85, and 76.6% in women for re-experiencing, avoidance and arousal symptoms, respectively), suggesting that the three symptom dimensions are well represented by the latent construct in both genders.

Table 3 - Indirect effects of *MECP2* expression on PTSD symptomatology in the hypothesized and confirmatory models

Indirect effects	b (SE)	β	p-value
***MECP2* → PTSD symptoms (latent construct)**			
Hypothesized model	-0.204 (0.140)	-0.063	0.029
Chronic stress model	0.074 (0.193)	0.023	0.682
Men subsample	-0.003 (0.136)	-0.001	0.921
Women subsample	-0.626 (0.326)	-0.139	0.025
***MECP2* → re-experiencing**			
Hypothesized model	-0.235 (0.159)	-0.058	0.033
Chronic stress model	0.085 (0.219)	0.021	0.687
Men subsample	-0.003 (0.157)	-0.001	0.917
Women subsample	-0.677 (0.358)	-0.122	0.027
***MECP2* → avoidance**			
Hypothesized model	-0.204 (0.140)	-0.056	0.029
Chronic stress model	0.074 (0.193)	0.021	0.682
Men subsample	-0.003 (0.136)	-0.001	0.921
Women subsample	-0.626 (0.326)	-0,128	0.025
***MECP2* → arousal**			
Hypothesized model	-0.240 (0.162)	-0.059	0.032
Chronic stress model	0.087 (0.228)	0.022	0.674
Men subsample	-0.003 (0.162)	-0.001	0.919
Women subsample	-0.698 (0.348)	-0.122	0.022

Abbreviations: *MECP2* - methyl-CpG binding protein 2; PTSD – post-traumatic stress disorders; b - unstandardized coefficient; SE - standard error; β - standardized coefficient. Symbols: underlined – significant results.

Importantly, in both samples, *MECP2* expression failed to be directly associated with the severity of childhood adversities, which, conversely, significantly predicted PTSD symptoms selectively in women (β=0.528, p=0.001). Of note, the total indirect effect of *MECP2* expression on PTSD symptoms was significant in the female (β=-0.139, p=0.025), but not in the male subsample (see Tables 3 and Supplementary Table S3 for further details).

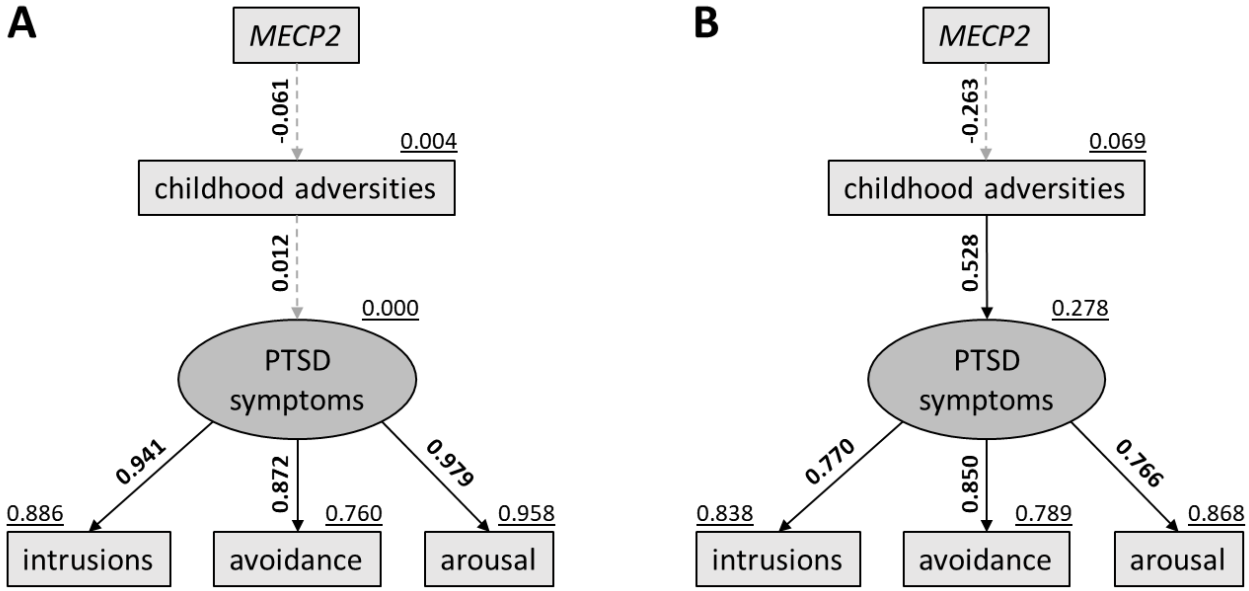

Figure 3 – Childhood adversities mediate the association between *MECP2* expression and PTSD symptomatology selectively in women. Expression of *methyl-CpG binding protein 2* (*MECP2*) is indirectly associated with post-traumatic stress disorder (PTSD) symptomatology via the mediation of childhood adverse experiences in women (B), but not in men (A). Symbols: directed arcs (nonsignificant); directed arcs ($p<0.05$); black numbers - standardized coefficients; black underlined numbers - variance explained (R^2).

Discussion

The present study provides evidence that *MECP2* expression in the blood of male and female participants appears to be differentially regulated by stress exposure, with traumatized men overexpressing *MECP2*, while women exposed to childhood adversities showing *MECP2* underexpression. Most importantly, decreased *MECP2* levels were indirectly associated with severe PTSD symptomatology in people who experienced adverse events at childhood, especially among females. Taken together, the present results suggest that *MECP2* might differentially contribute to disease pathogenesis in men and women, and that its underexpression may mark the severity of PTSD condition, particularly in women characterized by early life stress.

The present results highlight a putative involvement of *MECP2* in PTSD pathogenesis, and suggest that *MECP2* underexpression may represent a useful marker of disease severity, at least in a subset of patients. This perspective is of major importance for PTSD, since the lack of a precise understanding of the pathophysiological mechanisms at its basis challenges the identification of effective therapeutic interventions, leading to major difficulties in addressing the huge burden represented by the detrimental consequences of trauma exposure worldwide (5,49). In this line, uncovering reliable markers of disease may be of help in the process of clarifying PTSD pathogenesis and related physiological dysfunctions, and might aid in identifying novel targets for possible pharmacotherapies to take into account (6).

Importantly, a reduced amount of *MECP2* mRNA in the blood of traumatized participants was indirectly associated with more severe and frequent symptoms of PTSD, especially in case of stress exposure during childhood. The present findings are supported by extensive evidence, in rodent studies, of an interplay between MeCP2 function and stress in early life, ultimately leading to a persistent re-programming of the stress axis responsivity to future stressors (15,17,50). Importantly, such lasting physiological consequences were also described to influence rodents' behavioral response to stress in adulthood, triggering either vulnerability or resilience, depending on the severity of early experiences (15,17,50). In this light, MeCP2 appears to be a critical environmental sensor involved in the transduction of early external events in lasting epigenetic memory, ultimately modulating an individual's ability to cope with future challenges. Further reinforcing the existing functional link between MeCP2 and early life stress, multiple studies showed lasting modulations of MeCP2 expression in the brain of perinatally stressed rats and mice (32,51–54). In line with this, we observed that higher stress load during childhood associated with *MECP2* underexpression in adulthood. This looks promising, since experiencing adverse events at childhood is a risk factor common to multiple mental disorders, including depression and schizophrenia (55,56), with whom

PTSD shares a substantial proportion of genetic variance (9,57,58). In this light, a better understanding of the role of MECP2 in the pathophysiology of mental disorders may benefit from a research centered on the individuation of genetic and epigenetic associations with traits related to mental disorders, rather than based on strict diagnostic categories, as advocated by the Research Domain Criteria (RDoC) initiative. In support of this, profound differences have been evidenced in the DNA methylation profiles of PTSD patients exposed or not exposed to childhood adversities (21), suggesting that people experiencing stress in early life may constitute a distinct and specific PTSD biotype (5,59). MECP2 might thus be involved in the pathophysiology of a quite specific biological subtype of PTSD, with shared mechanisms among different mental disorders of stress origin.

Importantly, our results point to women as the gender most affected by reduced levels of *MECP2* in case of stressful experiences during childhood. Consistently, females were described to be more vulnerable than males to the detrimental and lasting consequences of childhood adversities (60,61). It is thus conceivable that MECP2 may take part in gender-dependent biological mechanisms that make females more vulnerable than males to stress-related disorders (62). Unfortunately, although reporting sex-dependent changes in MeCP2 expression following stress in early life, the existing evidence in rodent studies is inconsistent with regard to the direction of this alteration, and the most affected sex (32,33). This issue needs to be systematically addressed in further studies aimed at dissecting the factors involved in the sex-specific modulation of MeCP2 upon early challenges.

An intriguing aspect of the present results is that *MECP2* expression appears to be differentially regulated in men and women in case of exposure to stress in different periods of life, with women displaying reduced levels of *MECP2* following childhood adversities, while men may be overexpressing it in the aftermath of adult traumas. Interestingly, peritraumatic MeCP2 overexpression in rodent brains has previously been associated with the maintenance of good memory performance under stress, which is fundamental to prevent the onset of symptoms of fear generalization and avoidance that may instead be associated with a lack of modulation of MeCP2 levels (16). Based on this, we propose that decreased MECP2 function may be involved in the maladaptive outcomes of stress exposure, while its increase might be protective. Although further studies are needed to substantiate this hypothesis, an appealing possibility that emerges from these considerations is that MECP2 may take part in divergent or dymorphic pathways leading to gender differences in stress vulnerability or resilience. We indeed found an increase in *MECP2* expression specifically in males, who are known to be more resilient to the detrimental outcomes of trauma exposure compared to females (30,63), who appear, instead, prone to *MECP2* downregulation. In this line, it is important to underline that developmental sex differences in MeCP2 expression and its contribution to the emergence of sex dimorphisms have been previously acknowledged (28,29),

which may strengthen our hypothesis of an involvement of MECP2 in the establishment of gender differences in risk or resilience to PTSD.

Although rodent studies have provided evidence of a sex-dependent regulation of MeCP2 after stress exposure, the mechanisms involved are far less clear. A possibility is that the *MECP2* gene might be differentially methylated in males and females, which is supported by emerging work suggesting the establishment of gender-specific patterns of DNAm following stress (30,64). Whether these differences are triggered by the activational effects of gonadal hormones, or are the result of developmentally-organized dymorphisms at the level of signalling pathways, remains to be established (65). Of course, gender specific mechanisms involving *MECP2* trans-regulatory elements might also take place, with transcription factors or miRNAs involved in MECP2 expression regulation being selectively altered in males or females following stress (55,66). Gaining further insight into the mechanisms involved in the sex-divergent regulation of MeCP2 expression following stress exposure may be of great help for the identification of vulnerability or pathogenic pathways to be targeted with the aim of increasing resilience.

The present results should be considered in light of some limitations. Indeed, the cohort considered here was primarily composed of participants of European ancestry, thus limiting the possibility to extend our findings to multiple ethnicities. This is important, given that the genetic and epigenetic underpinnings of PTSD have previously been demonstrated to differ among ethnic groups (67). Furthermore, it is important to clarify that, while analyzing human blood samples allowed us to provide the first evidence of an association between *MECP2* levels and PTSD symptoms, gene expression in blood does not necessarily reflect the molecular processes that may take place within the brain. Although there is evidence that peripheral epigenetic responses might, in some instances, reflect brain-related states (30,68), the present findings need to be reinforced by animal studies addressing brain *MECP2* levels.

In summary, the present study suggests that *MECP2* underexpression may represent a step in the pathogenic process leading to PTSD onset in patients, especially women, exposed to childhood adversities. Studies focusing on dissecting the mechanisms involved in the regulation of *MECP2* expression could shed new light on the biological pathways underlying the gender bias in trauma vulnerability, and could provide a more detailed mechanistic understanding of the pathophysiology of the disorder, hopefully leading to more effective, individualized interventions.

Conflicts of interest

The authors declare no competing interests.

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Supplementary methods

Study participants

Participants received a reimbursement for participation (10€/h) plus travel expenses and patients were offered treatment in the clinics of the Central Institute of Mental Health in Mannheim. Data on subsamples of this combined sample were reported in (Siehl et al., 2020; Steiger et al., 2015; Wicking et al., 2016). No significant differences were observed for age or education among groups. Predictably, participants diagnosed with Post-traumatic Stress Disorder (PTSD) (37 subjects, of which 17 women) scored higher in the Center for Epidemiological Studies Depression Scale (CES-D) (Hautzinger & Bailer, 1993) and in the trait version of the State-Trait Anxiety Inventory (STAI-T) (Spielberger et al., 1970) measuring depressive and anxiety symptoms, respectively. PTSD patients reported higher levels of chronic stress, measured by the means of the Trier Inventory for Chronic Stress (TICS) (Schultz & Schlotz, 1999) and of childhood adversities, as demonstrated by the significantly higher scores obtained in the Childhood Trauma Questionnaire (CTQ) (Bernstein & Fink, 1998). See Table 1 for further details.

Psychometric measures

Posttraumatic Diagnostic Scale. Traumatic events were assessed by the means of the German version of the Posttraumatic Diagnostic Scale (PDS) (Ehlers et al., 1997; Foa, 1995; Griesel et al., 2006), a self-report instrument aimed at assessing the severity of post-traumatic stress symptoms. The first part of the questionnaire consists in a short checklist of potentially traumatizing events. Among the experienced events, respondents are required to indicate which has troubled them the most in the last month and to rate, on a 4-point scale (0 - never to 3 - daily), 17 items representing the frequency of cardinal PTSD symptoms occurrence in the last 30 days. Finally, respondents rate the degree of impairment caused by symptoms across different areas of life functioning. The symptoms severity score is obtained by adding up the responses to selected items and ranges from 0 to 51.

Structured Clinical Interviews for DSMIV-TR. PTSD diagnosis and possible comorbidities were evaluated by the Structured Clinical Interviews for DSMIV-TR axis I and axis II disorders (SCID-I and SCID-II) (Fydrich et al., 1997; Wittchen et al., 1997). They are both semi-structured clinical interviews, administered by trained clinicians, designed to yield psychiatric diagnoses consistent with DSM-IV-TR (American Psychiatric Association, 2000) diagnostic criteria for major mental and personality disorders, respectively.

State-Trait Anxiety Inventory. Trait anxiety was evaluated by the means of the trait scale of the german version of the State-Trait Anxiety Inventory (STAI-T) (Spielberger et al., 1970), which is

composed of 20 questions rated on a 4-point Likert scale ranging from 1 - nearly never to 4 - nearly always. Scores range from 20 to 80, with higher scores indicating greater trait anxiety.

Center for Epidemiological studies Depression Scale. Depressive symptoms were assessed using the the German version of the Center for Epidemiological Studies Depression Scale (CES-D; German Allegemeine Depressions Skala, ADS) (Hautzinger & Bailer, 1993), a self-report instrument assessing the frequency of 20 depressive symptoms in the last week using a 4-point scale ranging from 0 - rare to 3 - mostly. The score ranges from 0 to 60.

Supplementary tables

Table S1 - Direct and Indirect effects in the hypothesized model

Direct effects	b (SE)	β	p-value
Gender → *MECP2*	0.334 (0.049)	0.623	≤0.001
MECP2 → childhood adversities	-0.129 (0.062)	-0.236	0.037
childhood adversities → PTSD symptoms (latent construct)	1.578 (0.689)	0.268	0.022
PTSD symptoms (latent construct) → re-experiencing	1.153 (0.098)	0.916	≤0.001
PTSD symptoms (latent construct) → avoidance	1[a]	0.888	
PTSD symptoms (latent construct) → arousal	1.179 (0.097)	0.932	≤0.001
Indirect effects	**b (SE)**	**β**	**p-value**
Gender → childhood adversities	-0.043 (0.022)	-0.147	0.029
Gender → PTSD symptoms (latent construct)	-0.068 (0.047)	-0.039	0.024
Gender → re-experiencing	-0.078 (0.053)	-0.036	0.025
Gender → avoidance	-0.068 (0.047)	-0.035	0.024
Gender → arousal	-0.080 (0.054)	-0.037	0.025
MECP2 → PTSD symptoms (latent construct)	-0.204 (0.140)	-0.063	0.029
MECP2 → re-experiencing	-0.235 (0.159)	-0.058	0.033
MECP2 → avoidance	-0.204 (0.140)	-0.056	0.029
MECP2 → arousal	-0.240 (0.162)	-0.059	0.032
childhood adversities → re-experiencing	1.815 (0.803)	0.245	0.026
childhood adversities → avoidance	1.578 (0.717)	0.238	0.026
childhood adversities → arousal	1,860 (0.832)	0.250	0.026

Abbreviations: *MECP2* - methyl-CpG binding protein 2; PTSD – post-traumatic stress disorder; b - unstandardized coefficient; SE - standard error; β - standardized coefficient. Symbols: [a] - b was fixed to the value 1 in order to treat 'avoidance' as the reference variable for the latent factor 'PTSD symptoms'; underlined – significant results.

Table S2 - Direct and Indirect effects in the chronic stress model

Direct effects	b (SE)	β	p-value
Gender → *MECP2*	0.336 (0.049)	0.623	<0.001
MECP2 → chronic stress	0.116 (0.298)	0.046	0.697
Chronic stress → PTSD symptoms (latent construct)	0.642 (0.138)	0.510	<0.001
PTSD symptoms (latent construct) → re-experiencing	1.138 (0.098)	0.906	<0.001
PTSD symptoms (latent construct) → avoidance	1[a]	0.890	
PTSD symptoms (latent construct) → arousal	1.174 (0.098)	0.934	<0.001
Indirect effects	**b (SE)**	**β**	**p-value**
Gender → chronic stress	0.039 (0.103)	0.028	0.680
Gender → PTSD symptoms (latent construct)	0.025 (0.066)	0.014	0.656
Gender → re-experiencing	0.028 (0.075)	0.013	0.669
Gender → avoidance	0.025 (0.066)	0.013	0.656
Gender → arousal	0.029 (0.078)	0.013	0.660
MECP2 → PTSD symptoms (latent construct)	0.074 (0.193)	0.023	0.682
MECP2 → re-experiencing	0.085 (0.219)	0.021	0.687
MECP2 → avoidance	0.074 (0.193)	0.021	0.682
MECP2 → arousal	0.087 (0.228)	0.022	0.674
Chronic stress → re-experiencing	0.730 (0.142)	0.462	0.001
Chronic stress → avoidance	0.642 (0.134)	0.454	0.001
Chronic stress → arousal	0.753 (0.148)	0.476	0.001

Abbreviations: *MECP2* - methyl-CpG binding protein 2; PTSD – post-traumatic stress disorder; b - unstandardized coefficient; SE - standard error; β - standardized coefficient. Symbols: [a] - b was fixed to the value 1 in order to treat 'avoidance' as the reference variable for the latent factor 'PTSD symptoms'; underlined – significant results.

Table S3 - Direct and Indirect effects in men and women subsamples

	men			women		
Direct effects	**b (SE)**	**β**	**p**	**b (SE)**	**β**	**p**
MECP2 → childhood adversities	-0.035 (0.092)	-0.061	0.702	-0.217 (0.136)	-0.263	0.111
childhood adversities → PTSD symptoms (latent construct)	0.082 (1.094)	0.012	0.941	2.887 (0.881)	0.528	0.001
PTSD symptoms (latent construct) → re-experiencing	1.185 (0.129)	0.941	≤0.001	1.082 (0.146)	0.878	≤0.001
PTSD symptoms (latent construct) → avoidance	1[a]	0.872		1 [a]	0.922	
PTSD symptoms (latent construct) → arousal	1.212 (0.124)	0.979	≤0.001	1.116 (0.154)	0.875	≤0.001
Indirect effects	**b (SE)**	**β**	**p**	**b (SE)**	**β**	**p**
MECP2 → PTSD symptoms (latent construct)	-0.003 (0.136)	-0.001	0.921	-0.626 (0.326)	-0.139	0.025
MECP2 → re-experiencing	-0.003 (0.157)	-0.001	0.917	-0.677 (0.358)	-0.122	0.027
MECP2 → avoidance	-0.003 (0.136)	-0.001	0.921	-0.626 (0.326)	-0,128	0.025
MECP2 → arousal	-0.003 (0.162)	-0.001	0.919	-0.698 (0.348)	-0.122	0.022
childhood adversities → re-experiencing	0.097 (1.456)	0.011	0.940	3.124 (1.097)	0.463	0.001
childhood adversities → avoidance	0.082 (1.238)	0.011	0.934	2.887 (1.077)	0.487	0.001
childhood adversities → arousal	0.099 (1.493)	0.012	0.940	3.221 (1.107)	0.462	0.001

Abbreviations: *MECP2* - methyl-CpG binding protein 2; PTSD – post-traumatic stress disorder; b - unstandardized coefficient; SE - standard error; β - standardized coefficient. Symbols: [a] - b was fixed to the value 1 in order to treat 'avoidance' as the reference variable for the latent factor 'PTSD symptoms'; underlined – significant results.

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GENERAL DISCUSSION

Chapter 3

3.1 Summary of the aims and main results

Although affecting only a minority of people exposed to traumatic events, PTSD bears a significant impact on both the socio-economic and health systems (Watson, 2019) and is severely invalidating for patients, who chronically face extremely distressing symptoms, with a high rate of resistance to currently available treatments (Schmidt & Vermetten, 2018). Poor understanding of disease pathogenesis and pathophysiology is responsible for the absence of more effective interventions (Richter-Levin et al., 2019; Schmidt & Vermetten, 2018), and research on PTSD is largely dedicated to unravel the biological basis of this complex disorder, with the ultimate goal of developing novel targeted or preventive therapies. The present work fits into this framework and, based on the compelling evidence of an involvement of dysregulated epigenetic mechanisms in disease vulnerability and development (Rakesh et al., 2019; Zannas et al., 2015; Zovkic et al., 2013), aimed at translationally evaluating the contribution of the DNAm reader MECP2 to the pathogenesis and pathophysiology of PTSD.

Based on the association of stress risk or resilience to opposite DNAm profiles, a disrupted balance between activation and repression of gene expression has been proposed to interfere with the ability of successfully coping with traumatic events (Elliott et al., 2010; Zovkic et al., 2013). Therefore, we hypothesized that dysfunctions at the level of the transcriptional regulator and DNAm reader MeCP2 might provide vulnerability to maladaptive trauma outcomes. To test this, we exposed transgenic male mice carrying a hypofunctional form of MeCP2 (MeCP2-308, Shahbazian et al., 2002) to traumatic stressors, and evaluated whether they developed phenotypes comparable to patients' symptomatology from a behavioral, physiological and molecular perspective. We found that trauma-exposed mutant male mice exhibited lasting fear reactions when re-exposed to the traumatic context (Cosentino, Vigli, Medici, et al., 2019), mirroring patients' persistent distress in face of external reminders of the threat (American Psychiatric Association, 2013). The overgeneralization of fear afflicting patients, who usually transfer fear to stimuli unrelated to aversive experiences (Lis et al., 2020; Steiger et al., 2015; Wicking et al., 2016), is also modeled in traumatized mutants, which display enduring fear responses to non-threat-predicting salient cues (Cosentino, Vigli, Medici, et al., 2019). Traumatized mutants also displayed an increased avoidance of trauma reminders, a crucial aspect of PTSD symptomatology (American Psychiatric Association, 2013), and alterations in circadian rhythms (Cosentino, Vigli, Medici, et al., 2019), which are usually described in patients (American Psychiatric Association, 2013). This contributes to design a framework in which MeCP2 appears to be deeply involved in setting a threshold for the onset of behavioral alterations in the aftermath of traumatic events. Indeed, trauma hereby produces bidirectional behavioral changes as a

function of genetics (carriers of MeCP2 hypofunctional mutation or wt littermates). This suggests a role for MeCP2 functions in the establishment of interindividual differences in trauma vulnerability, which are responsible for disease onset in a minority of the trauma-exposed population (Borghans & Homberg, 2015; Yehuda & Antelman, 1993). Of utmost importance, mouse carriers of the MeCP2 hypofunctional mutation mirrored the transcriptional signatures previously described in cohorts of patients with different ancestries and trauma histories (Cosentino, Vigli, Medici, et al., 2019; de Kloet et al., 2008; Martini et al., 2013; Mehta et al., 2013). This provides further support to the involvement of MeCP2-related pathways in guiding trauma *sequelae* towards adaptive or maladaptive outcomes. The present findings support a model in which alterations in a specific epigenetic network, rather than in single systems, are required for replicating the wide complexity of PTSD-like conditions.

Of note, gender is a major contributor to the delineation of vulnerability or resilience to traumatic events (Bangasser & Valentino, 2014; Hodes & Epperson, 2019; Hu et al., 2017), and MeCP2 has been acknowledged to take part to the developmental process of sexual differentiation of the brain (Forbes-Lorman et al., 2012, 2014; Kurian et al., 2007; Kurian et al., 2008). It is thus likely that constitutive MeCP2 hypofunctionality might trigger sex-dependent outcomes with respect to stress responsivity and effects. We evidenced that MeCP2 mutations had similar behavioral consequences in male and female mice, since homozygous females, much alike hemizygous males, displayed exaggerated fear reactions when re-exposed to a threatening context (Cosentino et al., 2021; Cosentino, Vigli, Medici, et al., 2019), mirroring patients' intense distress in front of trauma reminders (American Psychiatric Association, 2013). Of note, such similar behavioral outcomes co-occurred with sex-dependent alterations in stress physiology, which entails crucial mechanisms guiding stress reactivity and regulation, usually altered in stress-related disorders (Doom & Gunnar, 2013; Liberzon & Ressler, 2016; van Zuiden et al., 2013). Indeed, MeCP2 hypofunctionality had the effect of abolishing the sex differences in HPA axis reactivity (Bangasser & Valentino, 2012, 2014), increasing stress-induced glucocorticoid surge in males, while causing its decrease in females (Cosentino et al., 2021; Cosentino, Vigli, Medici, et al., 2019). A deeper exploration of the HPA axis neuropeptide expression at the brain level revealed that a similar upregulation of *Avp* and *Crh* occurred in the hypothalamus of mutant males and females (Cosentino et al., 2021). This suggests that the sex-dependent effects of the MeCP2 mutation on glucocorticoid release may originate within the HPA axis, downstream the hypothalamus. Of note, sex differences in stress peptide sensitivity have been described at the level of both the pituitary and the adrenals (Bangasser & Valentino, 2014), whose developmental organization could have been affected by MeCP2 hypofunctionality (Forbes-Lorman et al., 2012; Kurian et al., 2007; Kurian et al., 2008).

Noteworthy, our studies also highlight an interesting sex-dependent alteration of *Fkbp5* expression in mutant mice (Cosentino et al., 2021; Cosentino, Vigli, Medici, et al., 2019). Although discouraging its role in shaping the outlined glucocorticoid profile, its differential regulation by MeCP2 holds promise for elucidating the mechanisms at place in PTSD pathogenesis and uncovering potential distinct risk biotypes. In fact, FKBP5 is profoundly involved in the biological processes underneath PTSD vulnerability and psychopathology, with controversial results with respect to its levels of expression (Castro-Vale et al., 2016; Klengel et al., 2013; Mehta et al., 2011; Sarapas et al., 2011; van Zuiden et al., 2012; Yehuda et al., 2009).

Based on these results, we suggest that constitutive MeCP2 hypofunctionality might exert a sexually convergent maladaptive effect on the behavioral response to stressful situations in transgenic mice. The mechanisms leading to these outcomes certainly need to be further explored. It is indeed conceivable that constitutive MeCP2 dysfunction may increase stress vulnerability via the exploitation of sex-dependent underlying effects on HPA axis functionality. In fact, an inverted-U-shape relationship between glucocorticoid levels and learning has been substantiated, which implies that either a reduction or an increase in glucocorticoids, relative to their optimal physiological levels, are associated with disruptions in memory performance (Salehi et al., 2010). In this line, mixed evidence exists relative to the association of altered cortisol levels in the immediate aftermath of an acute traumatic experience and later PTSD onset, with either negative and positive correlations being found (Bhattacharya et al., 2019).

Of course, other explanations may account for our results. Indeed, the systems , at the brain level, known to play crucial parts in PTSD risk in which MeCP2 functions are required are multiple. For instance, evidence is increasing regarding the synergistic action of glucocorticoid hormones and norepinephrine on emotional memory processing (Bahtiyar et al., 2020). Therefore, the fact that the norepinephrinergic system as well as LC excitability, are affected in the absence of MeCP2, points to the arousal system as a promising modulator of stress vulnerability in the the absence of proper MeCP2 functionality. Another possibility is that, at the circuit level, default mode network (DMN) deficits, previously associated with increased symptom severity among PTSD patients (Akiki et al., 2018; Lanius et al., 2020), might be responsible for poor processing of traumatic experiences in MeCP2 deficient mice, leading to the onset of PTSD-like exaggerated distress in front of trauma reminders. In support of this view, DMN hypoactivity has been formerly described in mice lacking MeCP2, where it is reversed by ketamine, a promising pharmacotherapy for the treatment of PTSD (Kron et al., 2012; Liriano et al., 2019).

Importantly, the abolishment, by constitutive MeCP2 dysfunction, of a well-known sex difference in HPA axis reactivity, in the context of a similar behavioral stress sensitization, also opens

an intriguing developmental perspective for the contribution of MeCP2 to stress susceptibility that needs further investigation. Indeed, by intervening in developmental processes aimed at establishing sex differences within the brain (Forbes-Lorman et al., 2012; Kurian et al., 2007; Kurian et al., 2008), MeCP2 represents a promising factor possibly involved in the organization of a sex-biased vulnerability to stress and traumas. Investigating the presence of critical time windows of MeCP2 effects on vulnerability programming and the mechanisms at place, might shed new light on the gender-dependent biology underneath PTSD pathogenesis.

Although fundamental for gaining insight into the pathophysiology of mental disorders, animal models often replicate only few pathological aspects and lack the profound complexity of human psychiatric conditions. On these grounds, we sought to substantiate the translatability of our preclinical findings by assessing the association of MECP2 function with trauma vulnerability in human cohorts, thus confirming the involvement of MECP2-related pathways in human psychopathology.

To examine whether reduced MECP2 functionality might mark increased susceptibility to stress-related disorders, the association of blood *MECP2* expression levels to anxiety and depressed mood was first assessed in a healthy adult population. To explore the possibility that MECP2 dysfunctions might represent long-term predisposing component contributed by the early environment, we also compared *MECP2* expression levels among healthy people exposed or not exposed to childhood adversities. Early adversities have been in fact demonstrated to increase vulnerability to later traumatic experiences (Heim & Nemeroff, 2001; Lanius et al., 2010; McLaughlin & Lambert, 2017; Provençal & Binder, 2015; Spalletta et al., 2020) and persistent signatures left by childhood stress exposure are recognised as promising biological markers predisposing susceptibility (Admon et al., 2013).

We found that *MECP2* downregulation was indirectly associated to increased depression and anxiety symptoms via the mediation of childhood adversities, confirming our hypothesis that decreased MECP2 functionality is associated with higher stress vulnerability, even in humans. Also, reduced *MECP2* expression associated with an increased severity of childhood adversities, selectively in women, the gender that usually appears more severely impacted by childhood stress (Cecil & Matson, 2001; Monteiro et al., 2015). Indeed, although no significant differences were present in the severity of childhood adverse experiences between genders, *MECP2* expression variation in men was completely unrelated to the magnitude of childhood stress. From our point of view, this opens to the appealing possibility that *MECP2* downregulation may be a gender-specific effect that stems from exposure to childhood adversities and contributes to increase the vulnerability

to future traumatic events. The gender-specificity of this modulation might provide a molecular explanation to the detrimental outcomes that childhood adversities hold especially for females and to the existing gender bias in the prevalence of trauma-related disorders. In this line, gender differences in DNAm, the epigenetic mark targeted by MECP2, have been previously suggested to play a role in the establishment of distinct thresholds of resilience to developing PTSD in males and females (Nugent & McCarthy, 2011; Uddin et al., 2013).

Noteworthy, preclinical findings support the hereby proposed role of MECP2 as a gender-specific marker of early life stress exposure (Blaze & Roth, 2013; Cassim et al., 2015; Eid et al., 2016; Glendining et al., 2018; Lewis et al., 2016; Schneider et al., 2013; Sobolewski et al., 2018). Studies are, however, inconsistent with respect to the directionality of MeCP2 expression modulation in males and females that seems to strictly depend on the type and intensity of the stressor as well as the developmental period of exposure. Indeed, sex differentiation during development has been proposed to contribute to the organization of different timing of vulnerability to early life perturbations in males and females (Mueller & Bale, 2008) that is consistent with findings of a sex-specific reprogramming of rodents' epigenetic machinery following exposure to perinatal stress. Further studies that systematically compare the effects of exposure to stressors at different periods of early life are needed to clarify the role of MeCP2 as a marker of early life stress. Dissecting the sex-specific regulatory mechanisms at place might shed new light on the gender-dependent biology underneath vulnerability to stress-related disorders.

Clearly, epigenetic signatures and, consequently, gene expression are responsive to environmental challenges, which results in the delineation of potentially distinct transcriptomic profiles in susceptible individuals pre- and post-trauma exposure (Pfeiffer et al., 2018). In the former study we focused on the potential role of MECP2 as a susceptibility mark, since uncovering the biological basis of PTSD vulnerability is crucial to shed light on the pathogenic mechanisms and to identify pathways to be targeted by preventive interventions (Rakesh et al., 2019; Walker F.R. et al., 2017). Alongside, however, uncovering biomarkers of pathological status is of paramount importance to increase diagnostic accuracy and dissect the pathophysiology underneath symptom persistence, with the ultimate goal of developing new and increasingly effective therapies (Yang et al., 2020; Zannas et al., 2015). Therefore, the final aim of the present work was to attest, in a human cohort comprising both traumatized and non-traumatized participants, whether *MECP2* expression represents a correlate of PTSD symptoms severity, thus representing a potential marker of the disorder.

Importantly, we found that peripheral *MECP2* expression shows a gender-specific alteration following trauma, since blood mRNA levels are selectively increased in male participants who reported traumatic experiences. Interestingly, stress-induced boosts in expression or DNA occupancy of MeCP2 have been previously associated with resilient phenotypes in rodent studies (Finsterwald et al., 2015; Lewis et al., 2013; Singh-Taylor et al., 2018). On this basis, we speculate that the selective increase of *MECP2* hereby outlined in traumatized men may represent a gender-specific protective mechanism underlying the existing bias in disease prevalence. Indeed one man every two women is reported to suffer from PTSD, although men are slightly more likely to experience traumatic events (Hu et al., 2017). Moreover, men were shown to experience shorter durations of post-traumatic stress symptoms and less severe re-experiencing, avoidance and hyperarousal compared to women (Hu et al., 2017).

Conversely, the present data confirm our former results with respect to the decrease in peripheral *MECP2* expression occurring in women, but not in men, experiencing childhood adversities. Importantly, this decrease was independent of later exposure to traumas. This suggests that, in females, *MECP2* downregulation may represent not only a lasting, but also a robust signature of childhood stress exposure and strengthens its potential significance as a gender-specific marker of susceptibility to stress-related disorders. Indeed, reduced *MECP2* was associated with increased re-experiencing, avoidance and arousal symptoms only if trauma-exposed female participants experienced adversities as children, which is in line with the finding of distinct DNAm profiles in PTSD patients according to gender and exposure to childhood maltreatment (Mehta et al., 2013; Uddin et al., 2013).

Altogether our results point to *MECP2* downregulation as a promising marker of PTSD-related symptom severity in trauma-exposed women who had a difficult childhood and suggest that *MECP2*-related epigenetic pathways may lie at the basis of the gender-biased prevalence of trauma and stressor-related disorders, by contributing to the onset and progression of PTSD in a subset of female patients. The suggestion of a potential protective role of MECP2, when overexpressed, also emerges, opening the possibility that the same epigenetic pathways may be differentially activated in vulnerability/resilience, depending on MECP2 regulation.

3.2 Limitations

We are aware that candidate gene studies have fallen out of favor in translational psychiatry, mainly because mental disorders have a polygenic nature, and single genes are expected to have small effect sizes. Despite these concerns, candidate gene research continues, since it entails an important

and unique strength which is the ability to test mechanistic hypotheses (Koenen et al., 2013; Morrison et al., 2019). Indeed, unlike single genes *per se*, gene-related mechanisms are likely to have strong effects and explain a great proportion of disease variance. This perspective is particularly valid for genes like *MECP2* which, given its centrality within brain epigenetic networks and its ability to drive the effects of multiple target genes, appears a very promising candidate for PTSD genetics.

Indeed, the present findings outline that MECP2 functionality is needed to face traumatic experiences, since its shrinkage associates with maladaptive trauma outcomes in mice and humans. It is important to underline that full comparisons between our results from preclinical and clinical studies are hereby challenged by the use of transgenic mice carrying a constitutively expressed hypofunctional mutation at the *MeCP2 locus*. Indeed, although extremely useful for uncovering the significance of MeCP2 functions throughout development, this mouse model does not allow identifying the most relevant time windows in which MeCP2 activity is fundamental for the development of the phenotypes under study, thus limiting possible mechanistic interpretations.

In this line, a future avenue of research in our laboratory regards the study of interindividual variability in peripheral *MeCP2* expression as a function of sex and stress vulnerability in non-mutant rodents, which would allow us to replicate and verify the results obtained in human cohorts and, most importantly, to explore the associated changes at the brain level. Indeed transcriptional profiles can be tissue-specific, and thereby peripheral *MECP2* downregulation may not reflect a similar modulation within the brain, thus challenging mechanistic interpretations relative to disease pathogenesis, although previous work has demonstrated associations between peripheral and central stress-induced epigenetic responses at MECP2-targeted *loci* (Klengel et al., 2013; Uddin et al., 2013). Also, overlaps in blood and brain transcriptomic profiles have been shown in *MeCP2* mutated mice (Sanfeliu et al., 2019), encouraging the relevance of MECP2 peripheral alterations for brain-related traits.

A further limitation of the present work resides in the correlational nature of our clinical results that narrows the possibility to draw definitive conclusions with respect to *MECP2* expression sensitivity to lifetime stressors as well as its prospective association to disease onset. In fact, to ultimately verify whether *MECP2* downregulation actually occurs in the aftermath of stressful experiences and can be predictive of PTSD onset in the aftermath of traumas, longitudinal studies need to be performed in mice as well as humans that track *MECP2* expression changes as a function of life events and clinical states throughout the development of individual subjects.

3.2 Outlook

Despite the strong efforts recently devoted to understanding the biological basis of trauma vulnerability, little clarification has been provided so far with respect to the mechanisms involved in PTSD pathogenesis (Daskalakis, Rijal, et al., 2018; Schmidt & Vermetten, 2018; Yang et al., 2020). The present results strongly support the relevance of decreased MECP2 functionality in boosting susceptibility to develop PTSD or PTSD-like symptoms in the aftermath of traumatic experiences, opening to the possibility that MECP2-related epigenetic pathways might be involved in the etiology of the disorder. Further studies aimed at verifying this association on separate, confirmatory cohorts, and devoted to dissect the related molecular mechanisms at the brain level are thus recommended.

Based on previous findings acknowledging MECP2 involvement in multiple mental diorders (Ausió, 2016; Bach et al., 2020), we propose that future studies are integrated into the RDoC framework, with the aim of uncovering possible MECP2-related pathophysiological processes at the basis of the expression of psychopathological symptoms that might be shared across different mental disorders. This perspective is particularly promising in this context, given the high comorbidity rates between PTSD and other mental disorders previously associated with MECP2 alterations such as depression and substance use disorders (Ausió, 2016). The discovery of common pathways of vulnerability might ultimately lead to the development of targeted interventions promoting resilience (Rakesh et al., 2019) that may significantly reduce the global burden represented by mental disorders.

Even though one of the strongest predictors of risk for the onset of stress-related disorders is female gender, the etiology of gender differences in PTSD is far from being understood (Uddin et al., 2013). Complementing existing knowledge with present results, we propose that MECP2 might represent a key factor involved in the gender-dependent regulation of PTSD susceptibility. Although multiple studies previously reported sex-specific alterations in *MeCP2* expression following stress exposure (Blaze & Roth, 2013; Sobolewski et al., 2018) the underlying mechanisms have not yet been addressed. Although we cannot exclude that the activational effects of steroid hormones might have played a role in the gender-dependent modulation of *MECP2* levels observed in our clinical studies, the sex-specific expression profiles previously shown in the early life of rodent models point to a crucial role of *MeCP2* in the developmental organization of sex differences (Forbes-Lorman et al., 2012; Kurian et al., 2007) and suggest that MeCP2 might take part in sexually dymorphic pathways involved in stress vulnerability. A better understanding of the mechanisms underlying the sex-specific regulation of *MeCP2* expression may be of fundamental importance to gain further insight into the biological underpinnings of gender differences in disease vulnerability and manifestation, hopefully contributing to setting the bases to the development of a gender-oriented medicine.

SUMMARY

Chapter 4

Exposure to trauma is a common experience worldwide, which may lead to multiple adverse consequences for physical and mental health, and is compulsory for the diagnosis of post-traumatic stress disorder (PTSD), a chronic mental disorder characterized by symptoms of persistent anxiety and hypervigilance. Considering the key role played by susceptibility in determining the outcome of traumatic experiences, and the well-documented contribution of epigenetic mechanisms in the establishment of interindividual differences in PTSD risk and resilience, this dissertation aimed at translationally addressing the potential contribution of the X-linked epigenetic regulator methyl-CpG binding protein 2 (MECP2) in shaping vulnerability to develop stress and trauma-related disorders.

With this goal, taking advantage of a transgenic mouse line, we evaluated whether disruptions in MeCP2 functionality may predispose to the onset of PTSD-like symptomatology in the aftermath of a traumatic event. Male mice constitutively expressing a hypofunctional form of MeCP2 protein and exposed to unescapable footshocks, displayed exaggerated fear and increased avoidance of trauma cues, overgeneralization of fear responses and altered circadian rhythms, mimicking the psychopathological symptoms experienced by patients. Also, traumatized mutants displayed peripheral alterations in gene expression mirroring patterns already evidenced in people suffering from PTSD. Interestingly, although mutant females displayed similar disruptions in fear memory compared to males, sex-dependent alterations in stress responsivity were evidenced among mutants, with females releasing lower and males higher levels of glucocorticoids compared to sex-matched wild type (wt) controls. At the brain level, a region- and sex- specific pattern of stress-related alterations in gene expression was found.

We also aimed at confirming that reduced MECP2 functionality was associated with increased stress vulnerability in human cohorts. This issue was addressed in two different studies. The first, including healthy participants, explored the link between blood *MECP2* expression and childhood adversities, and addressed its effect on symptoms of depression and anxiety, which could confer vulnerability to stress-related disorders. The second study focused on the evaluation of blood *MECP2* levels in PTSD patients and healthy traumatized and non-traumatized controls, with or without stress during childhood, and their correlation with the severity of PTSD symptomatology. In the first study we found that decreased *MECP2* expression was indirectly associated with increased depressed mood and trait anxiety in healthy participants, a correlation mediated by childhood stress and moderated by gender. In line with this, in the second study we observed that childhood stress mediated the association between decreased *MECP2* expression and increased PTSD symptom severity in traumatized participants, especially among women, further corroborating a role for *MECP2* downregulation in promoting trauma vulnerability. Interestingly, men traumatized at adulthood overexpressed *MECP2*, independently from the severity of childhood stress.

Overall, the present work presents strong evidence for the role of decreased MECP2 functionality in boosting susceptibility to develop PTSD in the aftermath of traumatic experiences, opening to the possibility that MECP2-related epigenetic pathways might be involved in the etiology of the disorder. Importantly, gender emerges as an important modulator of the association between MECP2 and PTSD susceptibility.

ABBREVIATIONS

Chapter 5

5HTT: serotonin transporter

5-HTTLPR: serotonin transporter variable number tandem repeat polymorphysm

aa: amino acid

ADCYAP1R1: pituitary adenylate cyclase-activating polypeptide receptor 1 gene

ASD: autism-spectrum disorder

AVP: arginine vasopressin

BF: behavioral profiling

BDNF*:* brain-derived neurotrophic factor

CAPS: Clinician-Administered PTSD Scale

CBC: cut-off behavioral criteria

CNS: central nervous system

CRH: corticotropin releasing hormone

CSF: cerebrospinal fluid

CTD: C-terminal domain

dACC: dorsal anterior cingulate cortex

DAT_1: dopamine transporter

DMN: default mode network

DSM: Diagnostic and Statistical Manual of Mental Disorder

DNAm: DNA methylation

EWAS: epigenome-wide association studies

FADS: Fetal Alcohol Spectrum Disorders

FKBP5: FK506 binding protein 51

F-R: fear resistant

F-S: fear susceptible

GABRA2*:* GABA type A receptor subunit α_2

GC: glucocorticoids

GILZ: glucocorticoid-induced leucine zipper

GR: mineralcorticoid (MR) and glucocorticoid receptors

GWAS: genome-wide association studies

H3K27me3: trimethylation of histone 3 at lysine 27

HPA; hypotalamic-pituitary-adrenal

IQ: intelligence quotient

ID: intervening domain

LTD: long-term depression

LTP: long-term potentiation

MBD: Methyl-CpG binding domain

MDS: MECP2 duplication syndrome

MECP2: Methyl-CpG binding protein 2

Met: Methionine

NE-LC: norepinephrine-locus coeruleus

NLGN1: neuroligin 1

NTD: N-terminal domain

PACAPR1: pituitary adenylate cyclase-activating polypeptide receptor 1

PBMs: peripheral blood mononuclear cells

PPM-X: X-linked syndrome of psychosis, pyramidal signs, and macro-orchidism

PRS: polygenic risk score

PTMs: posttranslational modifications

PTSD: post-traumatic stress disorder

PVN: paraventricular nucleus of the hypothalamus

RDoC: research domain criteria

RORA*:* retinoid-related orphan receptor- and -encoding genes

RTT: Rett Syndrome

SLC6A3: dopamine transporter gene

SLC6A4: serotonin transporter gene

SNS: sympathetic nervous system (),

SSRIs: selective serotonin-reuptake inhibitors

SNRIs: selective norepinephrine reuptake inhibitors

TRD: transcriptional repressor domain

Val: Valine

vmPFC: ventromedial prefrontal cortex

VNTR: variable number tandem repeat

WHO: World Health Organization

XCI: X inactivation

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Chapter 6

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CURRICULUM VITAE

Chapter 7

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11.2017 – today	Attending the PhD course in Behavioral Neurosciences, Sapienza University (Rome, Italy); external supervisor: Dr. Bianca De Filippis; internal supervisor: Prof. Dr. Andrea Mele
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PUBLICATIONS

Chapter 8

Urbinati C.*, **Cosentino L.***, Germinario E.A.P., Valenti D., Vigli D., Ricceri L., Laviola G., Fiorentini C, Vacca R.A., Fabbri A., De Filippis B. (accepted for publication). *Treatment with the bacterial toxin CNF1 selectively rescues cognitive and brain mitochondrial deficits in a female mouse model of Rett syndrome carrying a MeCP2-null mutation*. International Journal of Molecular Sciences. ***equally contributing authors.**

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Chapter 9

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