Transcriptional signatures of resilient and susceptible phenotypes in a novel arousal-based individual screening (AIS) PTSD-like model

Ph.D. Thesis

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Preface

Post-traumatic stress disorder (PTSD) is not a well-defined psychiatric disorder leading to a variable but durable psychic trauma. The definition and diagnosis of PTSD in humans is based on behavioral symptoms and self-reports, and there is a large overlap with other disorders, including mood disorders, anxiety disorders, as well as alcohol and drug abuse which exacerbates the outcome and complicates treatment. Moreover, only a subset of people experiencing a trauma will develop PTSD, underlining the importance of individual variation. Animal models for understanding the neurobiology of PTSD are expected to unravel the cellular and molecular mechanisms associated with this disorder and, accordingly, reveal novel targets for drug development. While the last two decades have seen a rapid progress in the development of non-invasive technologies to study human brain structure and function, there remain significant limitations in the ability to investigate details of the physiology and molecular biology of the human brain. The anatomical and physiological similarities between humans and other mammals, in particular rodents, have prompted researchers to investigate a large range of neurophysiological mechanisms and assess novel therapies in animal models before applying their discoveries to humans. Translational animal models for PTSD should encompass crucial features, including persistence of PTSD-like phenotypes, trauma susceptibility/resilience and predictive validity. Here we propose a novel arousal-based individual screening (AIS) model of PTSD that recapitulates all these features. The AIS model was designed by coupling the traumatization (24h restraint) of C57BL/6J mice with a behaviorally based individual screening consisting of z-normalization of post-trauma changes in startle reactivity, which is a measure of arousal depending on neural circuits present in all mammals. Through the AIS model, we identified susceptible mice showing long-lasting hyper-arousal (up to 56 days post-trauma), and resilient mice showing normal arousal. Susceptible mice further showed persistent PTSD-like phenotypes including exaggerated fear reactivity and avoidance of trauma-related cue (up to 75 days post-trauma), increased avoidance like behavior and social/cognitive impairment. Conversely, resilient mice adopted active coping strategies, behaving like control mice. The understanding the neurobiology of PTSD involves the insight of the mechanisms of susceptibility and resilience. A genetic background is not sufficient

to properly explain individual differences in sensitivity to stress and trauma, since individual biological diversities are also found within genetically homogeneous populations, such as inbred mice. Moreover, life experiences could interact with the genetic background producing long lasting alterations in coping abilities later in life. For this purpose, we investigated the transcriptional expression of specific PTSD-related genes and the relative targeting miRNAs in different brain areas (mPFC, HP, HT). Our bioinformatic analysis suggested four potentially targeting miRNAs (miR-15a-5p, miR-497a-5p, miR-511-5p, let-7d-5p) for two key genes: FKBP5 and BDNF. The expression analysis of mRNA targets and relative miRNAs showed alterations with a brain area specificity. These alterations were associated to susceptible and resilient phenotypes of traumatized mice, and linearly correlated with behavioral scores indicating the arousal levels, the avoidance of social interactions and the PTSD-like scores of animals. These data taken together contribute to the understanding of non-coding RNA role in AIS model and may help to identify novel biomarkers for resilience or vulnerability to stress which, in turn, could provide valuable information for the prevention and treatment of stress-related psychiatric disorders.

1. Introductory remarks

1.1. Introduction

How much can be stress *"the salt of life"*? Hans Selye, the pioneer of stress science in the 1930s, believed in good stress for which he coined the word "eustress" (Fig.1). He saw stress as "the salt of life". Although stress is a natural condition, today we know well that stress is a 2-sided medal. Stress can be bad, especially if you react to it exceeding the counterbalancing capacity of the body, acting anxiety, anger or depression. To date, the relationship between stress and disease is well established, but not always recognized (Musazzi, Tornese et al. 2017). Stress is difficult to define because it is so different for each of us (Tan and Yip 2018). However, stress can be broadly defined as a disruption of homeostasis, to which the organism responds by trying to reestablish the initial equilibrium or adopting an altered state in the new environment (Mannironi, Camon et al. 2013).

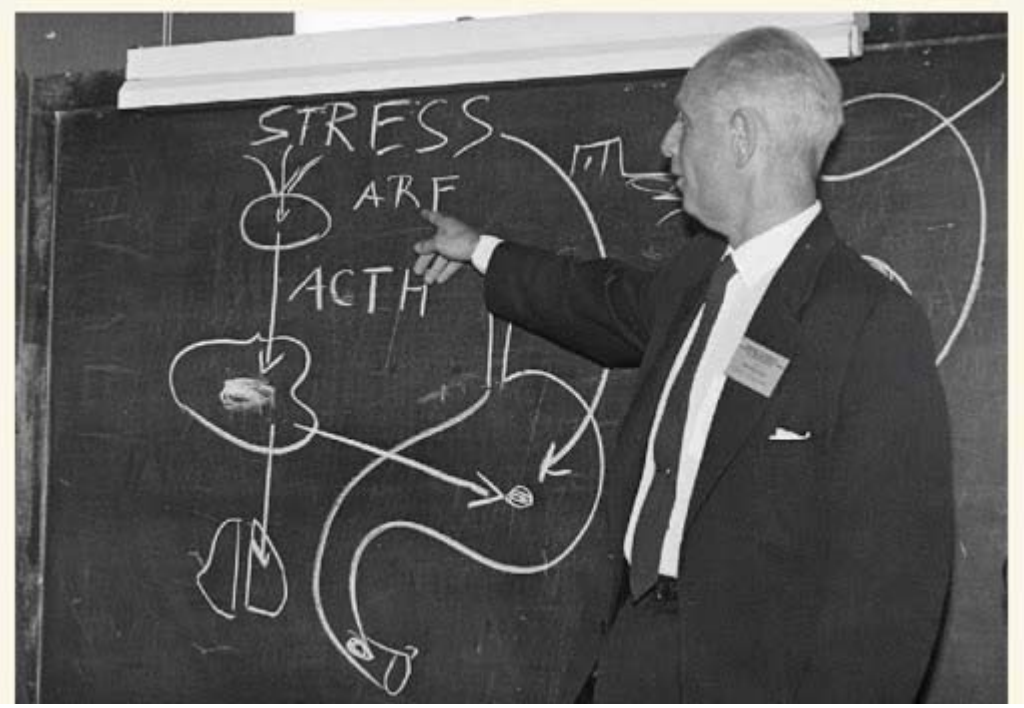

Figure 1. Hans Selye

Following its course through time, Selye's concept of biological stress evolved culminating in the modern concept of stress. Briefly, in the 1950's Geoffrey Harris observed that stress-induced adrenocorticotropic hormone (ACTH) secretion involves "neural control via the hypothalamus and the hypophyseal portal vessels of the pituitary stalk" (Harris 1950). Subsequently, another support came from Guillemin, a former Selye's Ph. D student, and Schally's group, who independently demonstrated the existence of hypothalamic factor(s) that triggered ACTH release from the rat pituitary (Guillemin and Rosenberg 1955, Saffran, Schally et al. 1955). In the 1970's, the intense studies of Guillemin and Schally, which obtained the Nobel Price, led to the identification of other hypothalamic releasing factors composed of 3–10 amino acid (a.a.) including thyrotropin-releasing hormone (TRH) and luteinizing hormone-releasing hormone (LH-RH) (Guillemin 2005). However, in 1981 Vale and his group contributed to major milestones with the identification of the 41-a.a. peptide, CRF, characterized from ovine hypothalami, and subsequently the cloning of CRF receptors and the development of specific CRF receptor antagonists (Vale, Spiess et al. 1981). Since then, different scientific groups explored the mechanism of hypothalamus-pituitary-adrenal axis noticing different morphological changes during biological stress compared to pathological stress.

1.2. From stress to disease: post-traumatic stress disorder

"It took years to get over it. Years! Long after, when you were working, married, had kids, you'd be lying in bed and you'd see it all before you. Couldn't sleep. Couldn't lie still. Many and many's the time I've got up and tramped the streets till it came daylight. Walking, walking – anything to get away from your thoughts . . . That went on for years, that did" (Fred White of the 10th Battalion King's Royal Rifle Corps) (Arthur 2009).

Descriptions like this of human responses to disasters, accidents and wars can be found in many historical documents, although described under different names, such as, "railway spine", "gas hysteria", "battle fatigue", "old sergeant syndrome" or "traumatic war neurosis " (Trimble). Understanding the history of the "post-traumatic stress" concept is essential for anyone working in the field. The fact that the diagnostic criteria have taken over 100 years to evolve in further revisions in the Diagnostic and Statistical Manual, represents a strong evidence of the active research and interest in this area. The term "post-traumatic stress disorder" (PTSD) came into use in the 1970s in large part due to the diagnoses of U.S. military veterans of the Vietnam War (Gersons and Carlier 1992), but was officially recognized by the American Psychiatric Association (APA) in 1980 in the third edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-III) (Scott 1990). To date, trauma and stress-related disorders, including PTSD (Table 1) ((2013) DSM-5 Task Force), are among the most prevalent and debilitating neuropsychiatric disorders in the world (Maren and Holmes 2016). The total disease burden (disability plus mortality) that is attributable to PTSD is extremely high (Kessler 2000), including suicide (Giesinger, Li et al. 2020). PTSD is a psychiatric condition that can develop following exposure to traumatic events such as interpersonal violence, combat, life-threatening accidents or natural disasters. PTSD is often induced by a single (acute) traumatic experience (Musazzi, Tornese et al. 2018). Symptoms of PTSD include: distressing/intrusive memories and nightmares of the trauma, dissociative "flashback", avoidance of trauma reminders, negative changes in mood and cognition, emotional withdrawal, irritability, hypervigilance, difficulty in sleeping and poor concentration ((2013) DSM-5 Task Force). These symptoms are relatively common in the immediate aftermath of a traumatic experience, but for most people, they will fade away within few weeks. Symptoms are not considered PTSD unless one month after the trauma (Bryant 2018). Moreover, the DSM-5 introduced an additional subtype of PTSD for children aged 6 years and younger, with a cluster of symptoms adapted to those young ages (McKinnon, Scheeringa et al. 2019). PTSD is associated with considerable

comorbidities, including heightened risk of dementia, substance abuse, mood and anxiety disorders, impulsive and dangerous behavior, chronic pain, inflammation and cardio-metabolic syndrome (Michopoulos, Rothbaum et al. 2015, Rosenbaum, Stubbs et al. 2015, Simmons and Suarez 2016). Sex differences in PTSD are well established, with the prevalence in women being approximately twice that found in men (Gill, Szanton et al. 2005). Men and women differ in the types of trauma most frequently encountered, i.e. sexual abuse is more frequent in women, while fights, accidents, and threats involving a weapon (and combat) are more frequent in men (Christiansen and Berke 2020). Moreover, across different populations and countries, differences in PTSD prevalence can be attributed to specific geographically distributions of trauma type and severity. Other factors, such as, the loss of personal, social and material resources and differences in culture are predictors of the development and the persistence of PTSD (Burri and Maercker 2014, Atwoli, Stein et al. 2015). Relevant component to stress response and PTSD development, have been identified at molecular level (Pitman, Rasmusson et al. 2012). Genetic and epigenetic effects are involved in the development of PTSD (Cornelis, Nugent et al. 2010, Dickson, Paulus et al. 2018). Genetic influences are estimated to be responsible for 30% of the variance in PTSD risk (True, Rice et al. 1993). In addition, studies have shown that adults with histories of childhood trauma have increased rates of PTSD (Nemeroff, Bremner et al. 2006). However, estimates suggest that of all individuals that will be exposed to a significant traumatic event in their lifetime, only the 5-10% of the general population will suffer from PTSD (Banerjee, Morrison et al. 2017)**.** This suggests that individual vulnerability and resilience are key factors to consider in this pathology. The resilience displayed by the majority of the population after traumatic events, suggests the existence of biological systems involved in adapting to severe stress (Dunlop and Wong 2019).

Table 1. Current diagnostic criteria for PTSD according to the Diagnosis and Statistical Manual of Mental Disorders 5th (DMS-5) and highlights areas of change from DMS-IV

Criterion*	Description	Specific examples	Requirements	Compared with DSM-IV
Criterion A	Exposure to stressor	• Direct exposure • Witnessing trauma • Learning of a trauma • Repeat or extreme indirect exposure to aversive details	DSM-5 recognizes that exposure to trauma can occur either by direct or indirect confrontation with extreme trauma	Specific definition of details of the stressor needed, including repeated experience or extreme exposure to details of events
Criterion B	Intrusion symptoms	• Recurrent memories • Traumatic nightmares • Dissociative reactions (flashbacks) • Psychological distress at traumatic reminders • Marked physiological reactivity to reminders	At least one of these five examples is required	No change, but further clarification of the dissociative quality of flashbacks needed
Criterion C	Persistent avoidance	• Trauma-related thoughts or feelings • Trauma-related external reminders such as people, places or activities	At least one of these two examples is required	DSM-IV did not separate the avoidance criterion
Criterion D	Negative alterations in cognitions and mood	• Dissociative amnesia • Persistent negative beliefs and expectations • Persistent distorted blame of self or others for causing trauma • Negative trauma-related emotions: fear, horror, guilt, shame and anger • Diminished interest in activities • Detachment or estrangement from others • Inability to experience positive emotions	At least two of these seven examples are required	DSM-IV noted social estrangement and restricted the range of affect; numbing redefined to positive rather than all affects
Criterion E	Alterations in arousal and reactivity	• Irritable and aggressive behaviour • Self-destructive and reckless behaviour • Hypervigilance • Exaggerated startle • Problems concentrating • Sleep disturbance	At least two of these six examples are required	Self-destructive and risk-taking behaviours were not defined in DSM-IV
Criterion F	Duration	Must experience criteria B, C, D and F for >1 month	Acute stress disorder is diagnosed for symptoms occurring for <1 month post trauma	No change
Criterion G	Functional significance	Impairment in social, occupational or other domains	Disability in at least one of these domains is required	No change
Criterion H	Exclusion	Not attributable to medication, substance use or other illness	Symptoms must not be secondary to other causes	Not stated in DSM -IV
Subtypes	• Dissociative subtype: used when depersonalization and derealization occur in tandem with other symptoms described above. • Delayed subtype: used to describe the emergence of symptoms following a period post trauma in which symptoms were not present or were present at a subthreshold level.			

1.3. HPA (hypothalamic–pituitary–adrenal) axis: the director of a big orchestra

The central biological pathway involved in the response to stress is the hypothalamic-pituitary-adrenal (HPA) axis (Fig.1), but also the brain noradrenergic system is able to control autonomic output (Young, Abelson et al. 2005). In 1930, Walter B. Cannon put forward the "fight or flight" model, which described the body response towards stress (Jacobs 2001). Around the 1950s, Selye's general adaptation syndrome was

introduced: chronic stress could induce a nonspecific response in the body, such as increase heart rate and blood pressure (Selye 1950). Subsequently, researchers illustrated association between the alterations in the HPA axis and the stress-related process. Thus, HPA axis, the primary hormonal mediator of stress responses, has been the first target for exploring the pathophysiology of PTSD (Dunlop and Wong 2019). Several brain regions modulate HPA axis activity. More specifically, the hippocampus (HIP) and prefrontal cortex (PFC) have an inhibitory effect on HPA axis, whereas the amygdala (Amy) and aminergic brain stem neurons stimulate it. The HPA axis consists of three hormonal cascade feedback cycles, that determine the level of circulating cortisol in the body. In processing experiences of stress, norepinephrine and indirect limbic inputs from HIP, mPFC and Amy act on neurons in the paraventricular nucleus (PVN) of the hypothalamus (HT) that contain corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) (Herman and Tasker 2016). Both peptides are stored in the medial eminence of HT. The activation of the HPA axis begins with the release of CRH and AVP, which travel across the hypophysial portal vessels in the infundibular stalk to the anterior pituitary, where they bind their own receptors (CRF1, V1) leading the release of adrenocorticotropin (ACTH) into the systemic circulation. CRH is the main activator of ACTH release, but its effects are amplified by AVP. Both ACTH and AVP are co-expressed and co-secreted from hypothalamic CRH neurons after stress. ACTH travels via the blood stream to the adrenals and, binding to melanocortin 2 receptors (MC2R) (zona fasciculata of the adrenal cortex), stimulates the release of cortisol in humans and corticosterone in animals, the primary effector molecule of the HPA axis. Cortisol induces a variety of effects throughout the body to support the stress response, such as inhibition of insulin and the enhancement of glucose availability, and regulation of the immunity system activity (Myers, McKlveen et al. 2014). A crucial feature of the HPA axis is the negative feedback signal elicited by cortisol. In healthy subjects, cortisol activity is time limited via its binding to glucocorticoid receptors (GRs) in the pituitary gland and HT, and act to reduce both CRH and ACTH release. Thus, cortisol is the primary molecule enabling stress response, as well as, the primary inhibitor of ongoing HPA axis activity in human (Hellhammer, Wust et al. 2009).

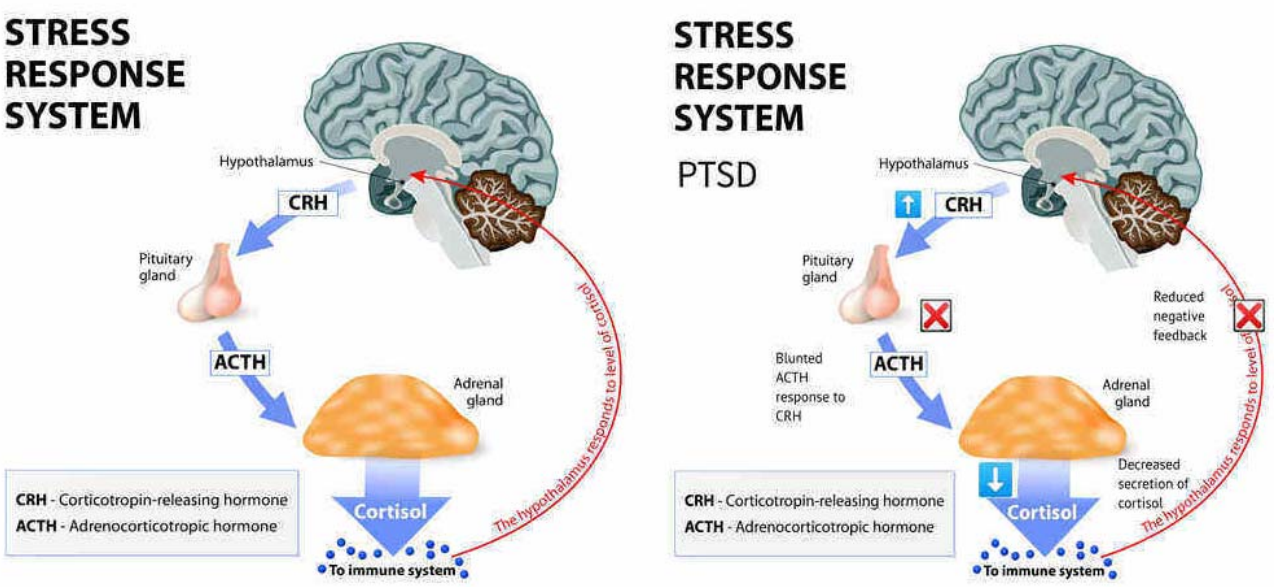

Figure 2. Schematic overview of the hypothalamic-pituitary-adrenal axis. In normal condition stress releases CRH from the hypothalamus which in turn releases ACTH from the anterior pituitary. ACTH stimulates release of cortisol from the adrenal cortex. Cortisol exerts a negative feedback control of the HPA axis. In PTSD there is dysregulation of glucocorticoid signaling with sensitised negative feedback of the HPA axis resulting in increased CRH and blunted ACTH responses to CRH which results in reduced cortisol secretion.

In PTSD, there exists a dysregulation of glucocorticoid signaling underlying of heightened negative feedback sensitivity of the HPA. As a consequence, low cortisol levels and blunted ACTH responses to CRH, due to elevated levels of CRH, result in the down-regulation of CRH receptors on the anterior pituitary (Yehuda 2006). Therefore, hypocortisolism might be a risk factor for maladaptive stress responses which in turn could be involved in the development of PTSD. This hypothesis is supported by the finding that exogenous administration of hydrocortisone, shortly after exposure to psychological trauma, can prevent PTSD (Delahanty, Gabert-Quillen et al. 2013). In addition, it has been shown that simulation of a normal circadian cortisol rhythm using hydrocortisone is an effective treatment in PTSD (Speer, Semple et al. 2019). Sensitivity to negative feedback induced by cortisol has been associated with a high availability of GRs on pituitary cells, leading a decrease in cortisol production (Yehuda 2006). Studies in PTSD patients showed a high density of GRs in their leukocytes, significantly correlated with PTSD symptoms (Yehuda, Lowy et al. 1991). We cannot

also exclude that altered pituitary and/or adrenal function, as well as alterations in vasopressin release could play a role in the pathophysiology of PTSD (de Kloet, Vermetten et al. 2006).

1.4. Genetic players in Post -Traumatic Stress Disorder

Epigenetic, molecular and endocrine studies confirmed distinct sets of HPA axis alterations that reflect exaggerated negative feedback sensitivity in PTSD. There is some evidence for the role of HPA axis-related genes, including: FKBP5 (encoding FK506-binding protein 5) (Leistner and Menke 2018), BDNF (brain derived neurotrophic factor) (Jeanneteau, Lambert et al. 2012), SGK1 (serum/glucocorticoid regulated kinase 1) (Licznerski, Duric et al. 2015), NR3C1 (nuclear receptor subfamily 3 group C member 1) (Vukojevic, Kolassa et al. 2014). Notably, altered expression of other genes, such as catechol-O-methyltransferase gene (COMT) (Winkler, Yue et al. 2017), serotonin transporter gene (SLC6A4) (Koenen, Uddin et al. 2011), neuropeptide Y (NPY) (Schmeltzer, Herman et al. 2016), dopamine receptor D2 gene (DRD2) (Comings, Muhleman et al. 1996), dopamine transporter (DAT) (Maul, Giegling et al. 2020), γ-aminobutyric acid A receptor α2 (GABRA2) (Nelson, Agrawal et al. 2009), cannabinoid receptor gene (CNR1) (Lu, Ogdie et al. 2008), pituitary adenylate cyclase-activating polypeptide (PACAP) (Ressler, Mercer et al. 2011), corticotropin-releasing hormone receptor 1 gene (CRHR1) (White, Acierno et al. 2013), has been associated with PTSD-like phenotypes.

1.4.1. FKBP5 gene

FKBP5 gene is located on the short arm of chromosome 6 (6p21.31) and consists of 13 exons (Zannas, Wiechmann et al. 2016). Immunophilin FKBP5 is a component of a multiprotein complex that retains the cytoplasmic form of GR in the cytoplasm (Denny, Prapapanich et al. 2005). This complex includes one GR molecule, a dimer of a HSP90 heat shock protein, and several other molecular chaperones and co-chaperones, (HSP70, DnaJ/HSP40, p23, Hop, FKBP5, etc.), that keep GR in the hormone binding conformation and protect it from proteolytic digestion (Cheung and Smith 2000, Pratt, Galigniana et al. 2004). When GR binds the

glucocorticoid hormone, FKBP5 in the complex is rapidly replaced by another immunophilin FKBP4. Subsequently the complex is translocated into the nucleus (Davies, Ning et al. 2002) due to direct interaction between FKBP4 and the motor protein dynein that can move along the microtubules toward the nucleus (Harrell, Murphy et al. 2004). It has been showed that expression of the FKBP5 gene in humans and animals is induced by GCs in all brain regions. (Scharf, Liebl et al. 2011). The human FKBP5 gene contains numerous sites for GR binding that are located in introns 2, 5 and 7 (Hubler and Scammell 2004). Binding of the hormone activated GR to these sites initiates an intracellular ultrashort feedback loop. More specifically, when GC induces activation of the FKBP5 gene transcription, a subsequently increasing in the FKBP5 protein at cytoplasmatic level, inhibits GR translocation to the nucleus, reducing the effect of GCs on the expression of GR target genes. Alterations in FKBP5, disrupt the negative feedback loop inducing "glucocorticoid resistance" (Merkulov, Merkulova et al. 2017). It has been studied in patients that single nucleotide polymorphisms (SNPs) in FKBP5 result in enhanced glucocorticoid receptor sensitivity, which leads to low basal cortisol levels and increased risk for PTSD (Binder, Bradley et al. 2008, Banerjee, Morrison et al. 2017, Leistner and Menke 2018). A growing body of evidence showed that FKBP5 dysregulation may contribute to a maladaptive stress response (Hartmann, Wagner et al. 2012). In this regard, studies showed that FKBP5 mRNA expression is strongly upregulated in the Amy following acute stress (Scharf, Liebl et al. 2011), and that FKBP5 knock-out mice are less affected by chronic social defeat stress (Hartmann, Wagner et al. 2012). In another study, BLA microinjections of SAFit2, FKBP5 antagonist, reduced anxiety-related behavior in wild-type mice (Hartmann, Wagner et al. 2015). Therefore, FKBP51 represents a promising therapeutic target for stress-related psychiatric disorders, but other studies are needed to elucidate its mechanism of action.

1.4.2. BDNF gene

BDNF is an important neurotrophic factor that enhances long-term potentiation and other forms of synaptic plasticity in the HIP and it is involved in the fear learning processes (Andero and Ressler 2012, Leal, Bramham et al. 2017). BDNF directly regulates the HPA axis and it is also an important modulator of CRH expression. More specifically, BDNF is able to induce expression of CRH in the paraventricular nucleus (PVN) by binding

to hypothalamic tropomyosin receptor kinase B (TrkB) receptors. TrkB activation in turn induces expression of cAMP response element-binding protein (CREB), which binds to the CRH promoter region and acts as a transcriptional activator (Jeanneteau, Lambert et al. 2012). This cross-talk between BDNF and CRH may be at least mediated by CREB and MAPK pathways and involved in the enhancement of fear memory under stress (Jeanneteau, Lambert et al. 2012). Some discrepancies have also been observed in clinical trials. For instance, Angelucci et al.; described low BDNF serum levels in subjects with PTSD diagnosis (Angelucci, Ricci et al. 2014); by contrast Martinotti et al.; reported high BDNF serum levels in PTSD patients (Martinotti, Sepede et al. 2015). The reason for these discrepancies is unclear, but it could be due to the complexity of BDNF gene structure. BDNF gene consists of at least eight 5' non-coding exons (i.e. from exon I to VIII) and one 3' exon (i.e. exon IX) encoding the mature BDNF protein (Timmusk, Palm et al. 1993). In the brain, different promoters of the BDNF gene and alternative splicing, control tissue-specific expression of BDNF mRNA isoforms, which could have different functions (Tsankova, Kumar et al. 2004). The complexity in the control of tissue-specific BDNF gene expression has been proven by several studies on DNA methylation (Takei, Morinobu et al. 2011). Furthermore, a polymorphism in the human BDNF gene (i.e., Val66Met) has also been observed among PTSD patients (Hori, Itoh et al. 2020). Overall, it seems that alterations of BDNF induced by stress exposure could be regulated by epigenetic mechanisms, which are dependent of brain regions, of gender, as well as of kind of stress (Miao, Wang et al. 2020).

1.4.3. NR3C1 gene

NR3C1 represents one of the most studied genes in the HPA axis and encodes the GRs. The binding of glucocorticoids to the GRs plays an important role in glucose homeostasis (Majer-Lobodzinska and Adamiec-Mroczek 2017) and regulates the stress response through both genetic (Derijk, van Leeuwen et al. 2008) and epigenetic mechanisms (Tyrka, Ridout et al. 2016). Several studies have shown that epigenetic modifications of the NR3C1 gene are associated with trauma (McGowan, Sasaki et al. 2009, van der Knaap, Riese et al. 2014, Palma-Gudiel, Cordova-Palomera et al. 2015). Evidence indicates that glucocorticoids can have effects on emotional processing. Thus, altered glucocorticoid signaling may have differential effects on the

development and symptoms of PTSD, depending on the emotional and cognitive processes affected. It has been suggested that the presence of elevated levels of glucocorticoids at the time of acute stress confers protection against the delayed enhancing effect of stress on amygdala synaptic connectivity and anxiety-like behavior (Rao, Anilkumar et al. 2012). However, gene expression analysis of NR3C1 in PTSD patients has been found heterogeneous (Gola, Engler et al. 2014, Logue, Smith et al. 2015), because of the genetic background of the analyzed population, the type of trauma, and the diversity of methodology employed (Dunlop and Wong 2019).

1.4.4. SGK1 gene

SGK1 is a protein involved in cellular stress response with a relevant role in neuronal activity, proliferation and apoptosis (Lang, Strutz-Seebohm et al. 2010), and a determinant key of susceptibility to mental illness (Han, Zhang et al. 2019). It has been shown that hippocampal SGK1 expression is altered by fear stimulation (Lang, Bohmer et al. 2006) and involved in long-term memory formation (Ma, Tsai et al. 2006). SGK1 is not only a downstream target of GR signaling, but also exerts a positive feedback on GR activation by regulating the long-lasting effects of glucocorticoids, by increasing the phosphorylation and nuclear translocation of GRs (Anacker, Cattaneo et al. 2013). Following the exposure to physiological stressors, the SGK1 gene is rapidly transcribed through the activation of GR and MR, and its protein undergoes constitutive phosphorylation via endogenous Phosphoinositide 3-Kinase (PI3-K) activity (Bhargava, Fullerton et al. 2001). In mouse PFC acute stress upregulates SGK1 that, in turn, increases the trafficking and function of NMDARs (N-methyl-D-aspartate receptors) and AMPARs (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors). This leads to a potentiation of synaptic transmission, facilitating cognitive processes mediated by PFC activation (Yuen, Liu et al. 2011). Anacker et al. showed that SGK1 mRNA was correlated positively with the mRNA levels of FKBP5 and negatively with BDNF transcripts (Anacker, Cattaneo et al. 2013). Since BDNF influences neuronal survival, mood, and long-lasting memory formation and intensifies synaptic plasticity with neuro-regenerative effects, SGK1 may contribute to several BDNF-dependent neuropsychiatric disorders (Lang, Strutz-Seebohm et al. 2010, Han, Zhang et al. 2019). However, the molecular mechanisms by which

SGK1 could be a convergence point between glucocorticoids and BDNF in neurogenesis are still not completely clear.

1.5. Stress effects on synaptic plasticity

Synaptic plasticity is a biological process that allows learning and memory through changes in the connections among synapses. Interactions with the external world induce synaptic activity that can lead to changes in the connections between specific neurons and neuronal networks (Citri and Malenka 2008). The first demonstration of a stress-induced suppression of LTP in the CA1 pyramidal cell region was reported in 1987 by Thompson, Levine and colleagues (Foy, Stanton et al. 1987). In particular, it has been shown that long-term potentiation (LTP) and long-term depression (LTD), are impaired in PTSD (Hayes, Vanelzakker et al. 2012, He, Wei et al. 2018). High levels of stress result in loss of synapses and changes in the neuroarchitecture (dendritic morphology, synaptic spines), affecting cognitive processes (Duman, Aghajanian et al. 2016). Stress compromises the integrity of signaling via glutamatergic synapses in several ways, including reduction of BDNF signaling and the reduction in the number of AMPA glutamate receptor. For this reason, PTSD might be considered a "synaptic disconnection syndrome" (Krystal, Abdallah et al. 2017). LTP has been shown in various brain structures and neural tissues (Sah, Westbrook et al. 2008, Cooke and Bear 2010); however, it occurs prominently in the HIP, the most important structure implicated in learning and memory (Eichenbaum 2000, Cobar, Yuan et al. 2017, Chen, Sun et al. 2018).

1.6. Anatomical regions of interest in the neurocircuitry of PTSD

Several studies indicate four brain regions which are considered to play an important role in the psychopathology of PTSD, including fear and learning, emotion regulation and executive function, contextual processing and threat detection. These brain regions include the Amy, the HIP, the HT and the anterior cingulate cortex (ACC). In particular, the Amy, an important structure for learning, is hyperresponsive in

PTSD, inducing an exaggerated fear response (Liberzon, Taylor et al. 1999, Tottenham and Gabard-Durnam 2017). The central nucleus of the amygdala (CeA) is the "output" of the fear-response system and triggers behavioral responses, such as, freezing and fear-potentiated startle and autonomic sympathetic nervous system responses. It has been observed that lesions of CeA prevent freezing to a tone exposure in the fear-conditioned test and increase active avoidance (Ressler 2010, Moscarello and LeDoux 2013). The lateral nucleus of the amygdala (LA) receives sensory information about conditioned stimuli from cortical and thalamic inputs. This information is processed through the basal amygdala (BA) and engages CeA projections to brainstem effector sites. CeA target regions, such as periaqueductal gray (PAG), driving freezing and other innate defensive reactions to learned threats (Choi, Cain et al. 2010, Lazaro-Munoz, LeDoux et al. 2010). Otherwise, active avoidance strategies appear to require signaling from the LA to the BA and then to the shell of the nucleus accumbens (NAc) (Ramirez, Moscarello et al. 2015). It has been suggested that "intrusive symptoms" represent a failure of the cortex to inhibit the limbic system (Lanius, Vermetten et al. 2010). In this regard, individuals with PTSD often showed decreased in mPFC activity when compared with individuals without PTSD (Bremner, Staib et al. 1999, Zubieta, Chinitz et al. 1999). More specifically, structures in the vmPFC, that normally inhibit the Amy, are hyporesponsive and underlie alterated fear extinction processes (Koenigs and Grafman 2009). In particular, prelimbic cortex (PL) in rodents (analogous to human dACC) is associated with fear expression; while, infralimbic cortex (IL) (analogous to human vmPFC) is associated with learning extinction (Kim, Kim et al. 2013, Do-Monte, Manzano-Nieves et al. 2015). In other words, PL acts as an "accelerator" during conditioning, while, the IL acts as "brakes" during extinction. In healthy individuals, rostral regions of the ACC (rACC) are activated during emotional states (Bishop, Duncan et al. 2004). In contrast, dorsal regions of the ACC (dACC) have traditionally been thought to be involved in multiple cognitive processes, as well as, performance monitoring, response selection, error detection and decision making (Mohanty, Engels et al. 2007). The HIP is a key structure in determining whether contextual cues are associated with danger or with safety. This region is crucial for memory and learning processes and, in particular, for declarative memory (Squire and Zola-Morgan 1991). Importantly, the HIP appears to interact with the Amy during the encoding of emotional memories, a process that is highly relevant in the study of PTSD (Shin, Rauch et al. 2006). Different studies have demonstrated a reduced hippocampal volume in individuals with PTSD (Pitman, Rasmusson et al. 2012). In rodent models, both acute and chronic

corticosterone secretions occur in response to traumatic experiences which can induce molecular changes in the HIP (Gould and Tanapat 1999, Segal, Richter-Levin et al. 2010, McEwen, Nasca et al. 2016). Several observations showed that the insula (or insular cortex) is functionally abnormal in PTSD and other anxiety disorders (Zhang, Xie et al. 2016). Furthermore, the re-experiencing symptoms in patients with PTSD have been associated with increased insula activity and decrease of rACC and inferior frontal cortex activity (Hopper, Frewen et al. 2007). The insula is thought to be involved in interoception and bodily awareness (Craig 2002). To date, despite huge efforts, a better understanding of the circuits involved in PTSD subjects is needed to identify other abnormalities in neuronal signaling.

1.7. Pharmacological treatments: the need for new targets

Even if PTSD is now recognized as a major health challenge, there are still only partially effective treatments for this disorder. Treatments for PTSD include drugs and psychotherapies, but a substantial proportion of patients exhibit symptoms resistant to treatment (Krystal, Rosenheck et al. 2011). In brief, to date there is no 'gold standard' pharmacological treatment in PTSD, and the available treatments have low efficacy (Ragen, Seidel et al. 2015). The only FDA (Food and Drug Administration) approved drugs for the treatment of PTSD are the selective serotonin reuptake inhibitors (SSRIs): sertraline and paroxetine. All other agents are used off-label, but are not FDA approved for PTSD treatment (Hoskins, Pearce et al. 2015, Saguil 2019). To date, the drugs in use are not different from those used more than 40 years ago (Hillhouse and Porter 2015), thus the advancements with regard to the PTSD will come likely only from the identification of novel pharmacological treatments (Feder, Parides et al. 2014, Mithoefer, Mithoefer et al. 2018). A critical factor contributing to this outcome is the poor understanding of the neural mechanisms underlying PTSD and the need to reproduce in animal models clinical evidence displayed by humans after trauma (Zhang, Hu et al. 2019). New animal models of trauma could be able to predict new treatment outcomes (Insel 2012).

1.8. The relevance of PTSD animal models: a big challenge

PTSD is not a well-defined disorder and also the nature of the trauma could be highly variable. The definition and diagnosis of PTSD in humans is based on behavioral symptoms and self-reports. Moreover, there is a large overlap with other disorders, including mood disorders, anxiety disorders, as well as, alcohol and drug abuse, which worse the outcome of the treatment (Brady, Killeen et al. 2000). Only a subset of people experiencing trauma will develop PTSD, underlining the importance of individual variation (Auxemery 2012). Most likely, the most important challenge is to understand the complexity of high brain functions, mostly for the ethical and practical difficulties to analyze living human brains. Even if the last two decades have seen a rapid progress in the development of non-invasive technologies to study human brain structure and function, significant limitations in the ability to investigate details of physiology and molecular biology of the human brain still remain (Nestler and Hyman 2010). The anatomical and physiological similarities between humans and animals, particularly rodents, have encouraged researchers to investigate a large range of neuronal mechanisms to assess new therapies in animal models with translational value (Barre-Sinoussi and Montagutelli 2015). However, the use of animals for scientific purposes is a frequent matter of debate in our society, even if the progress made through the use of animal models is unquestionable, and nearly 90% of researches done by Medicine Nobel Prize winners, used animal experiments in their discoveries (Liu, Wang et al. 2015). Animal models have been responsible for the most important knowledge advances in many scientific fields. Given the different approaches and the difficulties of validation, it is useful for the scientific community to share criteria to understand if a particular disease model is “good enough” to justify further investments. The main criteria to validate an animal model are based on: construct, face and predictive validity (Fig.2) (Belzung and Lemoine 2011).

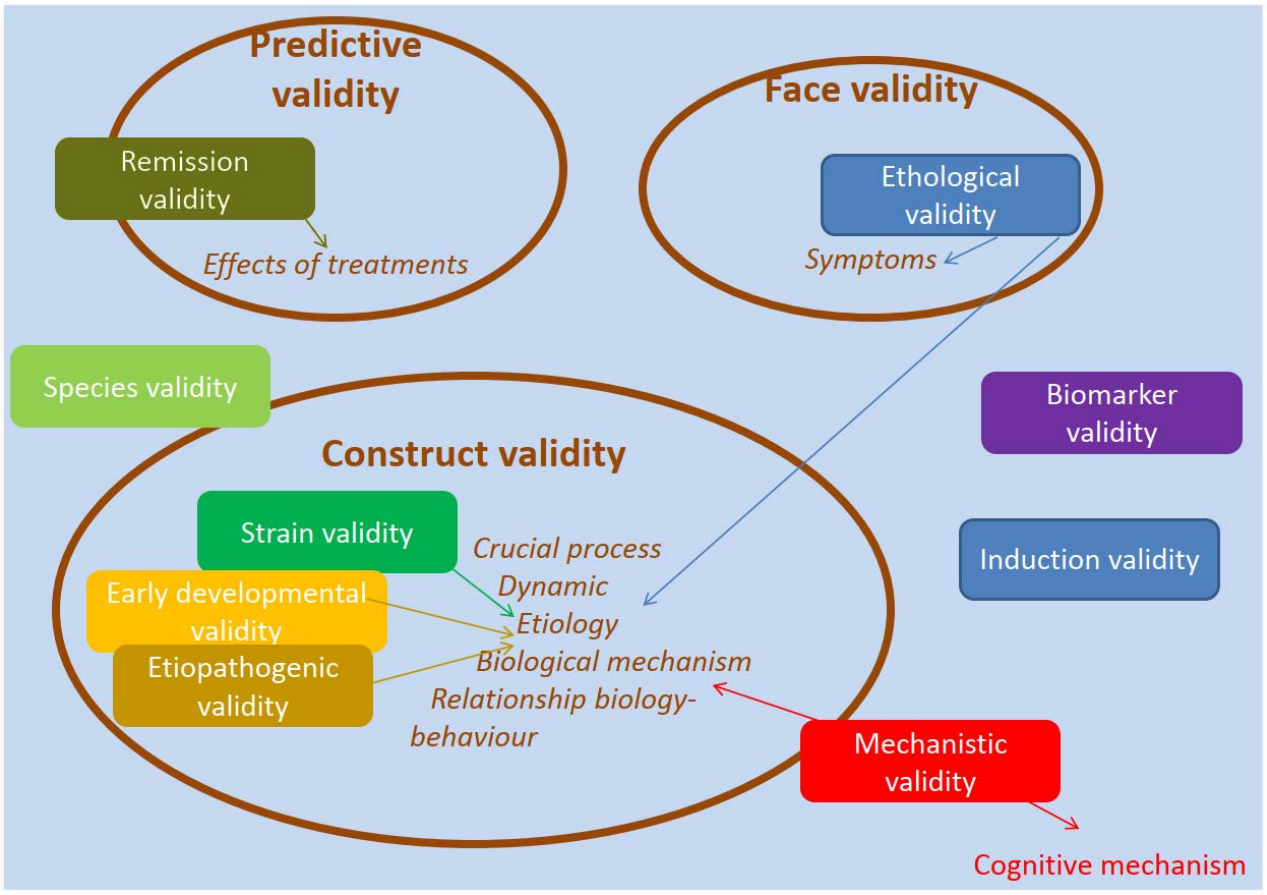

Figure 3. Criteria of validaty: all models are expected to display phenomenological resemblance, critical aspects of PTSD symptoms (face validity), causality of theoretical explanatory basis (construct validity) and a response to treatment similar to what is seen in humans (predictive validity).

Animal models for understanding the neurobiology of PTSD are expected to reveal cellular and molecular mechanisms associated with PTSD, which should serve to discover new targets for drug development. The neural circuitries involved in fear and anxiety, which are essential for the etiology of PTSD in humans, are highly conserved throughout evolution (Flandreau and Toth 2018). Furthermore, many symptoms can be modeled using behavioral tests (Belzung and Lemoine 2011). Essential requirements have been defined for models of PTSD with high translational value (Yehuda and Antelman 1993, Siegmund and Wotjak 2006):

- the trauma must be severe;

- a relatively short duration should be sufficient to provoke PTSD-like symptoms;

- the intensity of the trauma should predict the severity of outcome;

- the stressor should induce persistent or progressive PTSD-like alterations and significant interindividual variability in outcomes.

A key factor is that relevant symptoms are those that last more than 1 month (criterion F) ((2013) DSM-5 Task Force). PTSD is not defined by the immediate responses to the exposure to trauma. However, it is not clear if 1 month in human's life is translated exactly to 1 month in rodent's life, but clearly it is important to consider symptoms which last for a period of time after the exposure to trauma. Since PTSD originates in the brain, the most complex part of the human body, the different aspects found in PTSD are far from being easily recreated in animal models. Moreover, a model of PTSD based entirely on fear conditioning may be too narrow to explain the complexity of associated symptoms. Other PTSD symptoms listed in DSM-5 may not be consequences of dysregulated fear, such as, dysphoric arousal (difficulty concentrating, difficulty sleeping), anhedonia (detachment, loss of interest), and externalizing symptoms (anger, self-destructive, or reckless behavior) (Krystal, Abdallah et al. 2017). This is an important reason to look for a select group of symptoms, even if there are some of them, such as, intrusive thoughts, which are difficult or even impossible to measure (Richter-Levin, Stork et al. 2019) (Table2). Existing models apply stressors of physical, psychological, social, or combined nature. The most used are:

- physical stressor models (single-prolonged stress, restraint stress, foot shock, stress-enhanced fear learning, and underwater trauma);

- common social stressors (housing instability, social instability, early life stress, and social defeat);

- psychological models (predator scent stress).

Animal model for PTSD	DSM-5 criteria[1]
Single-prolonged stress	A, B, C, D, E, F, G, H
Restraint stress	A, B, C, D, E, F, G, H
Foot shock	A, B, C, E, F, G, H
Stress-enhanced fear learning	A, B, C, E, F, G, H
Underwater trauma	A, B, E, F, G, H
Predator-based psychosocial stress/predator scent stress	A, B, C, D, E, F, G, H
Housing instability	A, B, E, G, H
Social instability	A, B, E, F, G, H
Early life stress	A, B, C, D, E, F, G, H
Social defeat	A, B, C, E, F, G, H

[1]The listed criteria are: Presence of a stressor (A), intrusive symptoms (B), avoidance (C), negative changes in cognition and mood (D), changes in arousal and reactivity (E), persistence of symptoms (F), functional significance (G) and exclusion of other factors that may cause the displayed symptoms (H). PTSD: Posttraumatic stress disorder.

Table 2. A list of PTSD animal models and the separate criteria according to DMS-5 that each model has been reported to meet (according to PubMed literature search, individual references not listed) (Borghans and Homberg 2015)

1.8.1. Restraint Stress: a model of severe stress

Studies using this model showed increased HPA negative feedback similar to that observed in PTSD patients (Harvey, Brand et al. 2006, Maccari and Morley-Fletcher 2007). Both acute and chronic restraint stress, increase significantly behavioral anxiety and nociception (Gameiro, Gameiro et al. 2006, Whitaker, Gilpin et al. 2014). Restraint stress is performed by placing the rodents in an enclosed chamber allowing minimal or no movement. Total immobilization can be considered the most severe of the restraint methods. Two hours of complete immobilization has been shown to increase anxiety-like behavior in the elevated plus maze and open-field tests (Mitra, Jadhav et al. 2005, Andero, Brothers et al. 2013), reduce declarative memory performance in the water maze task, increase fear learning (Andero, Brothers et al. 2013), compulsive-like behavior and avoidance in the marble-burying test (Kedia and Chattarji 2014), inducing morphological changes in the brain (Andero, Brothers et al. 2013, Kedia and Chattarji 2014).

1.9. “What doesn’t kill you makes you stronger?”: a focus on Vulnerability and Resiliency

“Why do some individuals develop PTSD, while others exposed to the same trauma don’t?”

A crucial question is whether some components in the complex PTSD symptomology are pre-existent at the time of trauma and may be responsible for susceptibility of some individuals. The trauma is not a sufficient condition to induce PTSD. One of the most important aspects to consider in an animal model of PTSD, is that most people exposed to trauma do not actually develop PTSD. Understanding the neurobiology of PTSD involves the knowledge of susceptibility and resilience mechanisms. Individual behavioral traits could be indicative of an increased risk for future trauma-related pathologies (Richter-Levin, Stork et al. 2019). Only genetic background is not sufficient to explain individual differences in sensitivity to stress and trauma, since individual differences are also found within genetically homogeneous populations, such as, inbred mice (Hager, Jansen et al. 2014). Moreover, life experiences could interact with the genetic background producing long lasting alterations in coping abilities later in life (Dirven, Homberg et al. 2017). For this purpose, it is becoming increasingly evident the need to develop new animal models able to discriminate resilient traits from susceptible ones. Cohen et al. were one of the first groups to consider the approach to split into ‘maladaptive’ and ‘well-adaptive’ responses to trauma in rats (Cohen, Zohar et al. 2004). Embracing this approach, it was possible to demonstrate, as for humans (Bonanno and Mancini 2008), the prevalence rates of maladaptive responses to trauma (Cohen, Zohar et al. 2004). In addition, it is important to consider the difference between male and female rodent responses to stress and trauma (Cohen and Yehuda 2011), and the risk factors associated with the trauma, such as prepubertal, or juvenile pre-exposure to stress (Brydges, Wood et al. 2014). Behavioral profiling, which allows differentiating between susceptible and resilient mice both in male and female is a valuable tool that may help to identify pro-adaptive or maladaptive mechanisms.

1.10. Epigenetics

Epidemiological studies have reported associations between genetic and environmental factors, such as psychological or physiological stressors, stress-vulnerable phenotypes, behavioral adaptations to stress, as well as, synaptic plasticity, memory and cognitive processes (Kendler, Karkowski et al. 1999, Uchida, Hara et al. 2011) (Fig.4). In this regard, stress in utero or during early life has been shown that may led the brain to become more vulnerable to particular psychiatric disorders. However, also, stress in adolescence or later in adulthood may trigger the onset of such disorders (Boersma, Bale et al. 2014). Epigenetics has focused on how cellular traits can be inherited without a change in DNA sequence (Tsankova, Renthal et al. 2007). The epigenetic signal cascade begins with an "epigenator". Epigenator is a concept consisting of all signals, both environmental cues and intrinsic processes, usually transient, that end, with the recruitment of an "epigenetic initiator". The epigenetic initiator acts directly on chromatin and determines the site of epigenetic modification. An example of an epigenetic initiator is the REST protein (Berger, Kouzarides et al. 2009). The most described epigenetic mechanisms in the context of stress include histone modification (acetylation, methylation, phosphorylation, ubiquitination, and sumoylation), DNA methylation and post-transcriptional regulation by non-coding RNAs, such as microRNAs (miRNAs) (Tsankova, Renthal et al. 2007, Ivanova, Bozhilova et al. 2018). In particular, several studies have revealed that patients with psychiatric disorders showed abnormal miRNA expression profiles in the blood circulation and in relevant brain regions. Furthermore, animal studies have shown that manipulating the levels of particular miRNAs in the brain can affect the behavior (Issler and Chen 2015).

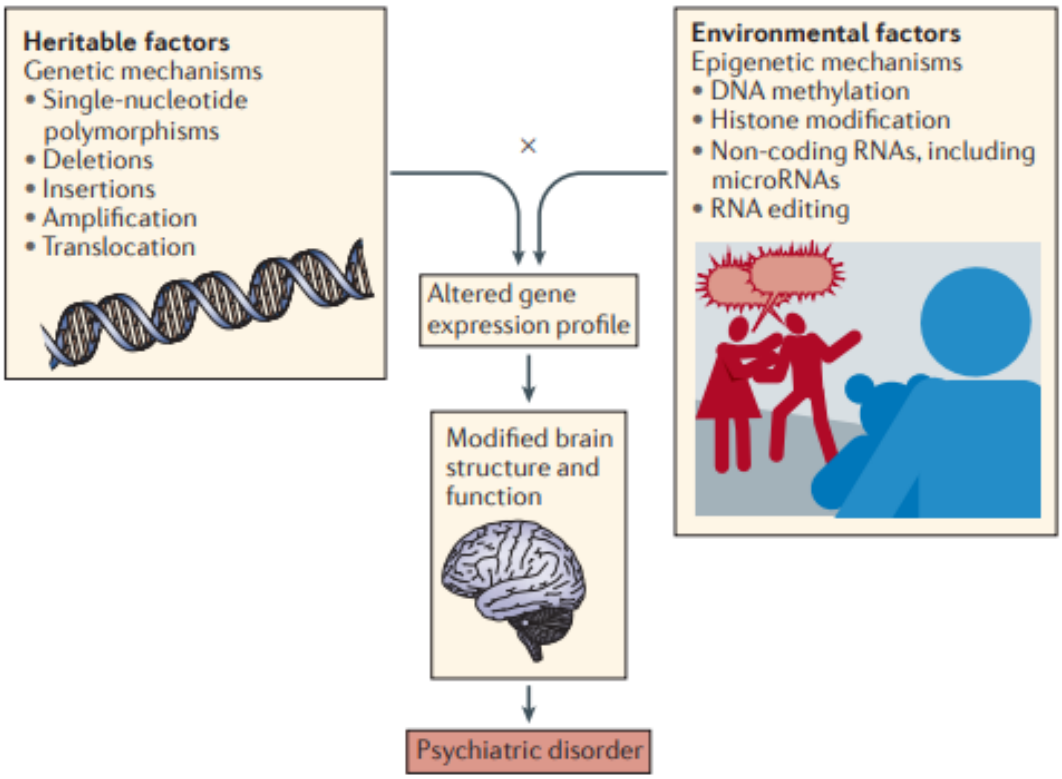

Figure 4. A combination of genetic factors and environmental factors may led to a predisposition for the development of psychiatric disorders

1.11. Small non-coding RNAs

Small non-coding RNAs, characterized by length equal or inferior to 200 nucleotides, have been widely studied for many years. By now, it is well known that they regulate many biological processes: i) RNA synthesis, processing and translation and transcriptional initiation [piRNAs, PASRs (promoter-associated small RNAs)]; ii) RNA maturation (snoRNAs); iii) RNA degradation or translation inhibition (miRNA, siRNA) (Ragusa, Barbagallo et al. 2015). Among all small non-coding RNAs, the most known and studied are definitely miRNAs, which biogenesis and function of post-transcriptional repressors have been well characterized.

1.11.1. MiRNA biogenesis

Originally thought of as “junk RNA”, miRNAs, has been revealed that may control the expression of up to 60% of the protein-coding genes in the human genome in a high complex network (Krol, Loedige et al. 2010). In 2001 the true potential of these small noncoding RNAs was revealed when their role as regulators of gene expression was demonstrated (Lau, Lim et al. 2001). MicroRNAs are a class of endogenous single-stranded RNAs consisting of approximately 18-25 nucleotides, expressed both in animals and in plants. MiRNA-coding genes are located at all chromosomes in humans, except for Y chromosome; frequently, genes coding for different miRNAs are located at adjacent loci on the same chromosome, forming clusters (e.g. let-7 cluster on chromosome 9, including hsa-let-7a-1, hsa-let-7f-1 e hsa-let-7d). Transcription of a cluster is simultaneous and generates a polycistronic primary transcript, successively processed into single miRNAs. MiRNAs belonging to the same cluster are often related to each other, suggesting that cluster derives from gene duplication; consequently, miRNAs from the same cluster are often functionally correlated, since function depends on sequence. Genomic localization of miRNA-coding genes is highly heterogeneous; indeed, they can be located at: i) intergenic regions, ii) protein-coding gene introns, iii) non-coding gene introns, iv) non-coding gene exons; moreover, some miRNA-coding genes have an independent promoter (Kim and Nam 2006). According to miRBase database (http://www.mirbase.org/), to date 1881 precursors and 2588 mature miRNAs have been identified, even if a recent study hypothesized a higher number of miRNA-coding genes in human genome (Londin, Loher et al. 2015). MiRNA biogenesis starts in the nucleus; transcription is prevalently performed by RNA polymerase II, rarely by RNA polymerase III; transcription produces a long (many kb) primary transcript called pri-miRNA, which folds into a double-stranded hairpin structure; the hairpin includes a loop and a double-stranded stem, made up of 33 complementary base pairs (bp), and ends with two long single-stranded traits (Cai, Hagedorn et al. 2004, Lee, Kim et al. 2004, Borchert, Lanier et al. 2006). Successively, pri-miRNA undergoes two sequential cleavages: the first cleavage occurs in the nucleus by Drosha, the second one in the cytoplasm by Dicer, two endonucleases including conserved RNAse III catalytic domains (RIIIDa, RRIIDb); these enzymes act in association with proteins containing double-stranded RNA-binding domains (dsRBDs) (Kim and Nam 2006). The first cleavage performed by a complex including Drosha and Pasha (DGCR8) converts the pri-miRNA into a 700-nucleotide long molecule with a hairpin structure called pre-miRNA, which

is exported to the cytoplasm by exportin-5 (Exp5), a GTP-dependent nuclear/cytoplasmic transporter (Lund, Guttinger et al. 2004). In the cytoplasm, pre-miRNA interacts with a big multiprotein complex called RISC loading complex (RLC), consisting of the endonuclease Dicer, TRBP (Tar RNA Binding Protein), which contains three dsRBDs, PACT (protein activator of PKR), and Argonaute protein Ago-2, an RNAse with catalytic function (Gregory, Chendrimada et al. 2005, Haase, Jaskiewicz et al. 2005, Lee, Hur et al. 2006, MacRae, Ma et al. 2008). Argonaute (Ago) proteins are expressed in all eukaryotes and contain specific domains: PAZ and MID domains to bind RNA targets at 3'-end, and PIWI domain al 5'-end to catalyse RNA cleavage (Filipowicz, Bhattacharyya et al. 2008). Dicer catalyses the cleavage of the hairpin loop, producing a 22-nucleotide long double-stranded miRNA characterized by mismatches and two protrusive nucleotides at both 3'-ends. This RNA duplex is released from RLC and splits into two strands, defined: i) guide strand, complementary to the targets, and therefore functional; ii) passenger strand (miRNA*), initially considered non-functional and degraded (Kim 2005) (Fig.5). Recent studies showed that passenger strand also is functional and accumulates in the cytoplasm (Ro, Park et al. 2007, Okamura, Phillips et al. 2008, Yang, Phillips et al. 2011): by now, it is known that every miRNA-coding gene originates two mature molecules, defined -5p and -3p depending on the duplex strand from which they derive.

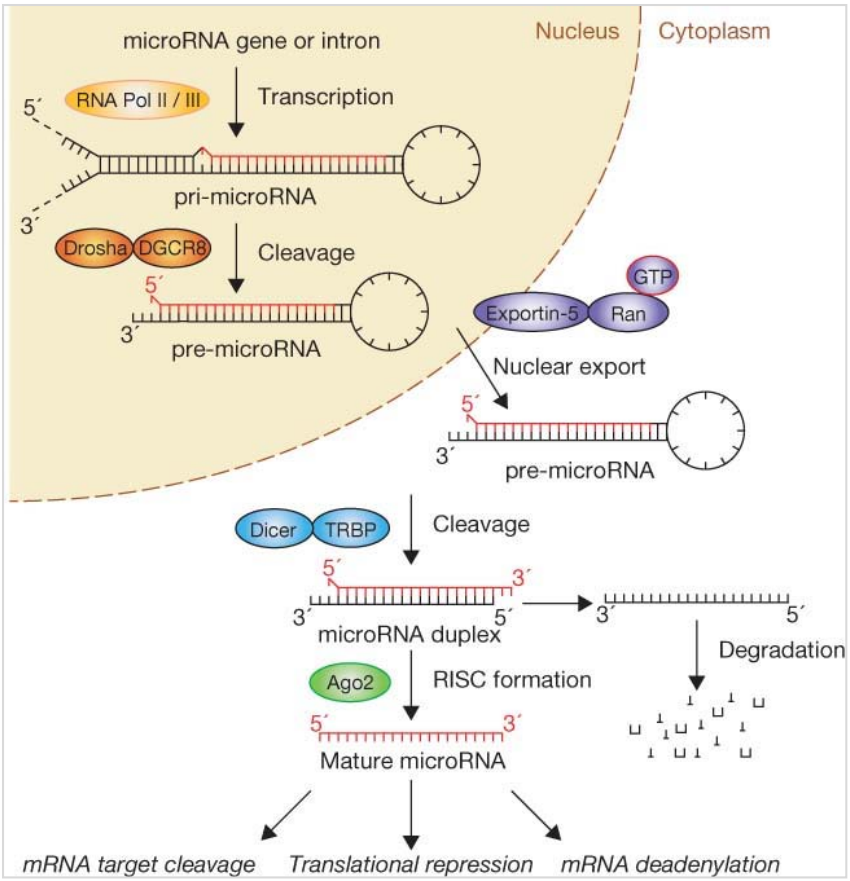

Figure 5. miRNA biogenesis starts in the nucleus with the transcription of a long pri-miRNA performed by RNA polymerase II or III; the pri-miRNA folds into a hairpin structure and undergoes an enymatic cleavage performed by Drosha, generating the pre-miRNA, characterizaed by a stem-loop structure. The pre-miRNA is exported to the cytoplasm;the hairpin structure undergoes a clevage performed by Dicer, generating a 22-nucleotide long double-stranded miRNA (Winter, Jung et al. 2009).

A particular miRNA subfamily includes genes located within protein-coding gene introns, which code for miRNA called mirtrons. Mirtron biogenesis is Drosha-independent: after splicing, they are processed into a hairpin RNA, which is exported to the cytoplasm by exportin-5 and directly cleaved by Dicer (Ruby, Jan et al. 2007) (Fig.6).

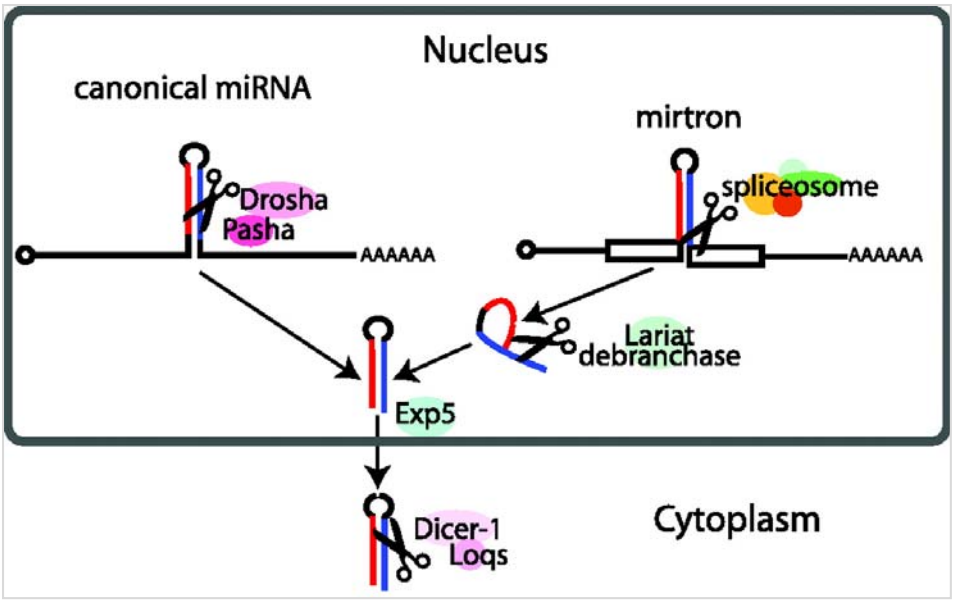

Figure 6. Mirtron biogenesis in Drosha-indipendent: after removal, the intron lariat is exported to the cytoplasm by exportin-5 and cleved by Dicer (Ruby, Jan et al. 2007).

1.11.2. MiRNA functions

All miRNAs perform the same function of post-transcriptional repressors of gene expression through the same molecular mechanism. Mature miRNAs bind Ago-2 protein and are led to the RNA-induced silencing complex (RISC), the true effector of miRNA-mediated silencing; RISC consists of several proteins, including Ago. Once associated to RISC, mature miRNA becomes active and recognizes its mRNA target: in animals, interaction occurs between the 3'-UTR of mRNAs, where many miRNA-binding sites can be located, and the seed region of miRNAs, included between the second and the eighth nucleotide of the 5'-end of mature miRNAs. MiRNA-mRNA complementarity determines mRNA destiny: i) a perfect match causes the degradation of the mRNA, which is first deprived of its poly(A) tail; ii) a partial complementarity induces the inhibition of mRNA translation (Carthew and Sontheimer 2009) (Fig.7).

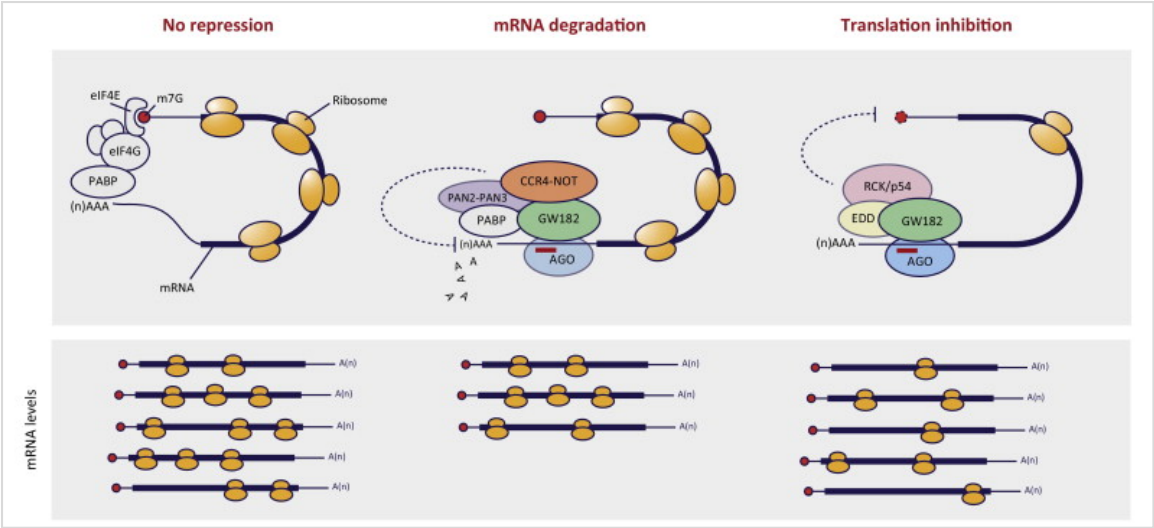

Figure 7. miRNA-mediated post-transcriptional silencing occurs throught mRNA degradation or through inhibition of mRNA translation (Vidigal and Ventura 2015).

MiRNA-mediated mRNA degradation mechanism is still not clear; it seems that RISC recruits on mRNA target the effectors of deadenylation and degradation (exonucleases). This process starts in the cytoplasm and continues in specialized organelles called P-bodies (processing bodies), subcellular structures representing transcript storage and decay areas (Nilsen 2007, Filipowicz, Bhattacharyya et al. 2008). Translation inhibition seems to be due to RISC-included Ago proteins, which compete with eukaryotic translation initiation factors (eIFs) to bind mRNA 5'-cap: during the first stages of protein synthesis, eIF factors bind the 5'-cap, while PABPC1 [poly(A) binding protein cytoplasmic 1] binds poly(A) tail; eIFs-PABPC1 interaction induces the approach of the two ends of mRNA, promoting its recognition by ribosomes. When mRNA is recognized by miRNA loaded on RISC, Ago proteins bind its 5'-cap and prevent translation start (Filipowicz, Bhattacharyya et al. 2008). It has been demonstrated that every miRNA can bind several mRNA targets, and, similarly, a single mRNA can be targeted by many miRNAs (Kim and Nam 2006); therefore, miRNAs can be considered small effectors of wider regulatory pathways, controlling fundamental processes, such as cell cycle, differentiation, apoptosis, and also more complex processes regarding the entire organism, such as embryonic development, immune response, and many others.

1.11.3. MiRNAs and stress

miRNAs show strong evolutionary conservation, and are involved in central nervous system (CNS) processes, such as, stress responses (Babenko, Golubov et al. 2012, Warnefors, Liechti et al. 2014), learning, memory (Lin, Wei et al. 2011, Wang, Kwon et al. 2012), and synaptic plasticity (Smalheiser and Lugli 2009, Wibrand, Pai et al. 2012). Given their central involvement in neural development and function; brain miRNA dysregulations have been identified in stress related disorders, such as, PTSD (Aten, Page et al. 2019, Sillivan, Jamieson et al. 2020). Today, it is well known that different brain regions are sensitive to acute and repeated stress (Arnsten 2009, Shoji and Mizoguchi 2010), which results in changes occuring in neural transmission and gene regulation (Rodgers, Morgan et al. 2013, Wingo, Almli et al. 2015). Some functional target genes of different miRNAs are associated with specific neurotransmitter/neuromodulator signaling, neurotrophin expression and other aspects of synaptic plasticity, as well as, stress regulatory hypothalamic-pituitary-axis function. For example, the epigenetic regulation of catechol-omethyltransferase (COMT), an enzyme which degrades dopamine, is associated with the impaired fear inhibition in PTSD (Norrholm, Jovanovic et al. 2013). The deletion of Dicer1 in the forebrain of mice caused a decrease in several miRNAs and enhanced learning and memory strength (Konopka, Kiryk et al. 2010). Several groups have reported other correlations between stress and miRNAs. In this regard, Volk et al. 2014, showed in a model of chronic social defeat stress (CSDS) an increase of miR-19b in the RISC RNA-Ago2 complex in the amygdala from stressed mice, together with a reduction of transcript and protein levels of its putative target, adrenergic receptor-1 (Adrb1) (Miyakawa, Oka et al. 1989). In another study, it has been found an increased expression of miR-128b in the infralimbic prefrontal cortices (PFCs) from mice in response to fear extinction training (Lin, Wei et al. 2011). A significant number of miRNAs have been observed in extracellular compartments, including blood plasma, serum, saliva, urine, sperm and cerebrospinal fluid (Taylor and Gercel-Taylor 2013, Raoof, Jimenez-Mateos et al. 2017, Wiegand, Savelsbergh et al. 2017, Lee, Baxter et al. 2019). It has been shown that, circulating miRNAs in biofluids may reflect cellular miRNA expression and/or dysfunction in the brain (Chen, Zhao et al. 2020). MiRNAs have several properties making them interesting candidates to be investigated as biomarkers: stability in various biofluids, sequences conserved among different species, specific-tissues expression, level of miRNAs easily assessed by various methods (Balakathiresan, Chandran et al. 2014, Snijders, Krauskopf et al.

2019). For example, exposure of male mice to early life stress altered the levels of specific sperm miRNAs which promoted stress-associated behaviors in their offspring. Moreover, in the same study, the reduction of miR- 449 and miR-34 in the sperm has been found also in men exposed to early life stress (Dickson, Paulus et al. 2018). It has become evident that studies on miRNAs, can offer novel and interesting perspectives. In this context, one of the first studies to confirm a role of miRNAs as pharmacological targets was conducted by Zhou et al. in 2009 (Zhou, Yuan et al. 2009). The potential biomarker power of miRNAs could be exploited to discriminate vulnerable and resilient to stress phenotypes. In another study, circulating miRNA profiles were examined 3 days before and 24 h following CSDS in rats (Chen, Kelly et al. 2015). Prior to the stressful event, four miRNAs (miR-4-2-5p, miR-27a-3p, miR-30e-5p, miR-362-3p) were significantly decreased only in those rats that later became vulnerable to stress. Following stress exposure, four different miRNAs (miR-139-5p, miR-28-3p, miR-326-3p, miR-99b-5p) were decreased in resilient animals. These results strongly suggest that different miRNAs potentially confer vulnerability to future stress or promote sustained resilience.

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2. A novel arousal-based individual screening reveals susceptibility and resilience to PTSD-like phenotypes in mice

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Abstract

Translational animal models for studying post-traumatic stress disorder (PTSD) are valuable for elucidating the poorly understood neurobiology of this neuropsychiatric disorder. These models should encompass crucial features, including persistence of PTSD-like phenotypes triggered after exposure to a single traumatic event, trauma susceptibility/resilience and predictive validity. Here we propose a novel arousal-based individual screening (AIS) model that recapitulates all these features. The AIS model was designed by coupling the traumatization (24 h restraint) of C57BL/6 J mice with a novel individual screening. This screening consists of z-normalization of post-trauma changes in startle reactivity, which is a measure of arousal depending on neural circuits conserved across mammals. Through the AIS model, we identified susceptible mice showing long-lasting hyperarousal (up to 56 days post-trauma), and resilient mice showing normal arousal. Susceptible mice further showed persistent PTSD-like phenotypes including exaggerated fear reactivity and avoidance of trauma-related cue (up to 75 days post-trauma), increased avoidance-like behavior and social/cognitive impairment. Conversely, resilient mice adopted active coping strategies, behaving like control mice. We further uncovered novel transcriptional signatures driven by PTSD-related genes as well as dysfunction of hypothalamic–pituitary–adrenal axis, which corroborated the segregation in susceptible/resilient subpopulations obtained through the AIS model and correlated with trauma susceptibility/resilience. Impaired hippocampal synaptic plasticity was also observed in susceptible mice. Finally, chronic treatment with paroxetine ameliorated the PTSD-like phenotypes of susceptible mice. These findings indicate that the AIS model might be a new translational animal model for the study of crucial features of PTSD. It might shed light on the unclear PTSD neurobiology and identify new pharmacological targets for this difficult-to-treat disorder.

Keywords: animal model, susceptibility, resilience, fear conditioning, stress, z-score.

Abbreviations:

5-trial social memory (5-trial SM), acoustic startle reactivity (ASR), amygdala (Amy), arousal-based individual screening (AIS), basal synaptic transmission (BST), brain derived neurotropic factor (BDNF), control (C), corticosterone (CORT), Diagnostic and Statistical Manual of Mental Disorders (DSM-5), elevated plus maze (EPM), field excitatory post-synaptic potentials (fEPSPs), FK506 binding protein 5 (FKBP5), Food and Drug Administration (FDA), forced swim test (FST), hypothalamus (HT), hypothalamic–pituitary–adrenal (HPA), hippocampus (HIP), medial prefrontal cortex (mPFC), open field (OF), post-traumatic stress disorder (PTSD), selective serotonin reuptake inhibitors (SSRIs), serum/glucocorticoid-regulated kinase 1 (SGK1), trauma-exposed (TE).

Highlights

- The AIS model includes highly requested features necessary to shape a translational PTSD animal model.
- Susceptible mice identified through the AIS model exhibited persistent PTSD-like phenotypes
- Resilient mice identified throught the AIS model adopted active coping startegies
- The AIS model revelead molecular adaptations underlying trauma susceptibility/resilience

2.1. Introduction

Post-traumatic stress disorder (PTSD) is a neuropsychiatric disorder developed by vulnerable individuals following a traumatic event. PTSD is considered a major health challenge (Shalev et al., 2017). The suicide risk associated with PTSD is very high (Kessler, 2000) and available treatments with the selective serotonin reuptake inhibitors (SSRIs) paroxetine and sertraline, which are the only two medications approved by U.S. Food and Drug Administration (FDA), are unsatisfactory (Malikowska-Racia and Salat, 2019; Torrisi et al., 2019). Thus, there is an urgent need to develop more effective treatments for PTSD. To this purpose, animal models are recognized essential tools for studying human diseases as well as for screening and identify new

potential drugs (Berardi et al., 2016; Everitt et al., 2018). Although available animal models for the study of PTSD have provided important insights, new models with a high translational value may be however useful. Indeed, several reports have outlined challenges that need to be addressed to shape a useful animal model for the study of PTSD (Berardi et al., 2014; Daskalakis et al., 2013; Deslauriers et al., 2018; Hendriksen et al., 2014; Richter-Levin et al., 2019). Because PTSD is often triggered by exposure to a single traumatic event (Musazzi et al., 2018), a single/acute traumatic procedure should be used rather than repeated/chronic stressful procedures, in order to trigger phenotypes closer to PTSD and diminish the probability of producing depressive-like phenotypes (Siegmund and Wotjak, 2006). The persistence of several behavioral phenotypes resembling PTSD symptoms, which are not only fear-related, is highly required for two reasons. First, according to criterion F for PTSD diagnosis of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), symptoms must last for more than one month (American Psychiatric Association DSM-5 Task Force, 2013). Second, whereas some individuals with PTSD recover soon after the diagnosis, many others can suffer from PTSD for several months or years (American Psychiatric Association DSM-5 Task Force, 2013; Kessler et al., 1995). More importantly, the long-term manifestation of susceptibility and resilience to the trauma is of high relevance for face validity of animal models (Richter-Levin et al., 2019; Sillivan et al., 2017). In this regard, some available models classify animals in susceptible and resilient (Cohen et al., 2004; Olson et al., 2011; Sillivan et al., 2017). However, in an experimental model is very difficult to reproduce the clinical evidence that only a subset of humans who experience a “traumatic event” are prone to develop PTSD (Hendriksen et al., 2014; Sillivan et al., 2017; Zhang et al., 2019). At the molecular level, it would be useful in a PTSD animal model, to have an overlapping with human findings showing biological changes in individuals with PTSD. Furthermore, useful animal models for the study of PTSD should include the predictive validity criterion, i.e. the prediction of treatment effects in individuals with PTSD on the basis of treatment effects on PTSD-like phenotypes observed in rodents (Hendriksen et al., 2014; Richter-Levin et al., 2019; Zhang et al., 2019). In an attempt to address altogether these challenges, we developed a novel arousal-based individual screening (AIS) model for the study of PTSD. To shape this model, we combined the traumatization of C57BL/6 J mice with a novel individual screening relying on long-term z-normalization of change in post-trauma acoustic startle reactivity (ASR), which is a well-validated measure of arousal depending on neural circuits conserved across mammals (Bale et al., 2019). Through the AIS model, we provide evidence that mice

exposed to 24 h of restraint, which is a single, long and severe traumatic procedure (Chu et al., 2016), can be segregated in susceptible and resilient subpopulations according to an "arousal score" obtained through the z-normalization. Interestingly, susceptible and resilient mice identified through the AIS model showed long lasting and persistent behavioral correlates of PTSD symptoms when tested in a battery of experimental paradigms. To support the validity of the segregation in subpopulations, several molecular and electrophysiological analyses were carried out. Moreover, mice were chronically treated with paroxetine to evaluate the predictive validity of this animal model. Because different complementary symptoms fluctuating over time characterize PTSD, we further applied the z-normalization to all behavioral tests in order to create composite scores for each behavioral dimension. Finally, we created a comprehensive "PTSD-like score", a single value originating from all the other scores. This PTSD-like score provided a general overview of the phenotypes as well as a general overview of the preclinical effects of paroxetine.

2.2. Materials and methods

Details regarding the AIS model, behavioral experiments [odor-cued fear conditioning test, open field (OF) test, elevated plus maze (EPM) test, 5-trial social memory (5-trial SM) test, forced swim test (FST)], analysis of gene expression and electrophysiological recordings are provided in supplementary materials and methods.

2.2.1. Animals

Male C57BL6/J mice (total n = 200, 8–16 weeks old at the beginning of the experiments, Charles River Laboratories Italia, Italy) were group-housed 3–5 per cage under controlled conditions (12-h light/dark cycle, 22 ± 2 °C, food and water ad libitum) and weighed once a week until the end of each experimental protocol. The experimenter handled animals on alternate days during the week preceding the stress procedure. Animals were acclimatized to the testing room at least 1 h before the beginning of the tests. All experiments were carried out according to EU Directive 2010/63/EU, the Institutional Animal Care and Use Committees of Catania and the Italian Ministry of Health (authorization n.110/2019 PR).

2.2.2. Experimental design

2.2.2.1. Experiment 1: The Arousal-based Individual Screening (AIS) model

Hyperarousal symptoms, including exaggerated startle reactivity and hypervigilance, are core symptoms of PTSD [criterion E, DSM-5; (American Psychiatric Association DSM-5 Task Force, 2013)]. They regularly occur early (Bremner et al., 1996) and have a major impact in the natural course of the disease, further influencing the development of other symptoms (Morena et al., 2015; Schell et al., 2004). For these reasons, here post-trauma changes of ASR were measured to detect hyperarousal (Fig. 1A) and identify trauma susceptibility/resilience. A pre-trauma ASR session (day −1) was carried out to measure ASR baseline the day before the traumatic procedure (24 h restraint stress, day 0, Chu et al., 2016). This was done to assemble two groups of mice [controls (C) and trauma-exposed (TE)] with similar average ASR baseline. C and TE mice were given two other ASR sessions, 14 (ASR 1, day 15) and 28 days (ASR 2, day 29) post-trauma respectively. Some mice were further tested two months post-trauma (day 56) in a third ASR session (ASR 3). The post-trauma change of ASR was analyzed both in terms of magnitude and latency and expressed as percentage of ASR baseline because of the high variability among animals (Longenecker et al., 2018). The change of startle magnitude was calculated by using the following formula: [(post-trauma magnitude – baseline magnitude) ×100/baseline magnitude]. The change of startle latency, whose decrease is a sign of hypervigilance (Lebow et al., 2012), was calculated by using the following formula: [(post-trauma latency – baseline latency) x 100/baseline latency]. To identify susceptibility and resilience of TE mice to the trauma, we developed a novel individual screening by using a simple mathematical tool, namely the z-normalization. This tool is widely used in clinical studies and also successfully employed in rodent studies (Guilloux et al., 2011) to measure emotionality dimensions that normally can diverge across time such as ASR (Longenecker et al., 2018). The z-scores originating from z-normalization reveal how many standard deviations an observation (X) is above or below the mean [(μ), with its standard deviation (σ)] of a control group: $Z = (X-\mu)/\sigma$. Here, the long-term changes of ASR of mice were z-normalized to obtain an individual score that we defined “arousal score”.

AROUSAL score = [(X-μ/σ startle magnitude, day 15, 29 + X-μ/σ startle latency, day 15, 29) /4].

Taking into consideration that the vast majority of C mice showed an arousal score below 1, we empirically segregated TE mice by choosing a susceptibility threshold of 1. TE mice that showed an arousal score ≥1 were classified as susceptible, while TE mice with an arousal score <1 were classified as resilient.

2.2.2.2. Experiment 2: Assessment of fear reactivity to and avoidance of a trauma-related cue

Intrusion symptoms such as marked physiological reactions to internal or external trauma-related stimuli [criterion B, DSM-5; (American Psychiatric Association DSM-5 Task Force, 2013)] and avoidance of trauma-related stimuli (criterion C, DSM-5; (American Psychiatric Association DSM-5 Task Force, 2013)] represent hallmark symptoms of PTSD. To model these symptoms, we evaluated fear reactivity to and avoidance of a trauma-related cue of susceptible and resilient mice, which were exposed to a neutral odor [lemon oil, the conditioned stimulus (CS) or trauma-related cue] during the trauma [24 h restraint stress, the unconditioned stimulus (US)], in an odor-cued fear conditioning paradigm (Fig. 1G).

2.2.2.3. Experiment 3: Assessment of avoidance-like behaviour social/cognitive function and depressive-like behavior

The general avoidance of situations that are not linked to the trauma (Sheynin et al., 2017) can be successfully modelled in rodents using approach-avoidance conflict paradigms. Here, the avoidance-like behavior of control, susceptible and resilient mice was assessed both in the OF (day 31) and EPM (day 32) tests (Fig. 2A). Social isolation and cognitive deficits [criterion D, DSM-5; (American Psychiatric Association DSM-5 Task Force, 2013)] characterize PTSD and contribute to the impairment in social, occupational, or other important areas of functioning (criterion G, DSM-5; (American Psychiatric Association DSM-5 Task Force, 2013; Morena et al., 2017)]. In particular, patients with PTSD may experience social cognition deficits, namely

disrupted processing (perception, attention or memory) of social information (Stevens and Jovanovic, 2019). In an attempt to model also this clinical aspect, here the same control, susceptible and resilient mice previously assessed for their avoidance-like behavior, were further tested in the 5-trial SM test (day 34–35) that evaluates social memory, namely the capacity to recognize novel versus familiar mice (Fig. 2A).

Many individuals with PTSD may also receive a diagnosis of major depressive disorder (Flory and Yehuda, 2015). For this reason, other control, susceptible and resilient mice were assessed in the FST [day 43 in line with (Chu et al., 2016); Fig. 3A], which provides measure of depressive-like behavior.

2.2.2.4. Experiment 4: Assessment of PTSD candidate genes mRNA expression in brain regions of interest in PTSD

Human findings indicate that PTSD is associated with altered gene expression (Smoller, 2016). To validate the segregation in susceptible and resilient mice obtained by the AIS model, we investigated the expression of four of the most promising and studied PTSD candidate genes, namely FK506 binding protein 5 (FKBP5), Serum/glucocorticoid-regulated kinase 1 (SGK1), the gene encoding for glucocorticoid receptor (NR3C1), and brain derived neurotropic factor (BDNF), which are important modulators of the stress system and have been found altered in individuals with PTSD (Binder et al., 2008; Breen et al., 2019; Girgenti and Duman, 2018; Lian et al., 2014; Yehuda et al., 2009; Zhang et al., 2014). The expression of these genes was evaluated in a triad of brain regions, medial prefrontal cortex (mPFC), amygdala (Amy), hippocampus (HIP), which according to neuroimaging studies are involved in triggering PTSD symptoms (Garfinkel et al., 2014; Karl et al., 2006; Lisieski et al., 2018; Tovote et al., 2015), as well as in the hypothalamus (HT) that coordinates HPA axis responses to stress (Smith and Vale, 2006). The day after behavioral procedures (day 36, Fig. 4A) mice were sacrificed in order to dissect brain regions.

2.2.2.5. Experiment 5: Assessment of PTSD candidate genes mRNA expression in the whole blood and HPA axis dysfunction

To further validate the segregation in susceptible and resilient mice obtained by the AIS model, possible variations in the expression of FKBP5 and SGK1, which have been found altered in blood of individuals with PTSD (Breen et al., 2019; Yehuda et al., 2009), were assessed in the blood of control, susceptible and resilient mice that were sacrificed the day after the segregation (day 30, Fig 5A).

As individuals with PTSD show long-term dysfunction of the HPA axis (Mehta and Binder, 2012), we further measured pre-trauma basal serum corticosterone level as well as post-trauma long-term basal serum corticosterone level in other control, susceptible and resilient mice (Fig 5A).

2.2.2.6. Experiment 6: Assessment of synaptic transmission and plasticity in the hippocampal CA1 region

PTSD has been associated with altered neuroplasticity, especially in the HIP (Abdallah et al., 2017), in line with evidences demonstrating that stress alters synaptic function in the hippocampal glutamatergic synapse (Pavlovsky et al., 2012). Thus, to further validate the AIS model, we investigated whether trauma susceptibility/resilience obtained by the AIS model was linked to changes in synaptic transmission and plasticity in the HIP. Because human data show hippocampal CA1 abnormalities (Chen et al., 2018) in individuals with PTSD and the CA1 subfield has the major influence on fear memory among the hippocampal subfields (Furini et al., 2014), extracellular electrophysiological recordings were carried out at CA3-CA1 synapses of slices from hippocampi obtained from control, susceptible and resilient mice (Fig. 6A).

2.2.2.7. Experiment 7: Effect of chronic treatment with paroxetine

To assess the predictive validity of our model, control, susceptible and resilient mice were chronically treated with a clinically relevant dose (10 mg/kg, i. p.) of paroxetine (first-line pharmacotherapy for PTSD) that was

shown to be effective in improving PTSD-like behaviors (Philbert et al., 2013, 2015), from the day after the segregation (day 30) to the end of behavioral experiments (day 48) as illustrated in the timeline (Fig. 7A). In particular, mice underwent post-segregation ASR sessions on the day 36 and 43 (ASR 3 and ASR 4), the OF on the day 44, the EPM on the day 45 and the 5-trial SM on the days 47/48. To evaluate whether or not paroxetine modified PTSD candidates gene expression, brain regions were dissected the day after behavioral procedures (day 49).

2.2.3. The AIS model (traumatic procedure)

C57BL6/J mice are more resilient to stress compared to other strains (Jacobson and Cryan, 2007; Mozhui et al., 2010; Savignac et al., 2011) and have been specifically reported resilient to acute restraint stress of short duration (Flint and Tinkle, 2001). To trigger long-term trauma susceptibility and avoid the possible occurrence of only long-term trauma resilience, we chose a restraint traumatic procedure of long duration (24 h), which also provides the advantage of being a traumatic procedure very easy to carry out compared to other commonly used traumatic procedures. Mice were gently put in Falcon 50 mL conical centrifuge cubes and exposed to 24 h of restraint from 3:00 pm (3 h before the beginning of the dark phase) to 3:00 pm of the next day.

2.2.4. Acoustic startle reactivity (ASR) sessions

Mice were tested during the light phase from 9.00 a.m. to 3.00 p.m. in illuminated, ventilated and sound-attenuated startle chambers (SR-Lab System, San Diego Instruments, San Diego, CA, United States) containing a Plexiglas cylinder equipped with a piezoelectric accelerometer to detect animal movement. Each chamber was calibrated according to manufacturer's guidelines before the start of each experiment. Mice were placed in the cylinders of the chambers for a 5-min acclimation period with a 65 dB(A) background noise, and then exposed to 10 acoustic startle stimuli [40 ms — 100 dB(A) noise bursts], which were delivered with variable inter trial intervals of 21, 7, 20, 9, 14, 21, 11, 8, and 23 s to avoid habituation and compensatory

mechanisms (Olson et al., 2011). Both magnitude (V max, peak of the response) and latency (T max, time at which the V max occurs) were considered for measurement of the ASR.

2.2.5. Behavioral paradigms

Behavior of mice was recorded (Sony Videocam PJ330E) and subsequently analyzed by two experts, well-trained researchers. All the apparatuses were cleaned with a 70% ethanol solution in between each test to prevent olfactory cues. All behavioral experiments were performed during the light phase from 9.00 a.m. to 3.00 p.m.

2.2.5.1. Odor-cued fear conditioning test

Mice were tested in an evenly illuminated (60 ± 1 lux) square open field (40 × 40 × 40 cm, Ugo Basile, Gemonio, Italy) after the segregation in susceptible and resilient subpopulations. The behavioral procedure consisted of a no cue exposure session, a cue exposure session and three cue re-exposure sessions, which were carried out at different time. Fear reactivity to the trauma-related cue was detected through the measurement of freezing behavior (% time), which was defined as the complete lack of movement except for that necessary for breathing. Avoidance of the trauma-related cue was identified by assessing explorative behavior of the trauma-related cue, which was defined as the mouse directing its nose toward the cap at a distance of < 2 cm.

2.2.5.2. Open field (OF) test

To assess avoidance-like behavior and locomotor activity, mice were tested in a square open field (40 × 40 × 40 cm, Ugo Basile, Gemonio, Italy) over a 5 min-period as previously reported (Torrisi et al., 2017).

Avoidance-like behavior was measured by counting numbers of entries and time spent in the center of the open field.

2.2.5.3. Elevated plus maze (EPM) test

To further measure avoidance-like behavior, mice were tested in the EPM test as previously described with minor modifications (Leggio et al., 2015). Number of entries (%) and time spent (%) in the open arms of the EPM were used as parameters.

2.2.5.4. 5-trial social memory (5-trial SM) test

To evaluate the social/cognitive domain, mice were tested as previously reported (Leggio et al., 2019b) with minor changes. If the social memory is intact, mice normally decrease their social interaction with a stimulus mouse (mouse 1) over the course of multiple exposures (trial 1–4, habituation), and then increase their social interaction with a different stimulus mouse (mouse 2) (trial 5, dishabituation) in the last trial of the test.

2.2.5.5. Forced swim test (FST)

To measure depressive-like behavior, immobility time as well as latency until the first episode of immobility of mice were assessed in the FST as previously reported (Gerhard et al., 2020).

2.2.6. Behavioral z-scoring: Fear score, avoidance like-score, social memory score and PTSD-like scores

Because PTSD is characterized by different complementary symptoms over time, we also applied z-normalization to all behavioral tests that mice further underwent, to create specific scores for each mouse (fear score, avoidance like-score, social memory score and PTSD-like score). Indeed, the z-normalization not only allows data integration deriving from different and complementary parameters of a specific behavioral paradigm in a single score, but it can also be used to obtain an overall score arising from the z-normalization of all the behavioral parameters observed in the same mouse exposed to a battery of different tests. Moreover, it decreases the behavioral noise related to the use of multiple tests, which enhances the reliability of behavioral phenotyping (Guilloux et al., 2011). The directionality of z-scores was adjusted such that maladaptive behavior is represented by positive standard deviations. A "fear score" was calculated for the odor-cued fear conditioning test by z-normalizing the % of freezing during the exposure and the re-exposure sessions and the % of time exploring the trauma-related cue:

FEAR score = [(X-μ/σ freezing, day 32 + X-μ/σ exploration time of trauma-related cue, day 32 + X-μ/σ freezing, day 40, 54, 75) /5].

Similarly, an "avoidance-like score" as well as a "social memory score "were calculated by z-normalizing the parameters we analyzed in the OF, EPM and 5-trial SM:

AVOIDANCE-like score = [(X-μ/σ center entries ratio + X-μ/σ time in the center + X-μ/σ entries open arms + X-μ/σ time open arms) /4].

SOCIAL MEMORY score = [(X-μ/σ trial 1,2,3,4,5) /5].

Finally, the z- normalization was further utilized to create a PTSD-like score that represents a single value originating from all the previous scores. Different PTSD-like scores were calculated according to the battery of tests each individual animal underwent.

PTSD-like score 1 = [(AROUSAL score + FEAR score) /2].

PTSD-like score 2 = [(AROUSAL score + AVOIDANCE-like score + SOCIAL MEMORY score) /3].

For the pharmacological experiments, the arousal score included in the PTSD-like score 2 was calculated considering the ASR sessions post trauma (ASR 3 and 4).

2.2.7. Pharmacological treatment

Mice were chronically treated with paroxetine hydrochloride (Cayman Chemical, Ann Arbor, Michigan 48108 USA) or vehicle. Paroxetine was dissolved firstly in dimethyl sulfoxide (DMSO) and then diluted with distilled water to obtain a final solution containing 3% of DMSO. Paroxetine and vehicle (3% solution distilled water/DMSO) were administered i. p. by using an injection volume of 10 ml/kg. On the day of the tests, paroxetine and vehicle were administered 2 h before the beginning of the test.

2.2.8. Analysis of gene expression by real-time quantitative RT-PCR

For analysis of gene expression in brain areas, mice were killed via cervical dislocation 24 h after the 5-trial SM, during the light phase from 10.00 a.m. to 2.00 p.m. HIP, mPFC, HT and Amy were microdissected according to established protocols (Leggio et al., 2019a; Zapala et al., 2005). One cohort of mice was used to microdissect the mPFC and HIP and another independent one was used to microdissect the HT and Amy. For analysis of gene expression in the whole blood, mice were killed via cervical dislocation and decapitated to collect trunk blood. Blood was directly collected in 4 µl-EDTA 0.5 M-containing Eppendorf and gently shaken to avoid coagulation. RT-PCR was performed as previously reported (Cosentino et al., 2019; Leggio et al., 2015).

2.2.9. Corticosterone measurement

A small amount of blood was collected from the same mice (C and TE) after bleeding of the submandibular vein as previously described (Golde et al., 2005), both 5 to 6 h before (9:00-10:00 a.m.) the start of the trauma and the day after (9:00-10:00 a.m.) the segregation in subpopulation. Blood was directly collected in a sterile Eppendorf and kept at room temperature for 3 h. Afterwards, it was centrifuged a 1000 × g for 15 min to isolate

serum. ELISA assay was performed using a Corticosterone ELISA kit (Cayman chemical, Michigan, U.S.A.) according to manufacturer's instructions and as previously reported (Cosentino et al., 2019). Each sample was run in triplicate.

2.2.10. Electrophysiological recordings

Mice were randomly selected and killed via cervical dislocation prior to the recording. These experiments were performed by an operator blind with respect to subpopulation. Extracellular electrophysiological field recordings were performed on 400 μm transverse hippocampal slices as previously described (Gulisano et al., 2019). We first measured basal synaptic transmission (BST) by stimulating with a series of increasing voltage pulses (from 5 to 35 V). In LTP experiments, baseline was recorded every minute for 15 min by a stimulus intensity evoking a response of 35% of the maximum evoked response in BST. LTP was induced by a theta-burst stimulation (trains of 10 × 100 Hz bursts with five pulses per burst with a 200 ms inter-burst interval at the test pulse intensity). After induction of LTP, every slice was recorded for 120 min. Triangular surface plots representing the individual slices in each condition were generated in Python 3 with Matplotlib 3.1.1 libraries.

2.2.11. Statistical analysis

Sample size was determined by using power analysis and was thus similar to that of studies using related methods (Lopez et al., 2017). Each experimental group consisted of a minimum of five mice. Data were analyzed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). To assess data distribution, the D'Agostino-Pearson omnibus normality test was carried out. The Levene's test was also used to verify equality of variances. All data assumed a normal distribution and then they were subjected to parametric tests (one- or two-way ANOVA and two-way ANOVA with repeated measures when appropriate). For all data analyses, upon confirmation of significant main effects, differences among individual means were assessed using Bonferroni post-hoc test. P values of <0.05 were considered significant. Pearson's correlation analysis was

performed to assess the linear correlation between the AROUSAL score and the PTSD-like score. Pearson's correlation analysis was further performed to assess the linear correlation between the AROUSAL score and mRNA expression of the PTSD-candidate genes. ANOVA with repeated measure was used to analyze BST curves and LTP for 120 min recording after tetanus. The estimate of dispersion is shown as the standard error of the mean (s.e.m.), and variances were found to be similar among groups. All data are presented as means ± s. e.m.

2.3. Results

2.3.1. Susceptible and resilient mice were identified through the AIS model

In line with the variable responses displayed by individuals exposed to the same traumatic event, we observed heterogeneity in the ASR of TE mice with a general significant increase of startle magnitude (Fig. 1B, Stress, $F(1, 91) = 6.244$, $P = 0.0143$) and a not significant decrease of startle latency (Fig. 1C), detectable in both post-trauma ASR sessions that were carried out on the same mice 14 (ASR 1) and 28 days (ASR 2) post-trauma respectively. To more finely capture the manifestation of hyperarousal over time, we used the z-normalization that allows integrating several converging and complementary data. The z-normalization allowed us to segregate TE mice in susceptible and resilient groups by calculating the arousal score (Fig. 1D, Stress susceptibility, $F(2, 90) = 35.66$, $P < 0.0001$). This approach was useful, as it uncovered a post-trauma hyperarousal only in susceptible mice. Indeed, susceptible mice (25%–35%, across experiments) showed a significant higher startle magnitude (Fig. 1E, Stress susceptibility, $F(2, 90) = 32.65$, $P < 0.0001$) as well as a faster reaction time (decrease of startle latency; Fig. 1F, Stress susceptibility, $F(2, 90) = 11.35$, $P < 0.0001$) to the startle stimuli compared to control mice during both ASR1 and ASR2. In contrast, resilient mice (65%–75%, across experiments) showed arousal level similar to control mice both in terms of startle magnitude (Fig. 1E) and latency (Fig. 1F) during both ASR1 and ASR2. Interestingly, the hyperarousal showed by susceptible mice was persistent, given that it was still present two months post-trauma (Fig. S1A startle magnitude, Stress susceptibility, $F(2, 17) = 4.03$, $P = 0.037$. Fig. S1B startle latency, Stress susceptibility, $F(2, 17) = 6.53$, $P = 0.0079$. Fig. S1C arousal score, Stress susceptibility, $F(2, 17) = 9.543$, $P = 0.0017$). Besides, we observed a persistent hyperarousal only in susceptible mice, which became significantly more pronounced over time (only in terms of startle magnitude) by adding a further stressor (chronic I.P. treatment; Fig. S1D startle magnitude, Stress susceptibility, $F(2, 19) = 13.46$, $P = 0.0002$; Treatment $F(3, 57) = 2.81$, $P = 0.048$. Fig. S1E startle latency, Stress susceptibility, $F(2, 19) = 6.76$, $P = 0.0060$). Also, a retrospective analysis of our data revealed low pre-trauma startle reactivity only in susceptible mice. They exhibited in fact, significant lower pre-trauma startle magnitude (Fig. S1F, Stress susceptibility, $F(2, 197) = 4.863$, $P = 0.0087$) and higher pre-trauma startle

latency compared to both control and resilient mice (Fig. S1G, Stress susceptibility, $F(2, 197) = 17.52$, $P < 0.0001$).

2.3.2. Susceptible mice exhibited long-lasting exaggerated fear responses to trauma-related cue

During the no cue exposure session (day 32), there was no difference in basal freezing time among groups (Fig. 1H). Importantly, only susceptible mice exhibited exaggerated fear responses to trauma-related cue during the cue exposure session (day 33), as indicated by the significant longer freezing time in comparison with control mice (Fig. 1I, Stress susceptibility, $F(2, 25) = 27.09$, $P < 0.0001$). By contrast, resilient mice behaved similarly to control mice (Fig. 1I). Both susceptible and resilient mice avoided to explore the trauma-related cue as displayed by the significant lower cue exploration time compared to the control mice (Fig. 1J, Stress susceptibility, $F(2, 25) = 10.27$, $P < 0.0001$). This latter observation indicates that also resilient mice learned to associate the cue with the trauma and thus they exhibited normal fear memory encoding/recall without showing abnormal fear responses. More importantly, the exaggerated fear responses displayed by susceptible mice were persistent, in fact they were further observed during cue re-exposure sessions (day 40, 54, 75), the last of which was performed two months and a half after the trauma (Fig. 1K, Stress susceptibility, $F(2, 25) = 21.27$, $P < 0.0001$; Time, $F(2, 50) = 25.69$, $P < 0.0001$). Overall, fear score (Fig. 1L, Stress susceptibility, $F(2, 25) = 28.8$, $P < 0.0001$) and PTSD-like score 1 (Fig. 1M, Stress susceptibility, $F(2, 25) = 30.62$, $P < 0.0001$) of susceptible mice were significantly higher in comparison with scores of both control and resilient mice.

2.3.3. Susceptible mice showed increased avoidance-like behavior and social/cognitive dysfunction, but they did not show depressive-like phenotypes

Susceptible mice exhibited a significant increase of avoidance-like behavior. In the OF test, they indeed significantly decreased the number of entries in the center compared to resilient mice (Fig. 2B, Stress susceptibility, $F(2, 39) = 5.957$, $P = 0.0055$) and they significantly decreased the time spent in the center compared to both control and resilient mice (Fig. 2C, Stress susceptibility, $F(2, 39) = 6.237$, $P = 0.0045$). Moreover, in the EPM test, susceptible mice significantly decreased the number of entries in the open arms (Fig. 2D, Stress susceptibility, $F(2, 39) = 5.526$, $P = 0.0077$) compared to control mice and they significantly decreased the time spent in the open arms compared to resilient mice (Fig. 2E, Stress susceptibility, $F(2, 39) = 3.629$, $P = 0.036$). Conversely, resilient mice adopted active coping strategies behaving as control mice (Fig. 2B–E). Of note, there was no basal difference in body weight among control, susceptible and resilient mice, and all groups significantly increased their body weight one month post-trauma (Fig. S2A, Time, $F(1, 39) = 219$, $P < 0.0001$). In addition susceptible mice showed a normal locomotion (total crossings) in the OF (Fig S2B), but a slight significant decrease of locomotion (total entries) in the EPM (Fig. S2C, Stress susceptibility $F(2, 39) = 3.888$, $P = 0.029$). As a whole, susceptible mice showed a significant higher avoidance-like score in comparison with both control and resilient mice (Fig. 2F, Stress susceptibility, $F(2, 39) = 22.24$, $P < 0.0001$).

Both control and resilient mice exhibited an intact social memory. They, indeed, progressively lost interest in the social investigation of a stimulus male mouse (mouse 1) over the course of the trials 1–4 (habituation) and then they were interested in the social investigation of a novel stimulus male mouse (mouse 2; dishabituation; Fig. 2G). Interestingly, susceptible mice showed a marked social memory impairment, as indicated by the delayed habituation to the mouse 1 that occurred in the last (fourth) trial, as well as a significant decrease of social investigation in the first trial compared to control mice (Fig. 2G, Trial, $F(4, 156) = 52.49$, $P < 0.0001$, Stress susceptibility x Trial, $F(8, 156) = 6.144$, $P < 0.0001$). Overall, susceptible mice displayed significant higher social memory score (Fig. 2H, Stress susceptibility $F(2, 39) = 8.604$, $P < 0.0001$) and PTSD-like score 2 (Fig. 2I, Stress susceptibility, $F(2, 39) = 47.31$, $P < 0.0001$) compared to both control and resilient mice. Interestingly, the development of a high arousal score predicted the development of a high PTSD-like score 2, as shown by a significant positive correlation between the arousal score and the PTSD-like score 2 of TE mice (Fig. 2J, $r = 0.85$, $p < 0.0001$). Conversely, there was no significant linear correlation between these two scores in C mice (Fig. 2K).

Neither susceptible nor resilient mice displayed long-term depressive-like behavior. Indeed, there were no significant differences between the three groups both in the latency to immobility time (Fig. 3B) and total immobility time (Fig. 3C).

2.3.4. Novel and divergent transcriptional signatures driven by PTSD candidate genes as well as peripheral marks of HPA dysfunction corroborated the segregation in subpopulations and correlated with trauma susceptibility/resilience

Susceptible and resilient mice showed divergent expression of PTSD candidate genes according to PTSD-related brain regions. FKBP5 was significantly up-regulated in the mPFC of susceptible mice, whereas it was significantly down-regulated in the Amy, HIP and HT of resilient mice (Fig. 4B, Stress susceptibility: mPFC, $F(2, 22) = 5.055$, $P = 0.0156$; HIP, $F(2, 22) = 4.931$, $P = 0.017$; Amy, $F(2, 13) = 5.132$, $P = 0.022$; HT, $F(2, 13) = 5.235$, $P = 0.0215$). There was also a positive significant correlation between the arousal score and the expression of FKBP5 exclusively in the HIP (Fig. 4C, $r = 0.52$, $P = 0.04$). Regarding SGK1, it was significantly up-regulated only in the HT of susceptible mice and unchanged in the other brain regions (Fig. 4D, Stress susceptibility: $F(2, 13) = 4.318$, $P = 0.0303$). No correlation between the arousal score and the expression of SGK1 was detected in any brain regions (Fig. 4E). Intriguingly, BDNF gene expression was found significantly up-regulated in the mPFC and HC (vs resilient) of susceptible mice and in the HT of resilient mice, but at the same time, it was down-regulated in the Amy of resilient mice (Fig. 4F, Stress susceptibility: mPFC, $F(2, 22) = 4.65$, $P = 0.0206$; HIP, $F(2, 22) = 3.50$, $P = 0.047$; Amy, $F(2, 13) = 27.79$, $P < 0.0001$; HT, $F(2, 13) = 10.7$, $P = 0.0018$). Moreover, there were positive significant correlations between the arousal score and BDNF expression in the HIP and Amy (Fig. 4G, HIP, $r = 0.77$, $P = 0.0006$; Amy, $r = 0.67$, $P = 0.03$). The expression of NR3C1 further changed depending on brain regions and subpopulations. It was found significantly up-regulated in the HT of susceptible mice and down-regulated in the mPFC and Amy of resilient mice respectively (Fig. 4H, Stress susceptibility: mPFC, $F(2, 22) = 7.429$, $P = 0.0034$; Amy, $F(2, 13) = 7.958$, $P = 0.0055$; HT, $F(2, 13) = 6.471$, $P = 0.0112$). Finally, there was a positive significant correlation between the arousal score and the expression of NR3C1 in the mPFC (Fig. 4I, $r = 0.78$, $P = 0.0006$).

2.3.5. Susceptible but not resilient mice exhibited impaired hippocampal synaptic plasticity

Basal synaptic transmission (BST) was not different among control, susceptible and resilient mice, either when analyzing fEPSP slope or fiber volley (FV) (Fig. 6B). Long-term potentiation (LTP), a type of synaptic plasticity thought to underlie memory formation, was significantly impaired in susceptible but not in resilient mice (Fig. 6C–D, Stress susceptibility, $F(1,13) = 16.505$, $P = 0.001$), as also visible from triangular surface plots representing potentiation of individual slices for each experimental group (Fig. 6E–G).

2.3.6. Chronic treatment with paroxetine resulted effective in susceptible mice but detrimental in control and resilient mice

Paroxetine-treated susceptible mice exhibited a trend of higher startle magnitude (Fig. 7B, ASR3: Treatment, $F(1, 37) = 6.024$, $P = 0.019$; Stress susceptibility, $F(2, 37) = 18.88$, $P < 0.0001$. ASR4: Treatment, $F(1, 37) = 3.84$, $P = 0.049$; Stress susceptibility, $F(2, 37) = 14.25$, $P < 0.0001$) and lower startle latency (Fig. 7C, ASR3: Treatment, $F(1, 37) = 12.02$, $P = 0.0014$; Stress susceptibility, $F(2, 37) = 6.64$, $P = 0.0034$. ASR4: Stress susceptibility, $F(2, 37) = 5.271$, $P = 0.0097$) compared to vehicle-treated susceptible mice, which significantly maintained their hyperarousal over time compared to control mice during both the ASR3 and ASR4. In addition, whereas paroxetine-treated control mice exhibited a trend of higher startle magnitude and a significant lower startle latency compared to vehicle-treated control mice during both the ASR3 and ASR4, paroxetine-treated resilient mice showed only a significant lower startle latency during the ASR4 compared to vehicle-treated resilient mice (Fig. 7B–C). As summarized through the calculation of the arousal score, paroxetine tended to worsen the hyperarousal of susceptible mice, induced a significant hyperarousal in control mice and marginally affect the arousal of resilient mice (Fig. 7D, Treatment, $F(1, 37) = 8.67$, $P = 0.0056$; Stress susceptibility, $F(2, 37) = 22.23$, $P < 0.0001$). Paroxetine-treated susceptible mice, subsequently tested in the OF and EPM tests, interestingly displayed a significant decrease of avoidance-like behavior compared

to vehicle-treated susceptible mice, as indicated by the significant augmented number of entries (Fig. 7E, Treatment x Stress susceptibility $F(2, 37) = 10.45$, $P = 0.0003$) and time spent (Fig. 7F, Treatment x Stress susceptibility $F(2, 37) = 7.45$, $P = 0.0019$) in the center of the OF, and by the significant augmented number of entries (Fig. 7G, Treatment $F(1, 37) = 25.14$, $P < 0.0001$; Treatment x Stress susceptibility $F(2, 37) = 22.68$, $P < 0.0001$) and time spent (Fig. 7H, Treatment $F(1, 37) = 5.29$, $P = 0.0272$; Treatment x Stress susceptibility $F(2, 37) = 8.77$, $P = 0.0008$) in open arms of EPM. Of Note, vehicle-treated susceptible mice further showed an increased avoidance-like behavior compared to vehicle-treated control mice (Fig. 7E–H). By contrast, paroxetine-treated resilient mice showed an almost significant decrease of number of entries and time spent in the center of the OF (Fig. 7E–F) and a significant decrease of number of entries and time spent in the open arms of the EPM compared to vehicle-treated resilient mice (Fig. 7G-H). In addition, paroxetine-treated control mice exhibited a significant decrease of number of entries and time spent both in the center of the OF (Fig. 7E–F) and open arms of the EPM (Fig. 7G-H). Overall, the avoidance-like score clearly showed the significant beneficial effect of paroxetine in susceptible mice as well as the detrimental effect in both control and resilient mice (Fig. 7I, Treatment $F (1, 37) = 18.3$, $P < 0.0001$; Treatment x Stress susceptibility $F (2, 37) = 30.48$, $P < 0.0001$). Of note, paroxetine did not affect the locomotion of control, susceptible and resilient mice in the OF test (Fig. S2D), but affected the locomotion of resilient mice in the EPM test (Fig. S2E, Treatment, $F (1, 37) = 7.648$, $P = 0.0088$). Regarding the 5-trial SM test (day 47–48), paroxetine significantly ameliorated the impaired social memory of susceptible mice. In fact, paroxetine-treated susceptible mice did not exhibit social memory impairment, as indicated by a striking habituation to the mouse 1 during the first four trials and a dishabituation after the exposure to the mouse 2 on the fifth trial compared to vehicle-treated susceptible mice, which instead exhibited a marked social memory impairment (Fig. 7J, Treatment x Trial, $F (20, 144) = 3.065$, $P < 0.0001$; Trial, $F (4, 144) = 74.6$, $P < 0.0001$). No effects of paroxetine on social memory of both control mice and resilient mice were observed (Fig. 7J). Likewise the avoidance-like score, the social memory score visibly displayed the significant beneficial effect of paroxetine in susceptible mice (Fig. 7K, Treatment x Stress susceptibility $F (2, 37) = 8.69$, $P = 0.0008$). Importantly, the further application of the z-normalization provided a comprehensive view of the effects of paroxetine through the creation of the PTSD-like score. Indeed, this score altogether summarized the general beneficial effect of the p harmacological treatment in susceptible mice as well as the general detrimental effect of the same treatment in both control and resilient mice (Fig. 7L,

Treatment F (1, 37) = 15.05, P = 0.0004; Stress susceptibility F (2, 37) = 14.31, P < 0.0001; Treatment x Stress susceptibility F (2, 37) = 17.45, P < 0.0001). Finally, paroxetine significantly rescued the increased mRNA expression of BDNF (Fig. S3A, Treatment, F (2, 15) = 6.93, P = 0.0074) and FKBP5 (Fig. S3B, Treatment, F (2, 15) = 4.3, P = 0.033) in the mPFC of susceptible mice.

2.4. Discussion

The present data indicate that the AIS model includes many key features required to shape a translational animal model for the study of PTSD. Starting from the type of trauma, the 24 h restraint stress is a single, long and severe traumatic procedure endowed with ecological validity in that a similar threatening trapping situation can happen in the natural environment of rodents (Goswami et al., 2013; Kondrakiewicz et al., 2019). This trauma may be also translationally relevant. Indeed, it would model the trapping situations experienced by survivors of natural disasters, who are at high risk of developing PTSD (Basoglu et al., 2002). With respect to the duration and severity of the trauma, a traumatic procedure of short duration should be sufficient to provoke PTSD-like phenotypes (Siegmund and Wotjak, 2006). However, C57BL6/J mice are mice generally resilient to stress and specifically resilient to single restrain stress of short duration (Flint and Tinkle, 2001; Mozhui et al., 2010). Here we show for the first time that a single severe restraint of long duration represent a traumatic procedure able to go successfully beyond the coping abilities of mice by triggering long-term and persistent PTSD-like phenotypes. This trauma was coupled to the z-normalization that firstly allowed us to capture the individual trauma susceptibility/resilience according to long-term change of startle reactivity. Startle circuits are highly conserved in connectivity and function across most species (Bale et al., 2019). Also, animals and humans are tested in a similar way. Thus, the probability of gaining translational information focusing on startle reactivity is very high. Importantly, compared to other models employing the ASR to divide animals in susceptible and resilience (Olson et al., 2011), the z-normalization had the advantage to capture the temporal fluctuation of ASR both in term of magnitude and latency at different post-trauma time points. The discrimination between susceptible and resilient individuals seems particularly relevant given that only few PTSD preclinical studies involving mice have included this aspect (Olson et al., 2011; Sillivan et al., 2017). In fact, in numerous other studies trauma susceptibility/resilience is not reported and all comparisons are made

between naive vs. trauma-exposed animals (Cohen et al., 2004; Flandreau and Toth, 2018; Goswami et al., 2013; Hendriksen et al., 2014; Richter-Levin et al., 2019; Zhang et al., 2019). Furthermore, many preclinical models are based on fear-related aspects of PTSD, whereas the AIS model cover multiple aspects listed in DSM-5 for PTSD diagnosis (American Psychiatric Association DSM-5 Task Force, 2013). Indeed, PTSD cannot be symptomatically restricted only to re-experiencing of the trauma in terms of maladaptive retention of fearful intrusive memories, and other PTSD symptoms may not be linked to dysregulated fear processes (Krystal et al., 2017). The segregation obtained through the AIS model was performed long post-trauma, consistent with PTSD diagnosis that relies on long-term symptoms, rather than on acute physiological symptoms appearing in the aftermath of the trauma (American Psychiatric Association DSM-5 Task Force, 2013). Another important aspect of PTSD diagnosis is related to the duration of the symptoms that must last for more than one month (criterion F of DSM-5). In line with this criterion, we found that susceptible mice identified through the AIS model showed several long-lasting PTSD-like phenotypes, resembling PTSD symptoms, belonging to all criterions of DSM-5.

Of note, different than the study of Chu and colleagues (Chu et al., 2016) showing that the 24 h restraint stress produces decreased performances in mice tested in the FST, susceptible and resilient mice identified through the AIS model did not display depressive-like phenotypes. This may be due to differences in experimental settings. Despite a similar timing of experiments, both the experimental protocols and the battery behavioral tests carried out were different.

The exposure to a trauma is not sufficient to trigger PTSD. Other risk factors are involved in shaping susceptibility to develop this pathology and a useful animal model should include the study of factors predicting susceptibility/resilience to trauma/stress (Richter-Levin et al., 2019). For instance, it has been reported that preexisting differences in social rank predicts vulnerability/resilience to chronic social defeat stress (Cherix et al., 2020; Larrieu et al., 2017). Here we found attenuated startle reactivity only in susceptible mice before the trauma. Thus, our data suggest that a pre-trauma low startle reactivity might represent a risk factor predicting the development of PTSD, and also that the AIS model is a tool that can potentially identify risk factors predicting trauma susceptibility/resilience.

We uncovered novel transcriptional signatures driven by PTSD candidate genes that supported the segregation in subpopulations and correlated with trauma susceptibility/resilience. In particular, we found an upregulation and a downregulation of FKBP5 respectively in the mPFC and whole blood of susceptible mice respectively. These findings also validate the AIS model. Indeed, they are consistent with human results showing a cortical upregulation of FKBP5 (Young et al., 2015) and a downregulation of it in the whole blood of individuals with PTSD (Yehuda et al., 2009). Such an opposite trend of FKBP5 expression in the brain and in the blood has been already reported. Whereas in the brain an upregulation of FKBP5 after GR activation may subserve the formation of trauma susceptibility mechanisms (Zannas et al., 2016), a downregulation of this gene in the blood has been associated with disrupted glucocorticoid sensitivity in blood cells (Sarapas et al., 2011; Yehuda et al., 2009). We also found a significant negative correlation between the arousal score and the expression of FKBP5 in the whole blood of mice, in line with human findings (Sarapas et al., 2011). In contrast, FKBP5 was downregulated subcortically in resilient mice in agreement with findings reporting a pro-resilience effect after inhibition of FKBP5 (Zannas et al., 2016). Regarding BDNF, whereas its role is well-established in MDD (Duman et al., 2019; Tornese et al., 2019), its role in trauma susceptibility and in trauma and stressor-related disorders such as PTSD is still unclear (Notaras and van den Buuse, 2020). Here we found an upregulation of this gene in the mPFC and HIP of susceptible mice. These results are in line with the recently proposed BDNF stress–sensitivity hypothesis, which postulates that a glucocorticoids-induced enhancement of BDNF may guide the manifestation of trauma susceptibility by promoting fear memory consolidation (Notaras and van den Buuse, 2020; Revest et al., 2014). Moreover, the upregulation of BDNF in the HIP of susceptible mice is in line with previous studies showing an increased BDNF in the hippocampus of rodents exhibiting PTSD like phenotypes (Sharma et al., 2020; Zhang et al., 2014). On the other hand, these results do not corroborate previous findings reporting a decreased BDNF mRNA/protein in mPFC and HIP of rodents tested in other preclinical models of PTSD (Ni et al., 2020; Zhao et al., 2020). One possible explanation in this case is that this increased BDNF mRNA in the mPFC might represent a not sufficient compensatory mechanism aim at counteract a blunted cortical BDNF signaling, which has been linked to maladaptive fear memory responses/fear extinction deficits (Kataoka et al., 2019). BDNF was also detected upregulated and downregulated in the HT and Amy of resilient mice respectively. To our knowledge, this is the first evidence that such a long-term divergent pattern of BDNF expression in these subcortical stress-related brain regions

triggers resilience to trauma. In addition, our data showing that downregulation of BDNF in the amygdala produces a pro-resilience effect is indirectly consistent with the opposite evidence of an association between susceptibility to fear-related behavior and increased BDNF levels in the basolateral amygdala (Chou et al., 2014; Regue et al., 2019). These findings together with the other novel transcriptional signatures and correlations we found, indicate that the AIS model is a tool able to identify molecular adaptations underlying trauma susceptibility/resilience. In particular, we quantitatively unraveled more transcriptional changes in resilient mice than susceptible mice, in agreement with previous studies showing that the resilience phenomenon triggers more molecular changes than the susceptibility phenomenon (Lorsch et al., 2018). This is of high relevance because understanding the neurobiology of resilience is essential to develop novel resilience-promoting therapeutic treatments.

PTSD is commonly associated with HPA axis dysfunction and low peripheral cortisol levels (Yehuda, 2004). However, discrepancies remain in this regard with previous other studies reporting also increased (Lindauer et al., 2006) or unchanged peripheral cortisol levels in PTSD (Speer et al., 2019). We found long-term post-trauma higher basal level of serum corticosterone exclusively in susceptible mice, in agreement with recent rodent data obtained through an animal model for the study of PTSD (Sillivan et al., 2017), and more importantly in agreement with a clinical study reporting long-term higher serum cortisol levels in earthquake survivors suffering from PTSD (Song et al., 2008). These results further validate the AIS model and may also explain the long-term hippocampal CA1 LTP impairment found only in susceptible mice. Indeed, it has been reported that high level of circulating stress hormones impairs hippocampal synaptic plasticity (Popoli et al., 2011). Furthermore, an association between hippocampal structural/connectivity deficits and PTSD symptoms has been shown (Abdallah et al., 2017). These electrophysiological findings further validate the AIS model and support the hypothesis that synaptic plasticity deficits might be responsible for PTSD symptoms.

By using the AIS model, we found preclinical evidence of paroxetine efficacy in susceptible mice. To the best of our knowledge, this is the first time that the criterion of predictive validity is included in a model for the study of PTSD taking into account the validation in susceptible mice. Thus, the AIS model might represent a novel tool to identify novel pharmacological strategies for SSRI-resistant individuals with PTSD. In fact, in agreement with human data showing that hyperarousal symptoms may often persist after treatment with SSRIs

(Belkin and Schwartz, 2015), here we found that paroxetine tended to worsen the hyperarousal of susceptible mice. Moreover, in line with previous findings (Huang et al., 2014), paroxetine exerted anxiolytic-like effects in susceptible mice. We further showed for the first time that paroxetine ameliorated the social memory impairment of susceptible mice assessed in the 5-trial SM test, which is a hippocampal-dependent task (Hitti and Siegelbaum, 2014). This may be explained taking into consideration that a chronic treatment with paroxetine is able to reduce the stress-induced apoptosis of hippocampal neurons (Huang et al., 2014). We also discovered that paroxetine restored the mRNA expression of BDNF and FKBP5 in the mPFC of susceptible mice, to the level of control mice. This may further explain the beneficial effect of paroxetine in susceptible mice, and is in line with a previous work showing that FKBP5 expression increases in the mPFC after fear conditioning and that lowering its expression in this area could contribute to trauma resilience (Criado-Marrero et al., 2017). Of note, paroxetine had detrimental effect in both control and resilient mice. These results further differentiated resilient mice from susceptible mice and are in agreement with previous findings. Indeed, an increased ASR has been observed in control rats chronically treated with paroxetine at the dose of 10 mg/kg (Amodeo et al., 2015). We further found that chronic treatment with paroxetine increased general avoidance-like behavior both in control and resilient mice. This effect may be linked to the evidence that specifically in control mice, the chronic blockade of serotonin transporter by paroxetine is able to produce metabolic alterations (Zha et al., 2017), which have been indeed reported to trigger avoidance-like behavior (Zemdegs et al., 2016).

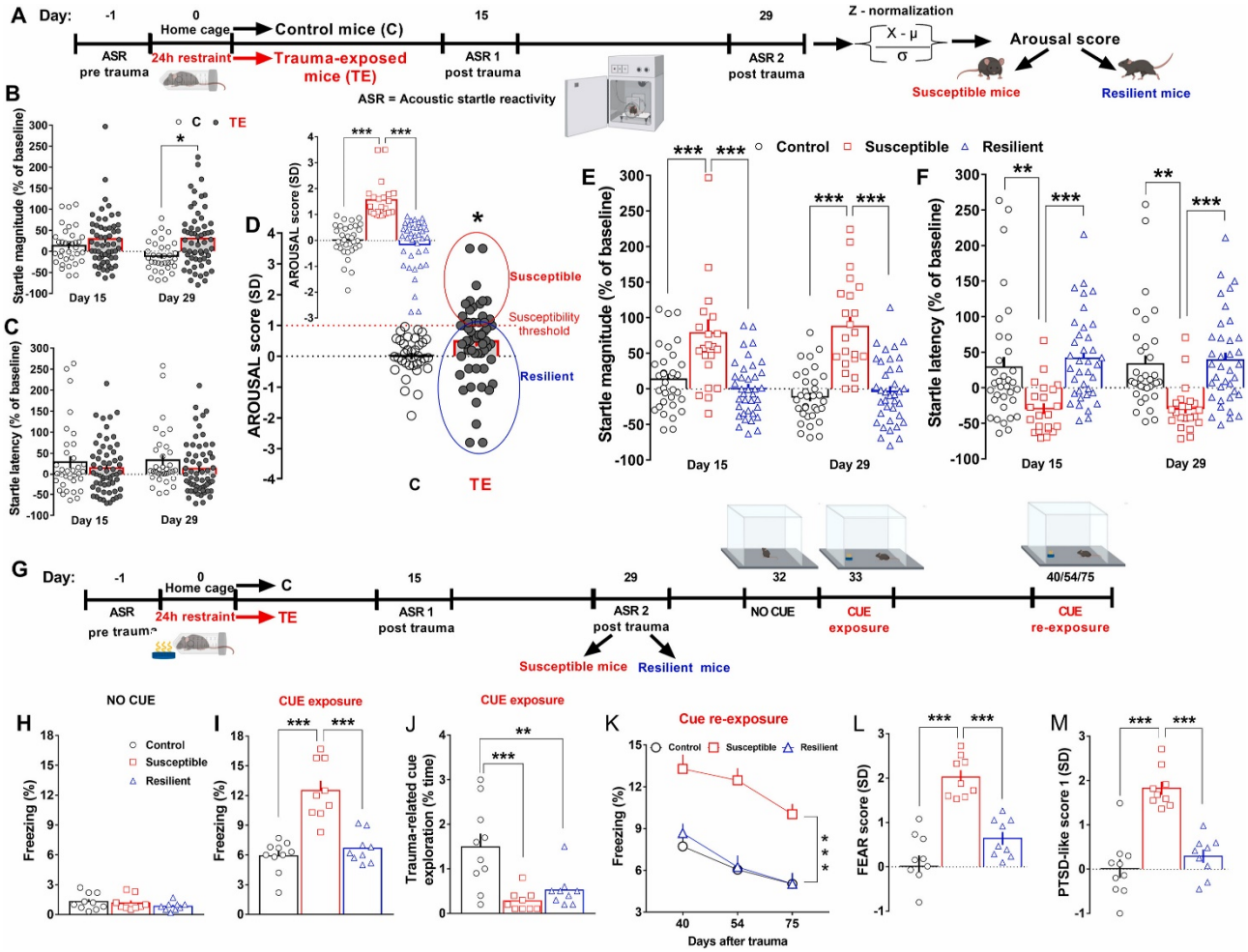
Although the evaluation of sex differences was not within the scope of this study, one potential limitation of this study is related to the lack of inclusion of a relevant risk factor for PTSD, namely the sex. As in human condition, male and female rodents display different responses to stressful and traumatic procedures (Cohen and Yehuda, 2011). However, we used male mice to avoid any confounding factor related to the hormonal status of females Thus, future studies should evaluate the effectiveness of the AIS model in female mice.

In conclusion, the AIS model is a translational and comprehensive tool that may serve for studying PTSD and, more in general, trauma susceptibility/resilience. It might be beneficial for the development of new and more effective pharmacological and psychological interventions for PTSD, for which there is a major unmet need.

2.5. Figure legends

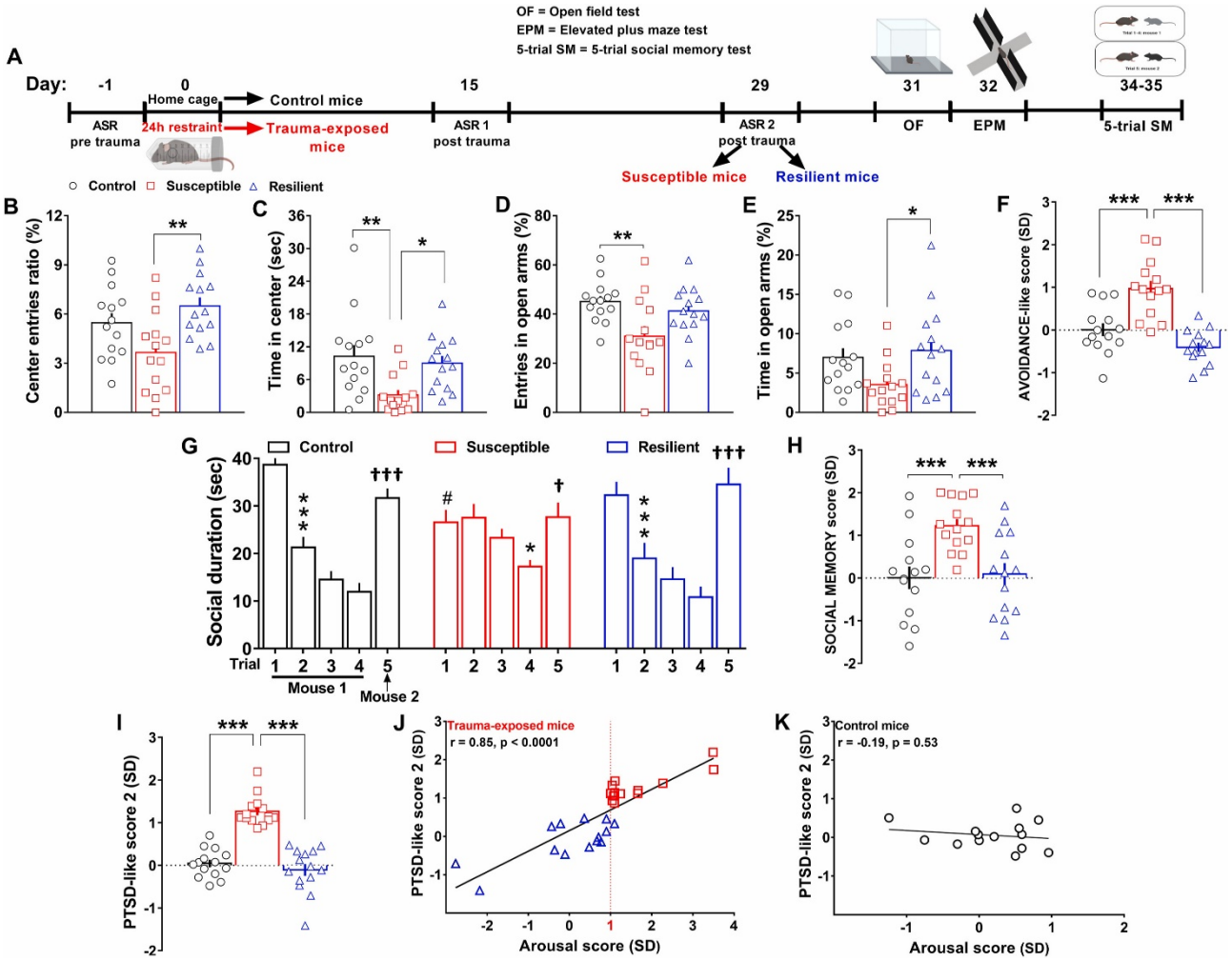
6.1. *Figure 1: Susceptible mice exhibited long-lasting hyperarousal and exaggerated fear responses to trauma-related cue, while resilient mice adopted active coping strategies showing normal behavior*

(A) Experimental procedure conceived to shape the AIS model, which identified susceptible and resilient subpopulations. **(B)** Startle magnitude (% of baseline, *Trauma,* $F(1, 91) = 6.244$, $P = 0.0143$) and **(C)** startle latency (% of baseline) of control mice (C, n = 34) and trauma-exposed mice (TE, n= 59) tested in the ASR 1 and ASR 2 post-trauma sessions. **(D)** AROUSAL score of C and TE mice. The red dotted line, indicating 1 standard deviation as susceptibility threshold, divides susceptible (red circle) from resilient (blue circle) mice (C vs TE: $P < 0.05$)**;** Inset: AROUSAL score after segregation in susceptible (n = 22) and resilient (n = 37) mice (*Subpopulation,* $F(2, 90) = 35.66$, $P < 0.0001$). **(E)** Startle magnitude (% of baseline, *Subpopulation,* $F(2, 90) = 32.65$, $P < 0.0001$) and **(F)** startle latency (% of baseline, *Subpopulation,* $F(2, 90) = 11.35$, $P < 0.0001$) of control mice, susceptible mice and resilient mice identified through the AIS model. **(G)** Experimental procedure conceived for the longitudinal assessment of control (n = 10), susceptible (n = 9) and resilient mice (n = 9) from a different cohort, which were exposed to a neutral odor [lemon oil, the CS or trauma-related cue] during the restraint procedure (US), and were then assessed in an odor-cued fear conditioning test post-trauma**. (H)** Freezing behavior (% time) expressed during the no cue exposure session. **(I)** Freezing behavior (% time) expressed during the cue exposure session (*Subpopulation*, $F(2, 25) = 27.09$, $P < 0.0001$). **(J)** Exploration (% time) of the trauma-related cue during the cue exposure session (*Subpopulation*, $F(2, 25) = 10.27$, $P < 0.0001$). **(K)** Freezing behavior (% time) expressed during the cue re-exposure sessions (*Subpopulation,* $F(2, 25) = 21.27$, $P < 0.0001$; *Time,* $F(2, 50) = 25.69$, $P < 0.0001$). **(L)** FEAR score (*Subpopulation*, $F(2, 25) = 28.8$, $P < 0.0001$). **(M)** PTSD-like score 1 (*Subpopulation*, $F(2, 25) = 30.62$, $P < 0.0001$). Unpaired t- test, two-way repeated measures (RM) ANOVA or one-way ANOVA followed by Bonferroni *post hoc* test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Values are expressed as means ± s.e.m.

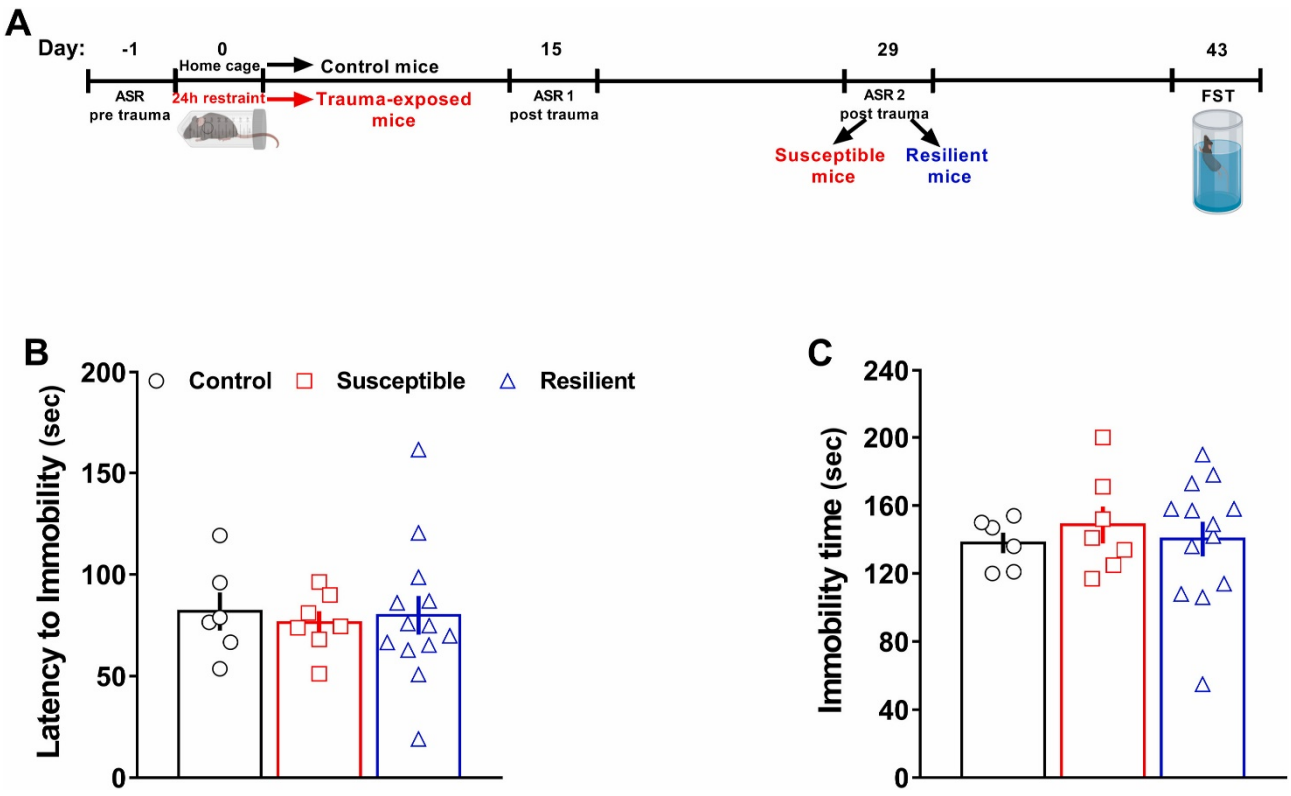

A
Day:
Home cage
Control mice (C)
ASR pre trauma
24h restraint
Trauma-exposed mice (TE)
ASR 1 post trauma
ASR 2 post trauma
Z - normalization
Arousal score
Susceptible mice
Resilient mice
ASR = Acoustic startle reactivity
B
Startle magnitude (% of baseline)
Day 15
Day 29
C
Startle latency (% of baseline)
D
AROUSAL score (SD)
Susceptible
Susceptibility threshold
Resilient
E
Control
Susceptible
Resilient
F
G
NO CUE
CUE exposure
CUE re-exposure
H
Freezing (%)
I
J
Trauma-related cue exploration (% time)
K
Cue re-exposure
Days after trauma
L
FEAR score (SD)
M
PTSD-like score 1 (SD)

6.2. Figure 2: Susceptible mice identified by the AIS model displayed increased avoidance-like behavior and social memory impairment, while resilient mice behaved similar to control mice.

(A) Experimental procedure conceived for the assessment of control (n = 14), susceptible (n = 14) and resilient mice (n = 14) in the OF test, EPM test and 5-trial SM test. **(B)** Center entries ratio (*Subpopulation*, F (2, 39) = 5.957, P = 0.0055) and **(C)** time spent in center of the OF (*Subpopulation*, F (2, 39) = 6.237, P = 0.0045). **(D)** Entries in open arms (*Subpopulation*, F (2, 39) = 5.526, P = 0.0077) and **(E)** time in open arms of EPM (*Subpopulation*, F (2, 39) = 3.629, P = 0.036). **(F)** AVOIDANCE-like score (*Subpopulation*, F (2, 39) = 22.24, P < 0.0001). **(G)** Social duration during the 5-trial SM test [*Trial,* F (4, 156) = 52.49, P < 0.0001, *Subpopulation* x *trial,* F (8, 156) = 6.144, P < 0.0001; Bonferroni post hoc test: *p < 0.05, ***p < 0.001 vs each specific trial 1 (habituation); #P < 0.05 vs the trial 1 of control mice, †p < 0.05, †††p < 0.001 vs each specific trial 5 (dishabituation)]. **(H)** SOCIAL MEMORY score (*Subpopulation*, F (2, 39) = 8.604, P < 0.0001). **(I)** PTSD-like score 2 (*Subpopulation*, F (2, 39) = 47.31, P < 0.0001). **(J)** Linear correlation between the arousal score and the PTSD-like score 2 of trauma- exposed mice (r = 0.85, p < 0.0001) and **(K)** control mice. Two-way RM ANOVA or one-way ANOVA followed by Bonferroni *post hoc* test *P < 0.05, **P < 0.01, ***P < 0.001. Values are expressed as means ± s.e.m.

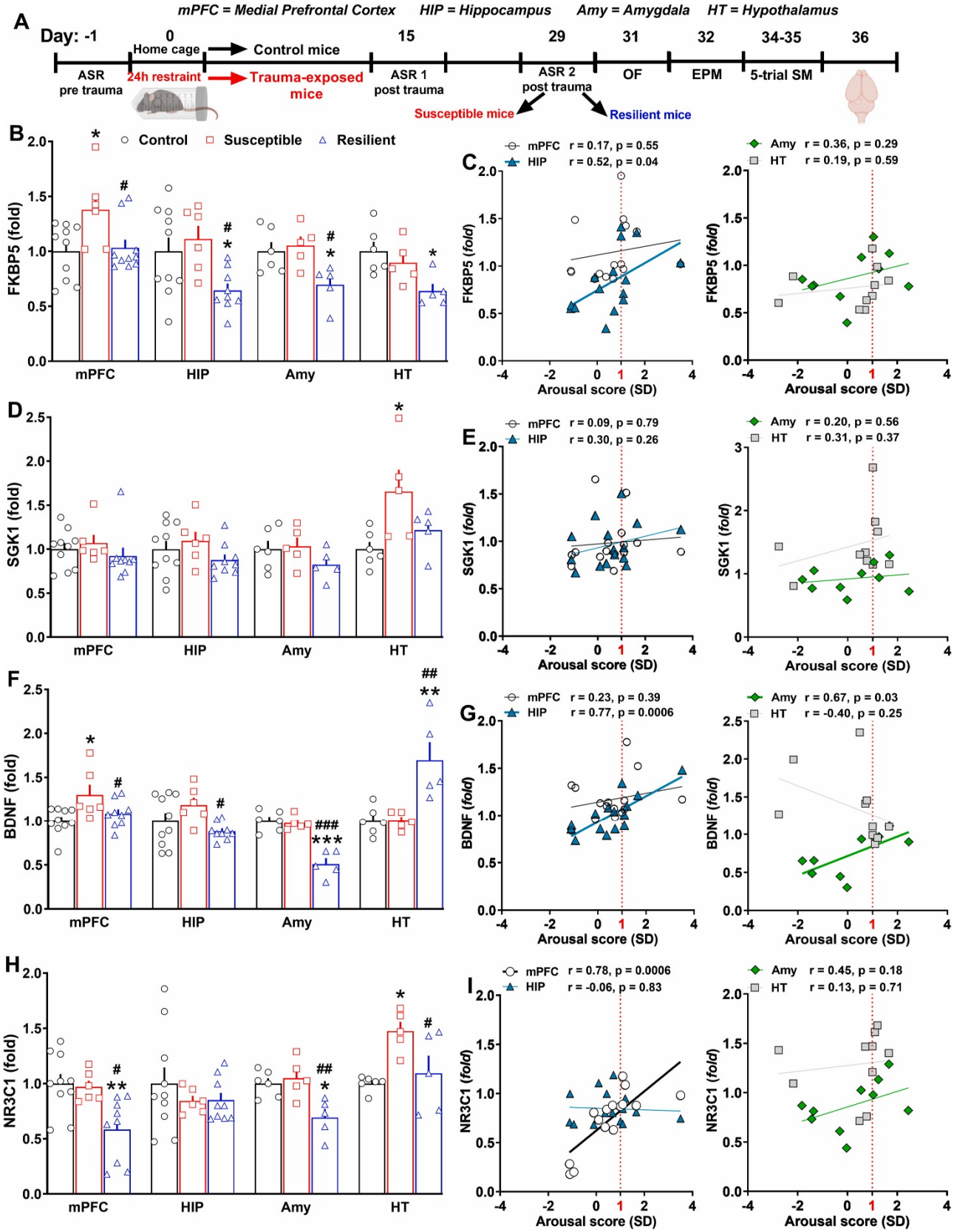

A
OF = Open field test
EPM = Elevated plus maze test
5-trial SM = 5-trial social memory test
Day: -1 0 15 29 31 32 34-35
Home cage
Control mice
ASR pre trauma
24h restraint
Trauma-exposed mice
ASR 1 post trauma
ASR 2 post trauma
OF
EPM
5-trial SM
Susceptible mice
Resilient mice
Control
Susceptible
Resilient
B
Center entries ratio (%)
C
Time in center (sec)
D
Entries in open arms (%)
E
Time in open arms (%)
F
AVOIDANCE-like score (SD)
G
Social duration (sec)
Trial 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
Mouse 1
Mouse 2
H
SOCIAL MEMORY score (SD)
I
PTSD-like score 2 (SD)
J
Trauma-exposed mice
r = 0.85, p < 0.0001
PTSD-like score 2 (SD)
Arousal score (SD)
K
Control mice
r = -0.19, p = 0.53
PTSD-like score 2 (SD)
Arousal score (SD)

6.3. Figure 3: Both susceptible and resilient mice did not exhibit depressive-like phenotypes. **(A)** Experimental timeline designed for the long-term assessment of control (n = 6), susceptible (n = 6) and resilient (n = 14) mice tested in the FST. **(B)** Latency to immobility and **(C)** total immobility time. One-way ANOVA followed by Bonferroni *post hoc* test. Values are expressed as means ± s. e.m.



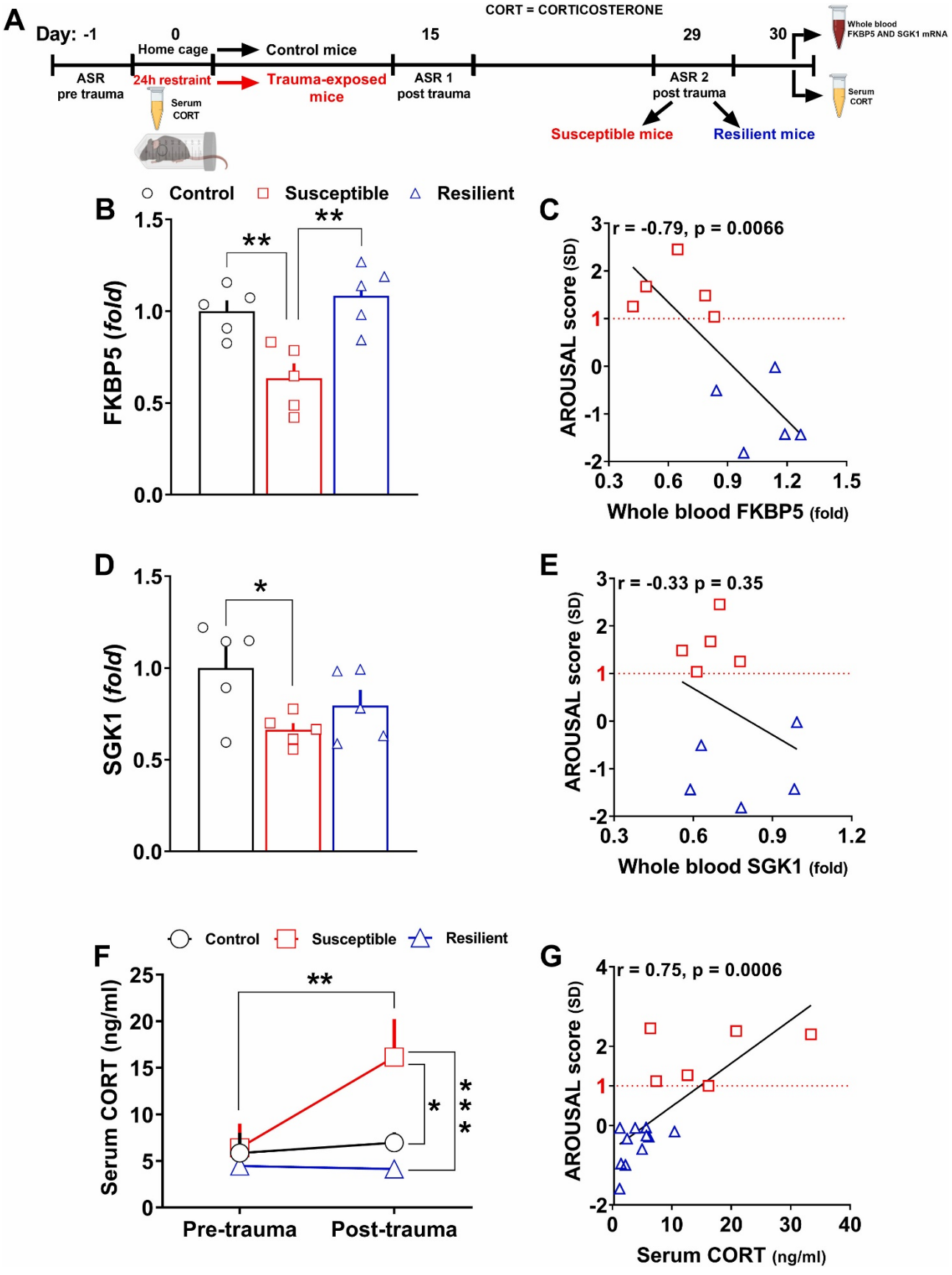
6.4. Figure 4: Divergent central transcriptional signatures driven by PTSD candidate genes corroborated the segregation in susceptible and resilient subpopulations revealed by the AIS model and correlated with trauma susceptibility/resilience

(A) Timeline: the day after the end of the 5-trial SM test, control (n = 10), susceptible (n = 6) and resilient mice (n = 9) from one cohort were sacrificed to microdissect mPFC and hippocampus. Other control (n = 6), susceptible (n = 5) and resilient mice (n = 5) from an independent cohort were sacrificed to microdissect amygdala and hypothalamus. Abundance of transcripts was assessed by qPCR. **(B)** FKBP5 mRNA expression in the mPFC (*Subpopulation*, F (2, 22) = 5.055, P = 0.0156), HIP (*Subpopulation*, F (2, 22) = 4.931, P = 0.017), Amy (*Subpopulation*, F (2, 13) = 5.132, P = 0.022) and HT (*Subpopulation*, F (2, 13) = 5.235, P = 0.0215). **(C)** Linear correlation between the arousal score and the expression of FKBP5 in the mPFC, HIP (r = 0.52, P = 0.04), Amy and HT. **(D)** SGK1 mRNA expression in the mPFC, HIP, Amy and HT (*Subpopulation*, F (2, 13) = 4.318, P = 0.0303). **(E)** Linear correlation between the arousal score and the expression of SGK1 in the mPFC, HIP, Amy and HT. **(F)** BDNF mRNA expression in the mPFC (*Subpopulation*, F (2, 22) = 4.65, P = 0.0206), HIP (*Subpopulation*, F (2, 22) = 3.50, P = 0.047), Amy (*Subpopulation*, F (2, 13) = 27.79, P < 0.0001) and HT (*Subpopulation*, F (2, 13) = 10.7, P = 0.0018). **(G)** Linear correlation between the arousal score and the expression of BDNF in the mPFC, HIP (r = 0.77, P = 0.0006), Amy (r = 0.67, P = 0.03) and HT. **(H)** NR3C1 mRNA expression in the mPFC (*Subpopulation*, F (2, 22) = 7.429, P = 0.0034), HIP, Amy (*Subpopulation*, F (2, 13) = 7.958, P = 0.0055) and HT (*Subpopulation*, F (2, 13) = 6.471, P = 0.0112). **(I)** Linear correlation between the arousal score and the expression of NR3C1 in the mPFC (r = 0.78, P = 0.0006), HIP, Amy and HT. One-way ANOVA followed by Bonferroni *post hoc* test: *p < 0.05, **P< 0.01, ***P < 0.001 vs control; #p < 0.05, ##P < 0.01, ###P < 0.001 vs susceptible. Values are expressed as means ± s.e.m. mPFC = medial prefrontal cortex; HIP = hippocampus; Amy = amygdala; HT = hypothalamus.


mPFC = Medial Prefrontal Cortex
HIP = Hippocampus
Amy = Amygdala
HT = Hypothalamus
A
Day: -1
0
15
29
31
32
34-35
36
Home cage
Control mice
ASR pre trauma
24h restraint
Trauma-exposed mice
ASR 1 post trauma
ASR 2 post trauma
OF
EPM
5-trial SM
Susceptible mice
Resilient mice
B
Control
Susceptible
Resilient
FKBP5 (fold)
mPFC
HIP
Amy
HT
C
mPFC r = 0.17, p = 0.55
HIP r = 0.52, p = 0.04
Amy r = 0.36, p = 0.29
HT r = 0.19, p = 0.59
FKBP5 (fold)
Arousal score (SD)
D
SGK1 (fold)
E
mPFC r = 0.09, p = 0.79
HIP r = 0.30, p = 0.26
Amy r = 0.20, p = 0.56
HT r = 0.31, p = 0.37
F
BDNF (fold)
G
mPFC r = 0.23, p = 0.39
HIP r = 0.77, p = 0.0006
Amy r = 0.67, p = 0.03
HT r = -0.40, p = 0.25
H
NR3C1 (fold)
I
mPFC r = 0.78, p = 0.0006
HIP r = -0.06, p = 0.83
Amy r = 0.45, p = 0.18
HT r = 0.13, p = 0.71

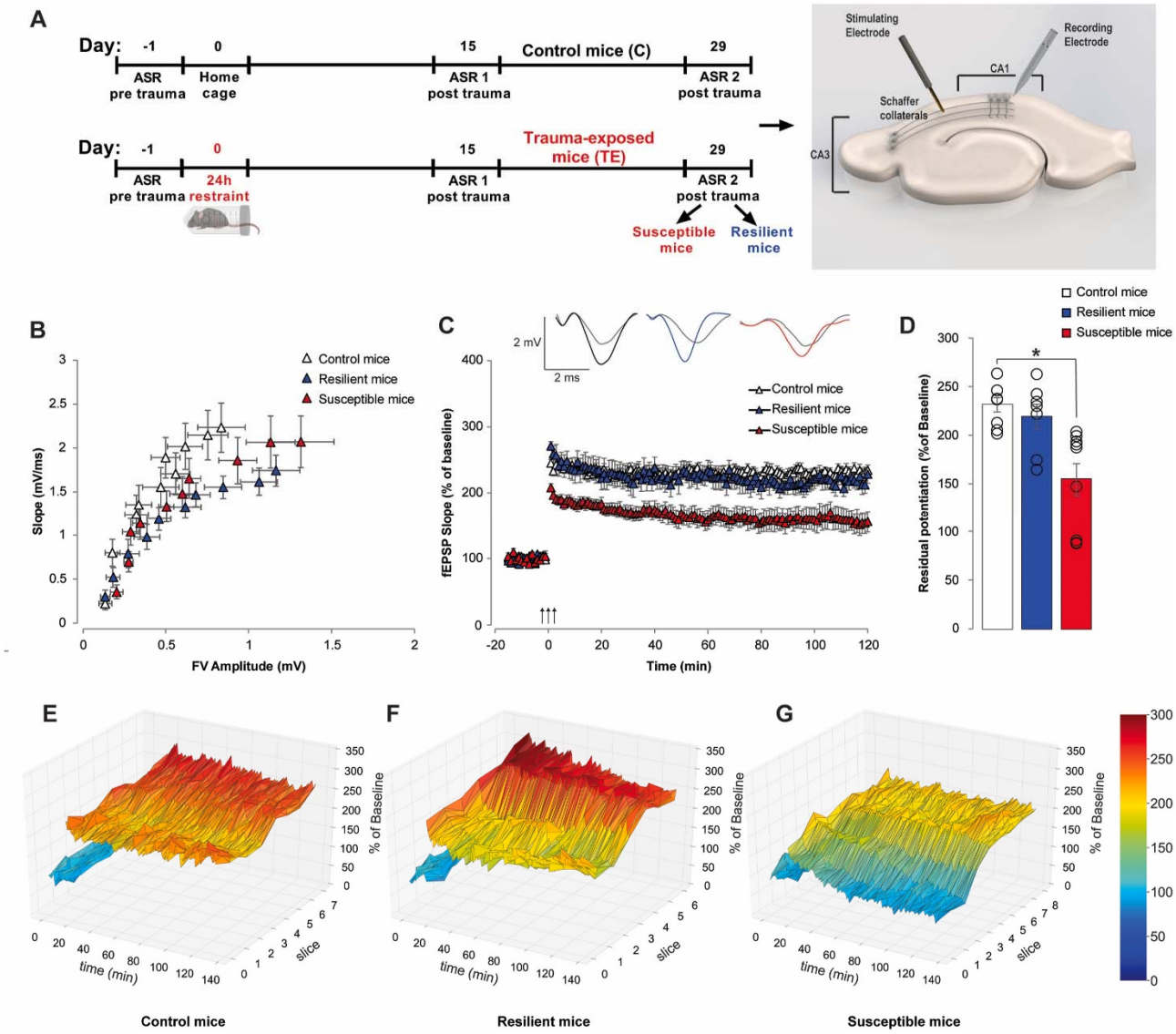
6.5. *Figure 5: Divergent peripheral transcriptional signatures driven by selected PTSD candidate genes as well as marks of HPA axis dysfunction further validated the segregation in susceptible and resilient subpopulations.*

(A) Timeline: the day after the segregation, control (n = 5), susceptible (n = 5) and resilient mice (n= 5) from one cohort were sacrificed to collect whole blood. Abundance of transcripts was assessed by qPCR. Other control (n = 8), susceptible (n = 7) and resilient mice (n = 7) from an independent cohort were sacrificed to collect serum. **(B)** FKBP5 mRNA expression (*Subpopulation*, F (2, 12) = 10.82, P = 0.0021) in the whole blood. **(C)** Linear correlation between the arousal score and the expression of FKBP5 in the whole blood (r = -0.79, P = 0.0066). **(D)** SGK1 mRNA expression (*Subpopulation*, F (2, 12) = 3.945, P = 0.048) in the whole blood. **(E)** Linear correlation between the arousal score and the expression of SGK1 in the whole blood. **(F)** Serum corticosterone levels (*Subpopulation*, F (2, 19) = 9.77, P = 0.0012). **(N)** Linear correlation between the arousal score and serum corticosterone level (r = 0.61, P = 0.02). Fold changes are expressed relative to transcript levels of control mice. One-way ANOVA followed by Bonferroni *post hoc* test: *P < 0.05, **P < 0.01, ***P < 0.001. Values are expressed as means ± s.e.m.


A
CORT = CORTICOSTERONE
Day: -1
0
15
29
30
Home cage
Control mice
ASR
pre trauma
24h restraint
Trauma-exposed
mice
ASR 1
post trauma
ASR 2
post trauma
Whole blood
FKBP5 AND SGK1 mRNA
Serum
CORT
Susceptible mice
Resilient mice
B
Control
Susceptible
Resilient
FKBP5 (fold)
C
r = -0.79, p = 0.0066
AROUSAL score (SD)
Whole blood FKBP5 (fold)
D
SGK1 (fold)
E
r = -0.33 p = 0.35
AROUSAL score (SD)
Whole blood SGK1 (fold)
F
Control
Susceptible
Resilient
Serum CORT (ng/ml)
Pre-trauma
Post-trauma
G
r = 0.75, p = 0.0006
AROUSAL score (SD)
Serum CORT (ng/ml)

6.6. Figure 6: LTP is impaired only in susceptible mice identified through the AIS model.

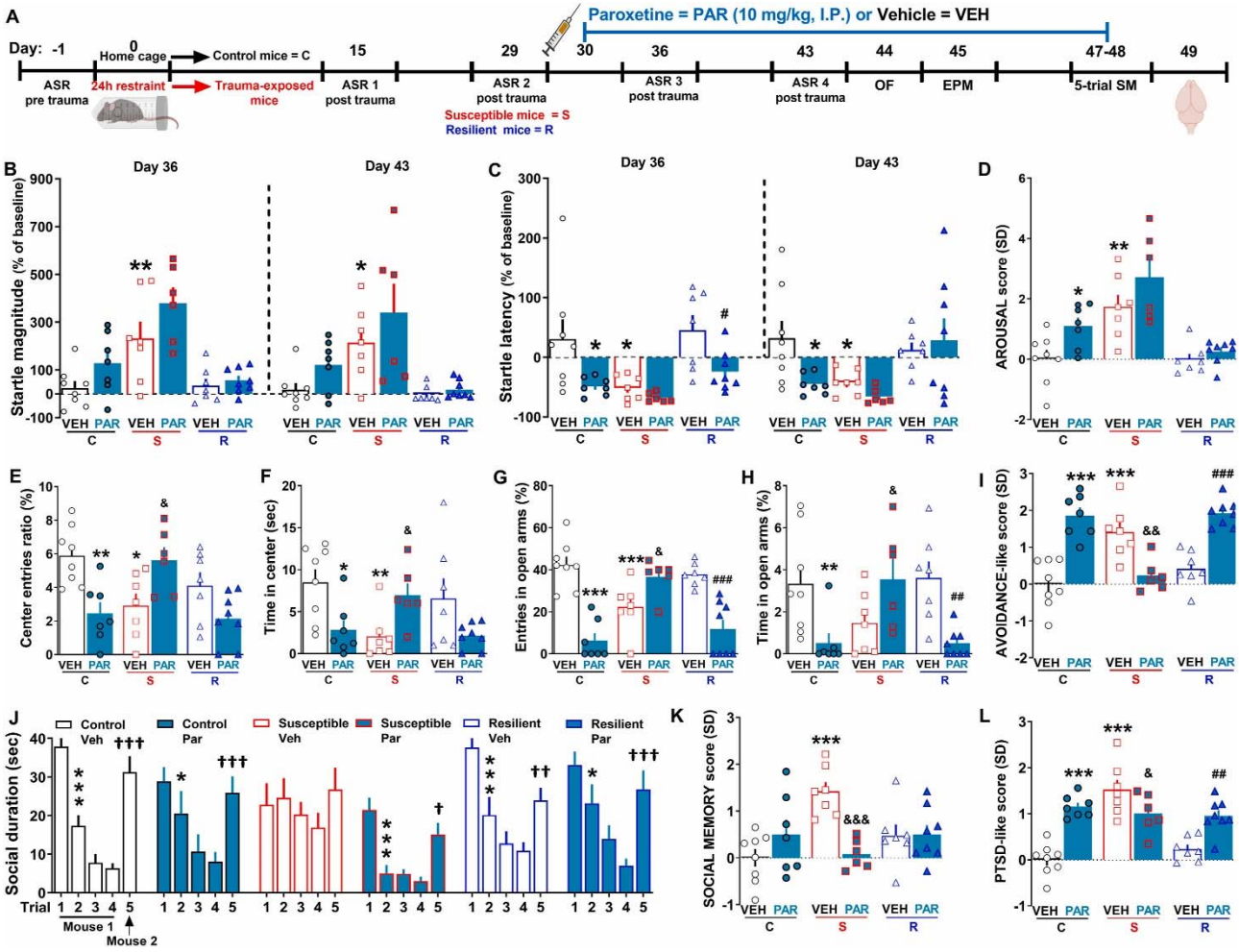
(A) Experimental design. On the right, cartoon representing electrodes placement within the hippocampal slice. **(B)** No differences in fEPSP slope or fiber volley (FV) are found when analyzing Basal Synaptic Transmission (BST) in control (n = 9 slices from 6 animals), resilient (n = 9 slices from 6 animals) and susceptible mice (n = 9 slices from 7 animals). **(C)** Long-Term Potentiation (LTP) is not impaired in resilient mice (234.68 ± 7.52 vs. 228.26 ± 13.77 % of baseline; n = 7/8 slices from 6/7 animals), whereas it is impaired in susceptible mice (155.94 ± 15.72 % of baseline; *Subpopulation,* F (1,13) = 16.505, P = 0.001, n = 9 slices from 7 animals). On top: representative traces of recoded fEPSPs comparing baseline (light grey) and last recording point (colored). **(D)** Residual potentiation (average of the last 5 min of LTP recording at 120 min after tetanus) analysis confirms the LTP impairment in susceptible mice (*Subpopulation,* F (2,21) = 9.403, P = 0.001 among all; controls: 231.39 ± 7.35% of baseline; resilient: 218.94 ± 12.60% of baseline; susceptible: 154.41±15.72% of baseline). **(E)** Triangular surface plot representing the individual traces of LTP recordings for each slice from control, **(F)** resilient, and **(G)** susceptible mice. Two-way repeated measures ANOVA or One-way ANOVA. Bonferroni *post hoc* test: *P < 0.05. Values are expressed as means ± s.e.m.



6.7. Figure 7: Chronic treatment with paroxetine was effective in susceptible mice but detrimental in control and resilient mice.

(A) Experimental timeline designed for the assessment of the effect of chronic treatment with paroxetine in control (vehicle n = 8, paroxetine n = 7), susceptible (vehicle n= 7, paroxetine n = 6) and resilient mice (vehicle n = 7, paroxetine n = 8). **(B)** Startle magnitude (% of baseline) (Day 36: *Treatment,* F (1, 37) = 6.024, P = 0.019; *Subpopulation,* F (2, 37) = 18.88, P < 0.0001. Day 43: *Treatment,* F (1, 37) = 3.84, P = 0.049; *Subpopulation,* F (2, 37) = 14.25, P < 0.0001). **(C)** Startle latency (% of baseline) (Day 36: *Treatment,* F (1,

37) = 12.02, P = 0.0014; *Subpopulation,* F (2, 37) = 6.64, P = 0.0034. Day 43: *Subpopulation,* F (2, 37) = 5.271, P = 0.0097). **(D)** AROUSAL score (*Treatment,* F (1, 37) = 8.67, P = 0.0056; *Subpopulation,* F (2, 37) = 22.23, P < 0.0001). **(E)** Center entries ratio (*Treatment x Subpopulation* F (2, 37) = 10.45, P = 0.0003) and **(F)** time spent in center of the OF (*Treatment x Subpopulation* F (2, 37) = 7.45, P = 0.0019). **(G)** Entries in open arms (*Treatment* F (1, 37) = 25.14, P < 0.0001; *Treatment x Subpopulation* F (2, 37) = 22.68, P < 0.0001) and **(H)** time in open arms of EPM (*Treatment* F (1, 37) = 5.29, P = 0.0272; *Treatment x Subpopulation* F (2, 37) = 8.77, P P = 0.0008). **(I)**. AVOIDANCE-like score (*Treatment* F (1, 37) = 18.3, P < 0.0001; *Treatment x Subpopulation* F (2, 37) = 30.48, P < 0.0001). **(J)** Social du ration during the 5-trial SM test [*Treatment* x *trial,* F (20, 144) = 3.065, P < 0.0001; *Trial,* F (4, 144) = 74.6, P < 0.0001. Bonferroni post hoc test: *p < 0.05, ***p < 0.001 vs each specific trial 1 (habituation); †p < 0.05, ††p < 0.01, †††p < 0.001 vs each specific trial 5 (dishabituation)]. **(K)** SOCIAL MEMORY score (*Treatment x Subpopulation* F (2, 37) = 8.69, P = 0.0008). **(L)** PTSD-like score 2 (*Treatment* F (1, 37) = 15.05, P=0.0004; *Subpopulation* F (2,37) = 14.31, P < 0.0001; *Treatment x Subpopulation* F (2, 37) = 17.45, P < 0.0001). Two-way ANOVA or one-way ANOVA followed by Bonferroni *post hoc* test *P < 0.05, **P < 0.01, ***P < 0.001 vs vehicle-treated control mice, §P < 0.05, §§P < 0.01 §§§P < 0.001 vs paroxetine-treated control mice, &P < 0.05, &&P < 0.01, &&&P < 0.001 vs vehicle-treated susceptible mice, #P < 0.05, ##P < 0.01, ###P< 0.001 vs vehicle-treated resilient mice. Values are expressed as means ± s.e.m.


A
Day: -1
0
Home cage
Control mice = C
15
29
30
36
43
44
45
47-48
49
Paroxetine = PAR (10 mg/kg, I.P.) or Vehicle = VEH
ASR pre trauma
24h restraint
Trauma-exposed mice
ASR 1 post trauma
ASR 2 post trauma
Susceptible mice = S
Resilient mice = R
ASR 3 post trauma
ASR 4 post trauma
OF
EPM
5-trial SM
B
Day 36
Day 43
Startle magnitude (% of baseline)
C
Startle latency (% of baseline)
D
AROUSAL score (SD)
VEH PAR
C
S
R
E
Center entries ratio (%)
F
Time in center (sec)
G
Entries in open arms (%)
H
Time in open arms (%)
I
AVOIDANCE-like score (SD)
J
Control Veh
Control Par
Susceptible Veh
Susceptible Par
Resilient Veh
Resilient Par
Social duration (sec)
Trial 1 2 3 4 5
Mouse 1
Mouse 2
K
SOCIAL MEMORY score (SD)
L
PTSD-like score (SD)

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3. Dysregulation of miR-15a-5p, miR-497a-5p, miR-511-5p in specific mouse brain areas is associated with modulation of BDNF, FKBP5 in PTSD-like susceptible and resilient mice.

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Abstract

Post-traumatic stress disorder (PTSD) is a neuropsychiatric disorder occurring in susceptible individuals following a traumatic event. Understanding the mechanisms subserving trauma susceptibility/resilience is essential to develop new effective treatments. In this respect, increasing evidence suggests that non-coding RNAs (ncRNAs) such as microRNAs (miRNAs) may play a prominent role in mediating trauma susceptibility/resilience. Here we investigated the transcriptional expression of two key PTSD-related genes

(FKBP5 and BDNF) and the relative targeting miRNAs (miR-15a-5p, miR-497a-5p, miR-511-5p*, let-7d-5p)* in PTSD-related brain areas of susceptible and resilient mice identified through a recently developed mouse model for the study of PTSD, namely the arousal-based individual screening (AIS), following a 24-h restrain-induced stress. We observed transcript levels of miR-15a-5p, miR-497a-5p and miR-511a-5p lower in hippocampus of susceptible mice than in that of resilient mice. Similarly, we detected hypothalamic levels of miR-15a-5p, miR-497a-5p and miR-511a-5p lower in susceptible mice than in resilient mice, suggesting that the expression of these miRNAs, at least in these two areas, could discriminate the two different phenotypes of stress-exposed mice. These miRNA variations could contribute alone or synergically to decreased levels of FKBP5 and BDNF in mice. Conversely, in the medial prefrontal cortex, downregulation of miR-15a-5p, miR-511-5p and let-7d-5p was observed in both susceptible and resilient mice, but it was not accompanied by changes in their mRNA targets. The level of hypothalamic let-7d-5p was increased in both susceptible and resilient mice, suggesting that, although sensitive to the stress exposure, it does not discriminate stress-related phenotypes. Interestingly, the levels of these miRNAs showed several significant correlations which might be due to common mechanisms affecting their expression, including neuron-related transcription factors and long non-coding RNAs. Furthermore, miRNA expression in the different brain areas correlated to stress-induced behavioral scores. Thus, these miRNAs may be part of a complex transcriptional and post-transcriptional ensemble, contributing to epigenetic modulation of stress-induced phenotypes.

In conclusion, we identified, for the first time, brain area-dependent changes of miRNAs, potentially shaping susceptibility/resilience following stress exposure, by targeting FKBP5 and BDNF, two key PTSD-related genes.

Keywords: AIS model, miRNA, PTSD, resilience, stress, susceptibility.

*Abbreviations:*ASR, acoustic startle reactivity; AIS, arousal-based individual screening; BDNF, brain derived neurotropic factor; BLC, basolateral amygdala complex; C, control**;** Cebpa, CCAAT/enhancer-binding protein alpha; Cebpb, CCAAT/enhancer-binding protein beta; CUMS, chronic unpredictable mild stress; FKBP5, FK506 binding protein 5; GEO, Gene Expression Omnibus; GR, glucocorticoid receptor; HIP, hippocampus; HPA, hypothalamic–pituitary–adrenal axis; HT, hypothalamus; Kcnq1ot1, KCNQ1 overlapping transcript 1; mPFC, medial prefrontal cortex; miRNAs, microRNAs; ncRNAs, non-coding RNAs; PTSD, post-traumatic

stress disorder; R, resilient; SAM, sympathetic-adrenal- medullary system; Sp1, specificity protein 1; S, susceptible; TFs, Transcriptional Factors; Trk, Tropomyosin receptor kinase.

3.1. Introduction

Intense stressful events evoke a plethora of biological responses, including neurochemical cascades altering the functioning of the sympathetic-adrenal-medullary (SAM) system, hypothalamic–pituitary–adrenal (HPA) axis and immune system. These biological changes can modify the neuronal connectivity, signaling and remodeling and are associated with the exacerbation or development of neuropsychiatric diseases, such as depression and post-traumatic stress disorder (PTSD) (Lupien, McEwen et al. 2009, McEwen, Bowles et al. 2015, Juruena, Eror et al. 2020). These biological changes are the result of the complex interactions between environment and gene function through epigenetic modifications such as DNA methylation, histone modifications, and changes in levels of non-coding RNAs (ncRNAs) (Pfeiffer, Mutesa et al. 2018, Park, Rosenblat et al. 2019, Ramo-Fernandez, Boeck et al. 2019), which play a critical role in adaptive and maladaptive processes, by regulating gene expression without changing the genome (Yeshurun and Hannan 2019). Among ncRNAs, microRNAs (miRNAs) seem to play a pivotal role in neural development and function, as well as in the response to stressors (Follert, Cremer et al. 2014, Rajman and Schratt 2017). Notably, as miRNA biogenesis involves several steps and regulatory proteins, there are numerous possibilities to fine-tune the levels of mature miRNAs (Siomi and Siomi 2010, Michlewski and Caceres 2019). The perturbation of cellular homeostasis may interfere with miRNA biogenesis, leading to deregulation of miRNA-controlled pathways and, accordingly, affecting the cell susceptibility to stress (Qiu, Tan et al. 2015, Olejniczak, Kotowska-Zimmer et al. 2018). Dysregulation of cellular and extracellular miRNA has been identified in several neurodegenerative and neuropsychiatric disorders, such as Alzheimer's disease, Parkinson's disease, major depressive disorder, Autistic Spectrum Disorders, and Tourette Syndrome (Rizzo, Ragusa et al. 2015, Cirnigliaro, Barbagallo et al. 2017, Sharma and Lu 2018, Fries, Zhang et al. 2019, Spampinato, Merlo et al. 2019, Barbagallo, Mostile et al. 2020).

The role of miRNA-based epigenetic mechanisms may also play a significant role in the onset and the progression of PTSD (Zannas, Provencal et al. 2015, Kim, Smith et al. 2018). Interestingly, several recent animal and human studies have shown that miRNAs are present in sperm and may be involved in non-Mendelian inheritance of stress behavioral phenotypes (Toth 2015, Dickson, Paulus et al. 2018).

Accordingly, miR-33 was considered as a mediator of state-dependent encoding and retrieval of contextual fear (Jovasevic, Corcoran et al. 2015). Another study demonstrated that exposure to acute traumatic stress in early adolescence involved miR-34c expression in hypothalamus inducing anxiety-like behavior as well as memory impairment (Li, Liu et al. 2016). Lin et al. showed that the level of miR-128b was increased in the infra-limbic pre-frontal cortex (PFC) of mice following fear extinction (Lin, Wei et al. 2011). In this respect, stress-induced alterations of specific miRNAs are not only temporally, but also spatially defined. Several animal studies reported that specific miRNAs in particular brain regions are involved in several stress-related behaviors, including fear memory consolidation, contextual fear memory and state-dependent fear (Griggs, Young et al. 2013, Wang, Phang et al. 2013, Dias, Goodman et al. 2014, Vetere, Barbato et al. 2014, Jovasevic, Corcoran et al. 2015). Because not everyone who experiences intense acute stress develops psychiatric disorders, epigenetic modifiers, especially miRNAs, have received increasing attention for their potential contribution in susceptibility and resilience to stress. Furthermore, the potential of miRNAs as biomarkers of vulnerability/resilience to stress is becoming a focus of particular interest. In a recent study, Chen et al. showed increased levels of miR-126a-3p and miR-708-5p in the medial pre-frontal cortex (mPFC) of susceptible compared to resilient rats (Chen, Kelly et al. 2015). Another study demonstrated that overexpression of miR-135b-5p in the basolateral amygdala complex (BLC) of stress-resilient animals enhanced remote fear memory expression. Conversely, inhibition of BLC miR-135b-5p in stress-susceptible mice promoted a resilient-like phenotype (Sillivan, Jamieson et al. 2020).

Although there is much evidence, research on resilience has to face several challenges, of which the most critical one involves the huge heterogeneity in defining and operationalizing resilience. Similar to humans, susceptible rodents can develop PTSD-like symptoms, while resilient ones can show less or no PTSD-like symptoms (Whitaker, Gilpin et al. 2014). With the help of a novel model we developed for PTSD, based on individual arousal screening (AIS) of mice, following a 24-h restraint, we evaluated a set of miRNAs targeting

FKBP5 and BDNF, two key PTSD-related genes. We provide evidence that a single traumatic event induced brain area-specific miRNA alterations, differentially associated with susceptible and resilient phenotypes, and related to behavioral scores.

3.2. Materials and methods

3.2.1. Animals

Male C57BL6/J mice (tot=46, 8-16 weeks, weight 28 ± 2 gr.) were purchased from Charles River Laboratories (Italy). Mice were group-housed (3 - 5 per cage) under controlled conditions (12 h light/dark cycle, 22 ± 2°C, 55± 5% humidity, food and water ad libitum) and weighed once a week

until the end of each experiment. All experiments were carried out according to EU Directive 2010/63/EU, the Institutional Animal Care and Use Committees of Catania and the Italian Ministry of Health (authorization n.110/ 2019 PR).

3.2.2. The Arousal-based Individual Screening (AIS) model, ASR sessions, Z-normalization score and behavioral paradigms

Details regarding the AIS model are described in our previous publication (Torrisi, Lavanco et al. 2021). Briefly, the day before the stress procedure (24 h restraint stress), a pre-stress acoustic startle reactivity (ASR) session was carried out to measure baseline ASR. The day after, mice were gently put into polyethylene Falcon 50mL centrifuge tubes and exposed to 24 h of restraint from 3:00 p.m. (3 h before the beginning of the dark phase) to 3:00 p.m. of the next day, without access to food and water. Tubes containing mice were randomly placed in conventional cages, while control mice remained in their home cages in a different room. After 24 h of restraint, mice were suddenly put back in their home cages, with free access to food and water. Two other ASR sessions, 14 (ASR 1) and 28 days (ASR 2) after the stress were given to control and stressed mice in

order to assess changes of arousal according to their post-stress ASR changes (% of ASR baseline). To segregate stressed mice into susceptible and resilient subgroups, we created an arousal score by applying a mathematical approach (Z-normalization) to ASR changes. Stressed mice that exhibited an arousal score ≥ 1 were classified as susceptible, while stressed mice with an arousal score < 1 were classified as resilient. We further created other composite scores (avoidance-like score, social memory score and PTSD-like score) related to other aspects of PTSD, by z-normalizing data from different behavioral tests that mice underwent post-segregation.

3.2.3. Selection of miRNA

The selection of miRNAs to analyze in control, susceptible and resilient mice was carried out by a two-step approach. Firstly, we performed computational data mining to identify protein-coding genes associated with PTSD. More precisely, we queried five different literature mining databases: DisGeNet (www.disgenet.org), PolySearch2 (polysearch.ca), Diseases (www.diseasesdatabase.com), DigSeE (http://gcancer.org/digsee), Psygenet (http://www.psygenet.org/web/PsyGeNET/v01/about) by using the keywords “Post-traumatic Stress Disorder”, “anxiety disorder” and “mood disorder”. We then combined the different outputs to obtain the most scored protein coding genes associated with PTSD. Secondly, we retrieved the miRNAs targeting the protein coding genes previously identified by interpolating the predicted and validated human and murine data from miRecords (http://miRecords.umn.edu/miRecords), miRTarbase (http://miRTarBase.cuhk.edu.cn/) and ENCORI tools (http://starbase.sysu.edu.cn/index.php). Finally, the miRNA list was further filtered by a manually curated search on PubMed, taking into account the dysregulation of these miRNAs in neuropsychiatric phenotypes.

3.2.4. Tissue collection

The day after the last behavioral experiment (36 days post-stress), forty-six mice were sacrificed via cervical dislocation. We used thirty mice to dissect hippocampus (HIP) and medial prefrontal cortex (mPFC) and sixteen to dissect hypothalamus (HT) according to previously described protocols (Spijker 2011, Leggio, Camillieri et al. 2014).

3.2.5. Total RNA extraction

Total RNA was isolated from HIP, HT and mPFC by using 1 ml Trizol Reagent/50-100 mg of tissue (Invitrogen Life Technologies, USA) following the manufacturer's instructions. Briefly, after homogenization 5 µg glycogen were added to improve RNA yield. Total RNA concentration and purity for each sample were evaluated by GeneQuant pro spectrophotometer (Biochrom). RNA was stored at −80 °C until cDNA synthesis.

3.2.6. cDNA synthesis and Real-time PCR

Single-stranded cDNA was synthesized from total RNA according to the manufacturer's instructions by using the SuperScript® III Reverse Transcriptase kit (Thermo Fisher Scientific, Massachusetts, USA). We investigated mRNA expression in brain tissues through RT-qPCR by using iTaq Universal SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions. All RT-qPCR reactions were performed on a 7900HT Fast Real Time PCR System (Applied Biosystems, Life Technologies™). cDNA expression was evaluated through SDS RQ Manager 1.2 software (Applied Biosystems); normalization was performed using GAPDH as the reference gene. No amplification samples were used as negative control. Relative quantity (RQ) for each transcript was calculated according to the 2-ΔΔCt method (Livak and Schmittgen 2001). Technical triplicates were performed for each reaction. All RT-PCR reaction were performed in

accordance with MIQE guidelines. PCR primers were designed by using the online tool Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences are shown in Table 1.

3.2.7. TaqMan microRNA Assays

Specific single assays were performed for each selected miRNA, exploiting the miRNA-specific reverse transcription (TaqMan microRNA Reverse Transcription Kit; Thermo Fisher Scientific) and Real-Time PCR (TaqMan Universal Master Mix II, no UNG; Thermo Fisher Scientific) by using Single TaqMan Assays (Thermo Fischer Scientific), according to the manufacturer's instructions. RT-qPCRs were performed on a 7900HT Fast Real Time PCR System (Applied Biosystems, Life Technologies™). The fold changes were calculated by the 2-ΔΔCt method by using RNA U6 as endogenous control. No amplification samples were used as negative control. Technical triplicates were performed for each reaction. All RT-qPCR reactions were performed in accordance with MIQE guidelines.

3.2.8. Computational Identification of miRNA regulators

To computational analyze the potential regulators of miRNAs, namely Transcription Factors (TFs) and long non-coding RNAs (lncRNAs), we screened the TransmiR v2.0 (http://www.cuilab.cn/transmir) and LncBase v2.0 (https://diana.e-ce.uth.gr/lncbasev3) databases. From TransmiR we retrieved the murine TF-miRNA regulations derived from ChIP-seq evidence, while LncBase provided the predicted interactions between miRNAs and lncRNAs in the mouse.

3.2.9. Statistical analysis

Statistical significance of different mRNA and miRNA expressions between groups was analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. To assess data distribution, the D'Agostino-Pearson omnibus normality test was carried out. The graphs were showed as box and whiskers plot (min to max). No animals or samples were excluded from the analysis. Pearson's correlation coefficients were calculated to statistically evaluate the strength and direction of the linear relationship among miRNA and mRNA expression values (RQ) and behavioral scores. Expression correlations among miRNAs were also investigated in mouse and human datasets from different tissues retrieved from Geo DataSets (https://www.ncbi.nlm.nih.gov/gds). For all analyses described in this section, p values are two-sided and alpha was set to 0.05. All statistical analyses were performed using GraphPad Prism v7.0 *- (GraphPad Software, San Diego, California, USA). To investigate the synergic effects of miRNAs on mRNA expression, linear regression models were built by using SPSS 23. The same statistical approach was used to evaluate the combined effect of expression variation of miRNAs+mRNAs, miRNAs only and mRNAs only on modulation of behavioral scores.

3.3. Results

3.3.1. Selection of miRNAs

By an automatic literature-based gene association analysis, we identified 558 protein-coding genes cited at least once in association with PTSD. Among them, BDNF and FKBP5 were the most cited. The number of the papers regarding BDNF and FKBP5 association with PTSD was about four folds than the median value of the papers regarding the other genes. BDNF and FKBP5 were used as targets to computational identify predicted and validated miRNAs both in humans and mice. By this approach, we found 239 miRNAs targeting BDNF and/or FKBP5 both in humans or mice. We further filtered this list by a manually curated search in biomedical literature to identify potential associations between these miRNAs and stress related disorders. Finally, we

focused our experimental analyses on four miRNAs that targeted both BDNF and FKBP5: miR-15a-5p, miR-497a-5p, miR-511 and let-7d-5p.

3.3.2. Expression of miR-15a-5p, let-7d-5p, miR-511-5p, miR-497-5p and their potential targets, BDNF and FKBP5

3.3.2.1. HIP

The expression of miR-15a-5p increased in the hippocampus of resilient mice compared to susceptible and control mice (Fig.1A). In the same area, both miR-497a-5p and miR-511-5p showed a downregulation in susceptible animals with respect to resilient mice (Fig.1C and 1D). These variations taken together suggested that the expression of these three miRNAs could discriminate the two different phenotypes of stressed mice. In contrast, no significant difference was found for hippocampal expression of let-7d-5p (Fig.1B). We observed a statistically significant reduction for both potential targets of miRNAs analyzed, BDNF and FKBP5, in resilient mice compared to susceptible mice, and also in resilient mice compared to control for FKBP5 (Fig. 1E and 1F).

FKBP5 hippocampal expression showed a significant negative correlation with those of miR-15a-5p, miR-511-5p and miR-497a-5p, as expected by functional miRNA:mRNA relationships. In contrast, we found no correlation between expression of BDNF and miRNAs (Table 2).

3.3.2.2. mPFC

We found a strong reduction of miR-15a-5p, let-7d-5p and miR-511-5p in mPFC (Fig. 2A, 2B, 2D) in both subpopulations of stressed mice compared to controls, except for miR-497a-5p, whose expression alteration was not statistically significant (Fig.2C). These findings suggest that low levels of these miRNAs in this

specific brain area is associated with a general reaction to the traumatic stress but not with the discrimination of resilient and susceptible phenotypes. On the other hand, both FKBP5 and BDNF increased their expression in susceptible mice with respect to control and resilient mice (Fig.2E, F). The inconsistent expression variations of miRNAs with respect to their mRNA targets was also demonstrated by calculated correlations. Indeed, we found no significant linear relationship between miRNAs and targets (Table 2).

3.3.2.3. HT

The hypothalamus expression of miR-15a-5p, miR-497a-5p and miR-511-5p (Fig. 3A, C, D) significantly increased in resilient mice compared to susceptible mice, but also compared to control mice for miR-15a-5p and miR-511-5p. However, both stressed groups of resilient and susceptible mice showed an evident up-regulation of let-7d-5p compared to control animals (Fig. 3B). We observed a decrease of FKBP5 mRNA expression in resilient mice compared to both susceptible and control mice, whilst an upregulation of BDNF was evident in the same comparisons (Fig. 3E, F). In this brain area, miRNAs showed an opposite trend of deregulation with respect to FKBP5, but not to BDNF, as supported by the significant negative correlations between FKBP5 and miR-15a-5p, miR-511-5p and miR-497a-5p (Table 2).

3.3.3. Evaluation of synergic effect of the selected miRNAs on regulating FKBP5 and BDNF expression

We also investigated whether the four PTSD-related miRNAs may have a synergic effect on regulating FKBP5 and BDNF expression, being both targeted by all the analyzed miRNAs. Different linear regression models were computed, each one including all 4 miRNAs, 3 miRNAs and 2 miRNAs, considering all possible combinations. For each model, we analyzed the combination of miRNAs considering only those ones with negative linear correlation, previously reported in Table 2. Table 3 lists the models showing the highest R-value for each target in each brain area. According to R2 values, which represent the proportion of the variance for the dependent variable that may be explained by the independent variables, this analysis suggested that part

of FKBP5 or BDNF expression may be explained by the synergic regulation performed by the 4 PTSD-related miRNAs. In particular, as regards FKBP5 expression, more than 41% in the hippocampus and 51% in the hypothalamus may be explained by PTSD-related miRNAs, with the best result obtained by the combination of all 4 miRNAs; with respect to BDNF, the 23% of its expression in mPFC may be globally regulated by PTSD-related miRNAs; again, the combination of all 4 miRNAs showed the best result.

3.3.4. Potential co-regulation of PTSD-related miRNAs

Because the analyzed miRNAs frequently showed similar variation trends in the same brain area in stressed mice, we investigated whether their expression was statistically correlated to each other in HIP, HT and, mPFC (Table 4).

In HIP we found a statistically significant positive correlation between the following miRNAs: miR-15a-5p and let7d-5p, miR-15a-5p and miR-511-5p, miR-511-5p and miR-497a-5p, and between miR-511-5p and let-7d-7p. In contrast, no significant correlation was observed between miR-497a-5p and, respectively, miR-15a-5p and let-7d-5p.

In HT we found a miRNA:miRNA correlation pattern consistent with the one calculated for HIP. In fact, miR-15a-5p was positively correlated with miR-511-5p and let7d-5p; while miR-511-5p showed the same

significant relationship with miR-497a-5p and let-7d-5p. No significant linear relation was observed between miR-497a-5p and miR-15a-5p, and miR-497a-5p and let-7d-5p.

In mPFC we found significant positive correlations for the pairs miR-497a-5p:miR-15a-5p and miR-497a-5p:let-7d-5p and a strong linear relationship between miR-15a-5p and let7d-5p, as previously observed in HIP.

We found several positive expression correlations among all miRNAs in all analyzed tissues. Therefore, we hypothesized that they may have the same regulation mechanism. To investigate this hypothesis, we retrieved miRNA expression data from different tissues, including brain, from mouse

and human datasets deposited in the Gene Expression Omnibus (GEO) database. Pearson's correlation coefficients confirmed that expressions of let-7d-5p, miR-15a-5p, miR-497a-5p, miR-511-5p were frequently co-linear in many rodent and human tissues, especially in brain, in agreement with results obtained in our PTSD-like model (Fig. 4 and 5).

Based on these data, we speculated on the possibility that these miRNAs could share some transcriptional and post-transcriptional regulators. For this purpose, we compared the TFs binding miRNA promoters and the lncRNAs predicted to decoy (bind and sequester) these miRNA sequences. From this comparative analysis we found that regulatory regions of miR-15a and miR-497a are potentially bound by 27 common TFs, as expected because they belong to the same miRNA family. The genomic loci of the three miRNAs are characterized by promoter regions that immunoprecipitate with TFs Cebpa/Cebpb and Spi1 (Table 5). Moreover, miR-15a-5p, let-7d-5p, miR-511-5p, miR-497a-5p share RNA-binding sites for several lncRNAs. More specifically, Kcnq1ot1 (KCNQ1 overlapping transcript 1) and Gm4117 are predicted to sequester all PTSD-related miRNAs (Table 6).

3.3.5. Correlation analysis between RNA expression and behavioral scores

Since we observed a dysregulated expression of mRNAs and miRNAs in the different brain areas in association with susceptible and resilient phenotypes, we evaluated whether RNA alterations were linearly related to the composite scores of behavioral tests in susceptible and resilient mice, including arousal score, avoidance-like

score, social memory score and PTSD-like score (Table 7). In HIP, we found a statistically significant inverse correlation between arousal score and expression levels of miR-15a-5p and miR-511-5p and, in contrast, a positive association between the same score and BDNF and FKBP5. These data suggest that the arousal level of mice segregated through the AIS model could be controlled by the hippocampal levels of BDNF, FKBP5 and their targeting miRNAs miR-15a-5p and miR-511-5p. On the other hand, we found no significant correlation between scores and expression of miRNAs and mRNAs in mPFC. These data were consistent with the observation that miRNA dysregulation in mPFC was associated with traumatic stress independently of susceptibility and that there was just a mild upregulation of BDNF and FKBP5 in susceptible mice.

Finally, we found a strong anti-correlation between avoidance-like score and hypothalamic expression of miR-511, while the same score showed a positive correlation with BDNF expression. The expression of miR-497a-5p in the hypothalamus was negatively correlated with arousal, avoidance score and PTSD-like scores.

Since we investigated the expression of multiple miRNAs and mRNAs, we evaluated whether they could be linearly associated with behavior. Linear regression analysis was performed considering all miRNAs and mRNAs together, or only miRNAs and only mRNAs. Similar to previous linear regression analysis, R2 evaluation showed that these miRNAs account for 96%, 78% and 83% of arousal, avoidance and PTSD-like score, respectively, in the hypothalamus. Moreover, in the hippocampus the arousal score may be regulated by mRNAs (27%) and, even more, by the combination of miRNAs and mRNAs (51%) (Table 8).

3.4. Discussion

A genetic background is not sufficient alone to properly explain individual differences in sensitivity to stress, since individual biological diversities are also found within genetically homogeneous populations, such as inbred mice (Hager, Jansen et al. 2014). Moreover, life experiences could interact with the genetic background producing long-lasting alterations in coping abilities later in life (Dirven, Homberg et al. 2017). For this reason, the development of behavioral and molecular profiling, discriminating susceptible and resilient mice to identify pro-adaptive or maladaptive mechanisms is fundamental. In the present observational study, we investigated

the expression of specific miRNAs and their potential targets in a long-term post-traumatic stress model. FKBP5 and BDNF were previously reported to play a role in the stress response as well as in post-traumatic stress disorder (Andero and Ressler 2012, Young, Inslicht et al. 2018, Duman and Girgenti 2019, Regue, Poilbout et al. 2019). FKBP5 belongs to the immunophilin protein family and it is known to play a role in GR (glucocorticoid receptor) transcriptional activation following the elevation of cortisol (Gillespie, Phifer et al. 2009) and its dysregulation may contribute to a maladaptive stress response (Scharf, Liebl et al. 2011, Hartmann, Wagner et al. 2012). In our previous study (Torrisi, Lavanco et al. 2021) and also in this study, we found a significant down-regulation of FKBP5 mRNA in hippocampus and hypothalamus of resilient mice, but not in mPFC, where its expression increased in susceptible animals. Previous studies have documented that FKBP5 knock-out mice exhibited a more resilient phenotype, characterized by lower corticosterone levels and better behavioral responses following restraint, social defeat stress, tail suspension test, forced swim, and enhanced cognitive flexibility in the radial arm water maze (O'Leary, Dharia et al. 2011, Touma, Gassen et al. 2011, Hartmann, Wagner et al. 2012, Sabbagh, O'Leary et al. 2014). On the other hand, FKBP5 expression in infralimbic mPFC (but not in the central prelimbic cortex) increased after fear conditioning in rats and remained elevated even after extinction, suggesting its active role in maladaptive stress response (Criado-Marrero, Morales Silva et al. 2017). Consistent with this animal study, Young et al. found increased levels of FKBP5 in the medial orbitofrontal cortex from post-mortem brain tissues of PTSD patients, which positively correlated with dendritic spine density (Young, Thompson et al. 2015). Our data, together with those previously cited, strongly suggest that FKBP5 plays distinct relevant roles in the different brain regions in stress-related phenotypes, but their complete functional cross-talking has to be still investigated.

BDNF is the most studied neurotrophin involved in neuroprotection, neurogenesis, neurodevelopment and synaptic plasticity processes required for long-term learning and memory (Minichiello 2009, Kowianski, Lietzau et al. 2018); consistently, its dysregulation was associated with stress-related disorders, such as PTSD (Duman and Monteggia 2006, Andero and Ressler 2012, Notaras and van den Buuse 2020). As previously reported (Torrisi, Lavanco et al. 2021), here we confirm that BDNF expression increased in hippocampus as well as in mPFC of susceptible mice, but not in hypothalamus, where its expression increased in resilient mice. Notwithstanding evidence showing that acute or chronic stress decreased BDNF expression, which could lead to the loss of synaptic function and increase vulnerability to insults (Kozlovsky, Matar et al. 2007, Niknazar,

Nahavandi et al. 2016), other studies reported BDNF overexpression in mice resulting in memory impairments (Cunha, Angelucci et al. 2009), while BDNF increased in the hippocampus, hypothalamus and pituitary gland from chronically stressed rats (Naert, Ixart et al. 2011). A progressive BDNF decrease at different time points after acute social defeat until 24 h after stress, with levels returning to baseline by day 5 post-stress was also demonstrated (Pizarro, Lumley et al. 2004). Taken together, these results, suggest a complex relationship between the intensity and duration of stress on BDNF levels, which would depend significantly also on the type of stress and the brain region being studied. The conflicting effects of stressors on BDNF expression and its related phenotypes show the complexity of stress-related behaviors. Moreover, this complexity could be found also in the structure of the BDNF gene. Use of different promoters and alternative splicing of the BDNF gene control tissue-specific expression of BDNF mRNA isoforms, which could have different functions in different brain areas (Tsankova, Kumar et al. 2004). Indeed, several studies demonstrated that epigenetic and post-transcriptional regulation of BDNF levels may depend on the type of neuron and stimulation (Bredy, Wu et al. 2007, Lubin, Roth et al. 2008, Chiaruttini, Vicario et al. 2009, Fuchikami, Yamamoto et al. 2010).

Animal studies have shown that dysregulation of miRNA levels on different brain regions can be associated with behavioral alterations (O'Connor, Gururajan et al. 2016). The MiR-15/107 family (also called miR-15/107 gene group) is highly expressed in brain tissues and includes multiple highly conservative miRNA members, including miR-497a-5p (Wang, Zhu et al. 2019). Our data would suggest that the consistent variations of miR-15a-5p, miR-497a-5p and miR-511a-5p and their targets in hippocampus and hypothalamus could potentially promote the resiliency processes by contributing to neuroadaptation (Russo, Murrough et al. 2012). On the other hand, miR-15a-5p, miR-511a-5p and let-7d-5p levels were reduced in mPFC of all stressed animals, suggesting that these miRNAs are not specifically involved in vulnerability and resiliency phenomena in this brain area, but rather in biological events related to the stress. Moreover, their expression was unrelated to BDNF and FKBP5, suggesting that mRNA modulation is controlled by other molecular factors in this brain area.

Although our molecular and computational data derive from a new and relatively unexplored PTSD-like model, accumulated evidence suggest the involvement of these miRNAs in a number of stress-related disorders, as well as in neurological diseases, thus supporting the results of this study (Li, Pan et al. 2020).

In this context Volk et al. showed reduced levels of amygdala miR-15a in mice exposed to chronic stress exhibiting an increase of anxiety-like behavior (Volk, Pape et al. 2016). Others groups confirmed the involvement of miR-511 associated with risk for stress-related disorders including major depression and PTSD, even if its role in susceptibility is still unclear (Zheng, Sabbagh et al. 2016, Xu, Wang et al. 2019). Depressed subjects had reduced miR-511 expression in the prefrontal cortex, as do adult rats following chronic unpredictable mild stress (CUMS) or dexamethasone administration in the adolescent period. On the other hand, long-term corticosterone treatment upregulated miR-511 expression in the cortex of mice (Zheng, Sabbagh et al. 2016, Xu, Wang et al. 2019, Yoshino, Roy et al. 2020). MiR-497 is considered a well know diagnostic biomarker in cancers, but to date, the role of miR-497 in PTSD remains unclear (Zhai, Liu et al. 2020). Some studies showed that miR-497 levels are low in the prefrontal cortex of depressed suicide patients but significantly increased in schizophrenic subjects inducing neuronal apoptosis, suggesting its involvement in psychiatric disorders (Yadav, Pandey et al. 2011, Smalheiser, Lugli et al. 2012, Banigan, Kao et al. 2013). Unlike the other analyzed miRNAs, levels of let-7d-5p did not change in the hippocampus, increased in the hypothalamus in stressed mice and decreased in mPFC of both groups. These results may suggest an involvement of let-7d-5p in stress related disorders but not in the discrimination of vulnerable and resilient phenotypes. Growing evidence indicates that let-7d might play a role in the neurobiology of psychiatric disorders (Maffioletti, Cattaneo et al. 2016). Let-7d is largely expressed in the brain and involved in modulating learning and memory (Shu, Qing et al. 2013, Bahi and Dreyer 2018); however, little is known about its role in the stress related disorders. Interestingly its upregulation was reported in the nucleus accumbens in a cocaine-conditioned place preference rat model and, in its circulating form, in animals that had undergone chronic social defeat (Chandrasekar and Dreyer 2011, Chen, Kelly et al. 2015).

Specific temporal and spatial expression of miRNAs in the brain is known, but the role of the area specificity in the gene regulatory networks has not yet been satisfactorily addressed (Shu, Qing et al. 2013). Our data, together with those previously published in other models, strongly suggest an involvement of these miRNAs in stress related disorders; their activity, in fact, seems to be brain-area specific and partially associated with the expression of their potential targets. This last observation would suggest that these miRNAs could also target different mRNAs other than BDNF and FKBP5, as well as that these mRNAs could be regulated by diverse miRNAs or other regulators. Moreover, these PTSD-related miRNAs showed several positive

correlations among their expressions in the different brain areas both in our model and in other human and mouse neuronal and not neuronal tissues, suggesting their co-regulation might be due to common transcriptional or epigenetic control. Indeed, the comparative analysis of miRNA promoters by screening of ENCODE chip-seq experiments showed a potential common regulation by transcription factors Cebpa/Cebpb (CCAAT Enhancer Binding Protein alpha/beta) and Spi1 (Transcription factor PU.1), at least for miR-497 and miR-511a. Cebpa/b transcriptionally control dozens of genes involved in neuro-differentiation and neuro-development in rat hippocampus, and is essential mediators of BDNF/Trk signaling in cortical neurons (Calella, Nerlov et al. 2007, Kfoury and Kapatos 2009). Inhibition of Sp1 function in transgenic Alzheimer's disease mice model increased memory deficits (Citron, Saykally et al. 2015); on the other hand, identification of lncRNAs predicted to bind miR-15a-5p, miR-497a-5p, miR-511a-5p and let-7d-5p showed several common lncRNAs potentially sponging and impairing their function. Among them, Kcnq1ot1 was reported to induce apoptosis or autophagy in neurons after different types of injuries by sequestering different miRNA species (Yu, Yu et al. 2019, Li, Liu et al. 2020, Wang, Tang et al. 2020). All these findings would suggest that these miRNAs are part of a complex transcriptional and post-transcriptional machinery that controls fundamental processes in neurons and, consequently, their dysregulation would contribute to molecular cascades induced by neuropsychological stress. Based on these considerations, the inverse correlation between arousal score and hippocampal expression of miR-15a-5p and miR-511-5p is not surprising and suggests the existence of a linear relationship between the arousal level of mice and miRNA levels that, in turn, coherently influences BDNF and FKBP5 transcripts. Moreover, the inverse correlation between miR-511-5p and avoidance-like score and, on the contrary, the positive relationship between BDNF levels and avoidance-like score, indicates that the proportional effects of BDNF on neuronal circuits regulating avoidance behaviors towards distressing memories would depend linearly on miR-511-5p expression in the hypothalamus. In the same brain area, miR-497a-5p expression was inversely related to arousal, social memory and PTSD-like scores, suggesting its functional role in PTSD-associated phenotypes. Linear regression models showed a synergic effect of expression of all miRNAs on modulation of behavioral scores in the hypothalamus. This observation, consistent with complex system biology, further indicates that complex phenotypes, such as specific psychiatric behaviors, are the combined result of expression modulation of different miRNAs, rather than the effect of an individual miRNA (Liufu, Zhao et al. 2017).

Because of the observational nature of this work, we didn't able to demonstrated the signaling from a mechanistic point of view, but only by using computational elaboration and statistical analysis.

However, this observational research had an important role in providing the information needed for further future validation in in vitro models.

In conclusion, we identified, in a new PTSD-like model, the brain area-specific alteration of four miRNAs, potentially regulating two PTSD critical genes, BDNF and FKBP5. MiRNA modulation could underlie the epigenetic mechanisms controlling BDNF and FKBP5 mRNAs that are associated with resilience or susceptibility to stress. Validation of these data on biological specimens from PTSD patients could provide valuable information for the prevention and treatment of stress-related psychiatric disorders.

3.5. Tables

Table 1. PCR primer sequences of mRNAs analyzed in this study

Gene	**Forward primer**	**Reverse primer**
FKBP5	5'-TGAGGGCACCAGTAACAATGG -3'	5'-CAACATCCCTTTGTAGTGGACAT-3'
BDNF	5'-GTTCGAGAGGTCTGACGACG-3'	5'-AGTCCGCGTCCTTATGGTTT-3'
GAPDH	5'-AGGTCGGTGTGAACGGATTTG-3'	5'-TGTAGACCATGTAGTTGAGGTCA-3'

Table 2. Expression correlation among miRNAs and BDNF and FKBP5.

Brain area	**Target**	**miR-15a-5p**	**miR-511-5p**	**miR-497a-5p**	**let-7d-5p**
HIP	BDNF	-0.09 (0.65)	-0.04 (0.82)	-0.04 (0.80)	-0.18 (0.36)
	FKBP5	**-0.40 (0.03)**	**-0.45 (0.02)**	**-0.43 (0.02)**	-0.07 (0.69)
HT	BDNF	**0.66 (0.005)**	**0.73 (0.01)**	**0.53 (0.03)**	**0.62 (0.01)**
	FKBP5	**-0.69 (0.002)**	**-0.60 (0.01)**	**-0.62 (0.022**)	-0.38 (0.20)
mPFC	BDNF	-0.10 (0.60)	-0.38 (0.10)	-0.01 (0.94)	-0.32 (0.09)
	FKBP5	0.03 (0.85)	-0.20 (0.29)	0.06 (0.76)	-0.16 (0.39)

Pearson coefficient (r) and p-values (between brackets) are reported. Bold typed values are statistically significant.

Table 3. **Linear regression models built on combinations of 4, 3 or 2 PTSD-related miRNAs.**

Brain area	Target	miRNA combination	**R**	**R^2**	**p-value**
HIP	FKBP5	let-7d-5p, miR-15a-5p, miR-497a-5p, miR-511-5p	**0.657**	**0.431**	**0.024**
		miR-15a-5p, miR-497a-5p, miR-511-5p	**0.652**	**0.425**	**0.01**
		miR-497a-5p, miR-511-5p	**0.641**	**0.411**	**0.004**
	BDNF	let-7d-5p, miR-15a-5p, miR-497a-5p, miR-511-5p	0.191	0.037	0.936
		let-7d-5p, miR-15a-5p, miR-511-5p	0.191	0.037	0.841
		let-7d-5p, miR-15a-5p	0.186	0.035	0.656
HT	FKBP5	let-7d-5p, miR-15a-5p, miR-497a-5p, miR-511-5p	**0.749**	**0.56**	**0.044**
		miR-15a-5p, miR-497a-5p, miR-511-5p	**0.741**	**0.549**	**0.019**

		miR-15a-5p, miR-511-5p	**0.719**	**0.517**	**0.009**
mPFC	FKBP5	let-7d-5p, miR-15a-5p, miR-497a-5p, miR-511-5p	0.429	0.184	0.322
		let-7d-5p, miR-15a-5p, miR-511-5p	0.42	0.177	0.206
		let-7d-5p, miR-15a-5p	0.323	0.105	0.251
	BDNF	let-7d-5p, miR-15a-5p, miR-497a-5p, miR-511-5p	**0.597**	**0.357**	**0.038**
		let-7d-5p, miR-15a-5p, miR-511-5p	**0.587**	**0.345**	**0.019**
		let-7d-5p, miR-511-5p	**0.487**	**0.237**	**0.039**

For each combination of miRNAs, were reported: the R-value, representing the coefficient of correlation, the R^2 value, representing the proportion of variance of a dependent variable that may be explained by an (or more) independent variable(s) and the p-value. Bold typed values are statistically significant.

Table 4. Correlation matrix of miRNA expression in a PTSD-like model

	HIP			HT			Mpfc		
	miR-15a-5p	let-7d-5p	miR-511-5p	miR-15a-5p	let-7d-5p	miR-511-5p	miR-15a-5p	let-7d-5p	miR-511-5p
let-7d-5p	**0.71 (<0.0001)**			**0.88 (<0.0001)**			**0.79 (<0.0001)**		
miR-511-5p	**0.38 (0.04)**	**0.39 (0.04)**		**0.70 (0.002)**	**0.63 (0.008)**		0.32 (0.09)	0.18 (0.36)	
miR-497a-5p	0.25 (0.19)	0.35 (0.06)	**0.64 (0.0003)**	0.39 (0.12)	0.38 (0.13)	**0.62 (0.01)**	**0.57 (0.001)**	**0.54 (0.003)**	0.14 (0.46)

Pearson coefficient (r) and p-values (between brackets) are reported. Bold typed values are statistically significant.

Table 5. Comparison Matrix of TFs shared by miRNAs based on ChIp data

	miR-15a	let-7d	miR-497a
let-7d	**Cebpa, Spi1**		
miR-497a	Bcl11b, Brd4, Cbfb, **Cebpb**, Ctcf, Ep300, Erg, Ets1, Hdac2, Ikzf1, Max, Med1, Myc, Nelfe, Nr1d1, **Nr3c1**, Otx2, Pou5f1, Pparg, Rad21, **Rela**, Smarca4, **Spi1**, Suz12, Tfap4, Zfp281, Zfp384	**Spi1**	
miR-511	Batf, Btaf1, **Cebpa**, **Cebpb**, Mafb, **Nr3c1, Rela**, **Spi1**, Tcf12	**Cebpa, Spi1**	**Cebpb**, Fli1, **Nr3c1**, **Rela**, **Spi1**, Stat5a

TFs typed in bold are shared by several miRNA pairs.

Table 6. Comparison Matrix of lncRNAs predicted to bind PTSD-related miRNAs

	miR-15a-5p	let-7d-5p	miR-497a-5p
let-7d-5p	**Gm20605**, **Kcnq1ot1**, **Ptprv**, **Gm4117**		
miR-497a-5p	**Gm20605**, **Ptprv**, **ENSMUSG00000093535**, **Gm11579**, **Rab10os**, **Kcnq1ot1**, **Gm4117**	**Gm20605**, **Kcnq1ot1**, **Ptprv**, **Gm4117**, Mecomos	

miR-511-5p	**<u>Kcnq1ot1</u>**, **ENSMUSG00000093535**, **<u>Gm4117</u>**, **Gm11579**, ENSMUSG00000085287, **Rab10os**	**<u>Kcnq1ot1</u>**, Gm26856, **<u>Gm4117</u>**, Zfp950	**<u>Kcnq1ot1</u>**, **ENSMUSG00000093535**, **<u>Gm4117</u>**, **Gm11579**, **Rab10os**

LncRNAs typed in bold and underlined are shared by all miRNA pairs; lncRNAs typed in bold are shared by several miRNA pairs.

Table 7. Correlations between RNA expression and behavioral scores

	HIP			
	Arousal	Avoidance	Social Memory	PTSD-like
miR-15a-5p	**-0.44 (0.02)**	-0.33 (0.08)	-0.12 (0.56)	-0.31 (0.11)
let-7d-5p	-0.08 (0.68)	0.26 (0.20)	0.30 (0.13)	0.16 (0.42)
miR-511-5p	**-0.44 (0.02)**	-0.14 (0.49)	-0.10 (0.61)	-0.34 (0.08)
miR-497a-5p	-0.22 (0.28)	-0.12 (0.56)	-0.10 (0.61)	-0.24 (0.22)
FKBP5	**0.39 (0.04)**	-0.04 (0.84)	-0.09 (0.65)	0.11 (0.61)
BDNF	**0.47 (0.01)**	-0.02 (0.89)	-0.10 (0.62)	0.27 (0.18)
	HT			
	Arousal	Avoidance	Social Memory	PTSD-like
miR-15a-5p	-0.26 (0.31)	-0.27 (0.30)	-0.11 (0.66)	-0.26 (0.31)
let-7d-5p	-0.12 (0.64)	-0.20 (0.45)	0.09 (0.72)	-0.07 (0.79)
miR-511-5p	-0.13 (0.62)	**-0.63 (0.007)**	-0.31 (0.23)	-0.39 (0.12)
miR-497a-5p	**-0.61 (0.01)**	**-0.57 (0.01)**	-0.37 (0.15)	**-0.67 (0.004)**
FKBP5	-0.02 (0.92)	0.22 (0.39)	0.27 (0.29)	-0.17 (0.51)

BDNF	-0.25 (0.33)	**-0.54 (0.02)**	-0.37 (0.15)	-0.46 (0.07)
	mPFC			
	Arousal	Avoidance	Social Memory	PTSD-like
miR-15a-5p	0.09 (0.65)	-0.10 (0.60)	0.10 (0.61)	0.04 (0.83)
let-7d-5p	0.11 (0.58)	-0.13 (0.53)	0.18 (0.38)	0.13 (0.52)
miR-511-5p	-0.09 (0.67)	-0.37 (0.07)	-0.09 (0.64)	-0.19 (0.34)
miR-497a-5p	-0.02 (0.92)	-0.27 (0.19)	-0.15 (0.45)	-0.15 (0.45)
FKBP5	0.27 (0.18)	0.16 (0.41)	0.18 (0.37)	0.30 (0.12)
BDNF	-0.27 (0.19)	0.16 (0.43)	0.28 (0.16)	0.02 (0.89)

Pearson coefficient (R) and p-values (between brackets) are reported. Bold typed values are statistically significant.

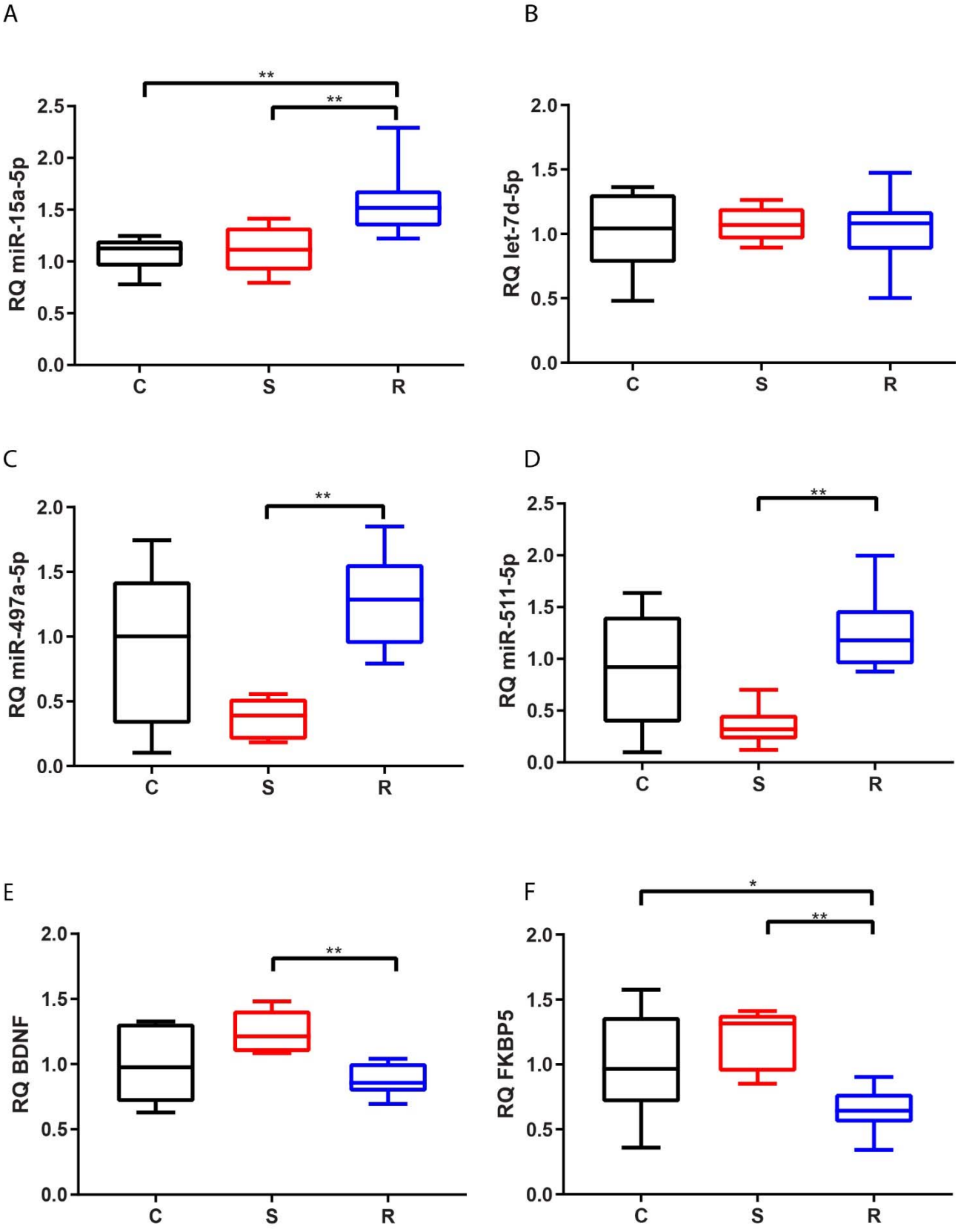
Table 8. Synergic effects of analyzed RNAs on behavioral scores.

		HIPPOCAMPUS			HYPOTHALAMUS			mPFC		
		R	R^2	p-value	R	R^2	p-value	R	R^2	p-value
Arousal score	miRNA+mRNAs	**0.72**	**0.519**	**0.032**	0.563	0.317	0.791	0.385	0.149	0.88
	MiRNA	0.442	0.196	0.335	**0.981**	**0.962**	**0.00022**	0.199	0.04	0.937
	mRNAs	**0.523**	**0.273**	**0.03**	0.274	0.075	0.761	0.103	0.011	0.885
Avoidance score	miRNA+mRNAs	0.529	0.28	0.402	0.728	0.53	0.449	0.503	0.253	0.632
	miRNA	0.349	0.122	0.606	0.711	0.506	0.304	0.488	0.238	0.247
	mRNAs	0.133	0.018	0.822	0.688	0.473	0.106	0.038	0.001	0.983
Social memory score	miRNA+mRNAs	0.691	0.477	0.058	0.884	0.782	0.094	0.541	0.293	0.526
	miRNA	0.512	0.263	0.172	**0.884**	**0.781**	**0.035**	0.207	0.043	0.928
	mRNAs	0.241	0.058	0.518	0.725	0.525	0.074	0.254	0.065	0.464

PTSD-like score	miRNA+mRNAs	0.699	0.488	0.05	0.69	0.476	0.541	0.531	0.282	0.554
	miRNA	0.502	0.252	0.194	**0.915**	**0.838**	**0.015**	0.33	0.109	0.681
	mRNAs	0.347	0.121	0.244	0.541	0.293	0.298	0.162	0.026	0.737

For each model, built on miRNAs+mRNA, only miRNAs or only mRNAs, the R-value, representing the coefficient of correlation, the R2 value, representing the proportion of variance of a dependent variable that is/may be explained by an (or more) independent variable(s) and the p-value are reported. Bold typed values are statistically significant.

3.6. Figures


A
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RQ let-7d-5p
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RQ miR-497a-5p
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RQ miR-511-5p
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RQ BDNF
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C
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R
F
*
**
RQ FKBP5
2.0
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0.0
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R

Figure 8. *Expression of miR-15a-5p (A), let-7d-5p (B), miR-497a-5p (C), miR-511-5p (D), BDNF (E), FKBP5 (F) in hippocampus of control (C=12), susceptible (S=7) and resilient (R=11) mice. *P-value <0.05, **P-value <0.01, ***P-value <0.001.*

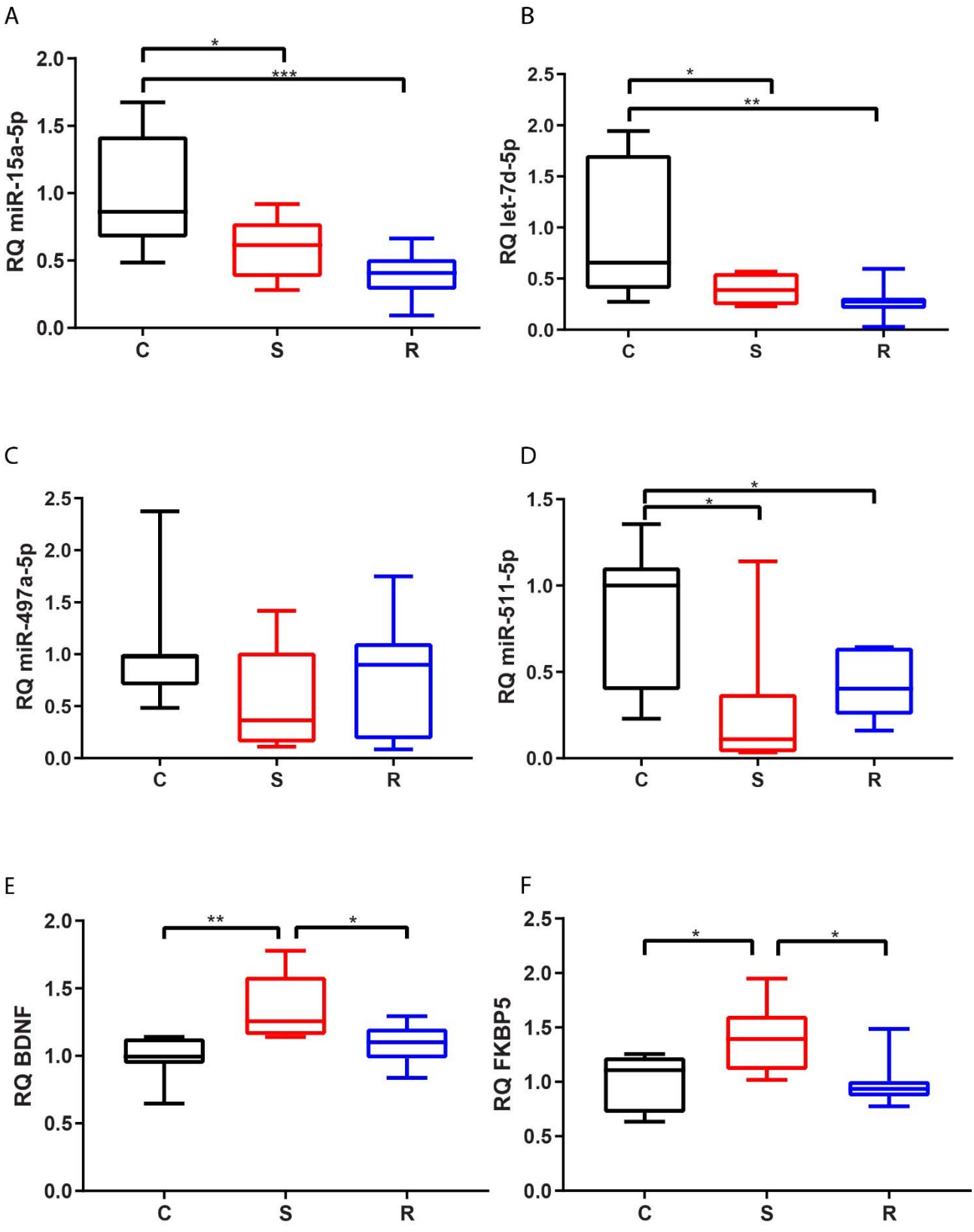
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Figure 9. *Expression of miR-15a-5p (A), let-7d-5p (B), miR-497a-5p (C), miR-511-5p (D), BDNF (E), FKBP5 (F) in mPFC of control (C=12), susceptible (S=7) and resilient (R=11) mice. *P-value <0.05, **P-value <0.01, ***P-value <0.001.*

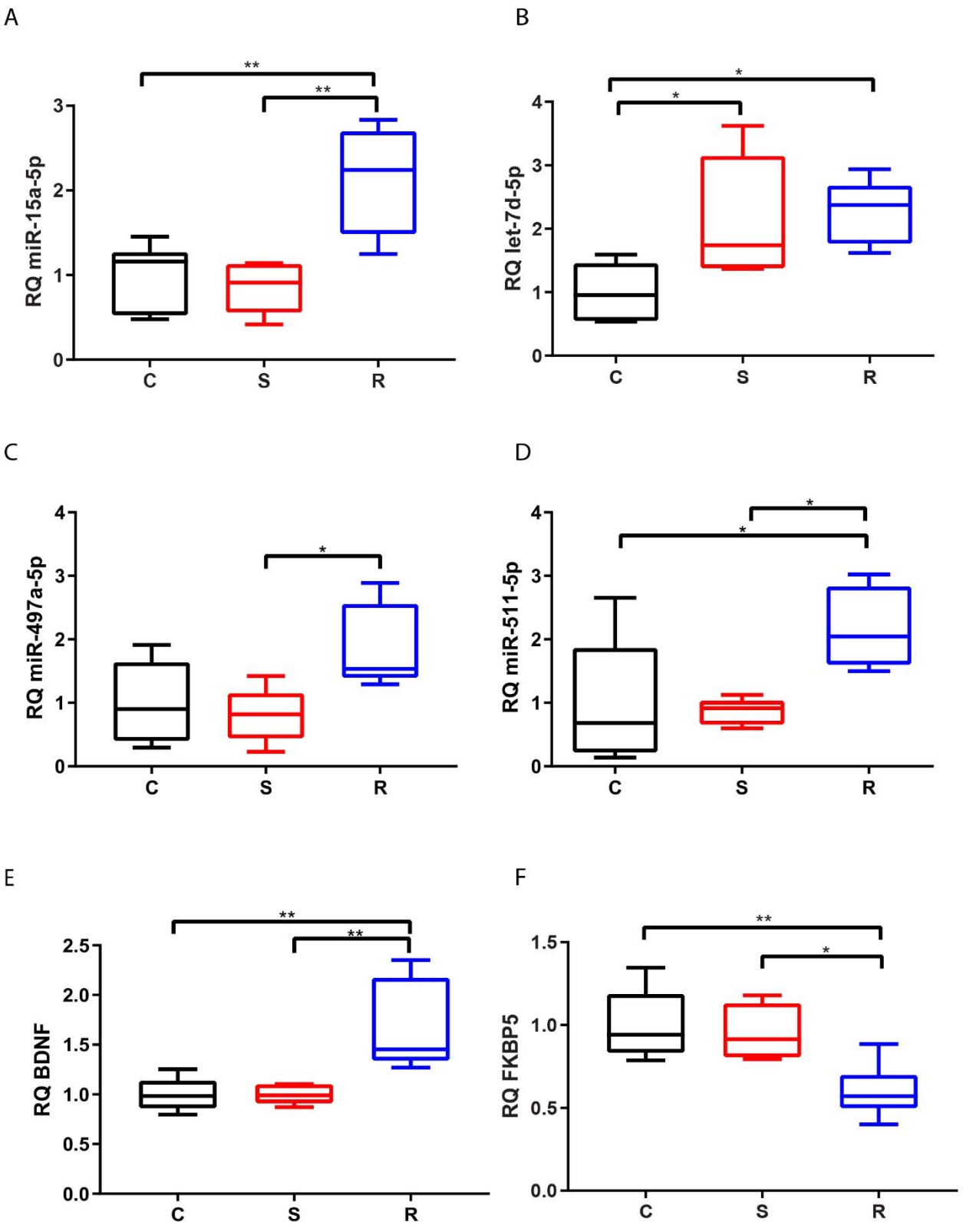

Figure 10. *Expression of miR-15a-5p (A), let-7d-5p (B), miR-497a-5p (C), miR-511-5p (D), BDNF (E), FKBP5 (F) in hypothalamus of control (C=6), susceptible (S=5) and resilient (R=5) mice. *P-value <0.05, **P-value <0.01, ***P-value <0.001.*

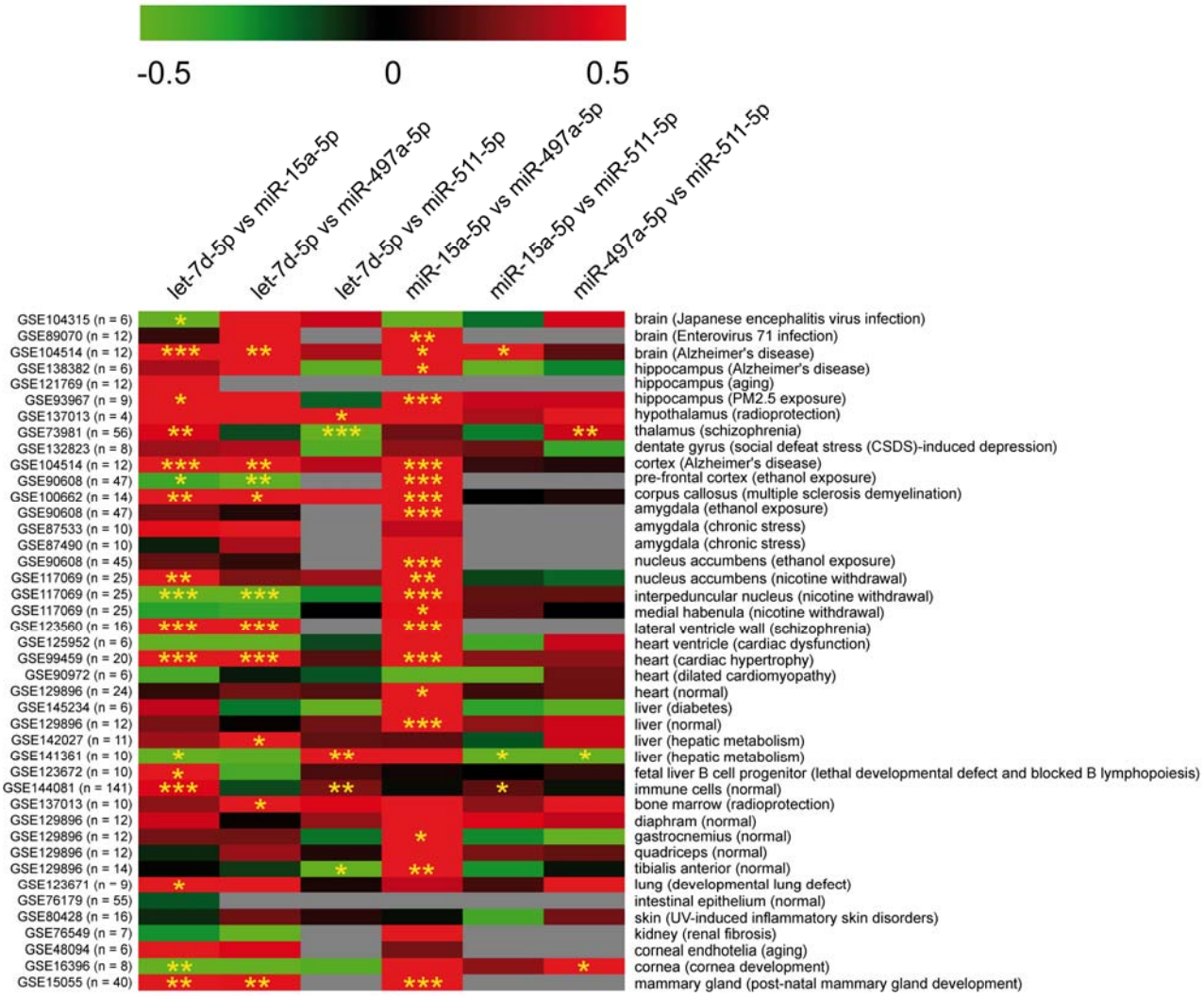

Figure 11*. Correlation matrix of miRNA expression from GEO data in Mus musculus.* The correlation coefficient is indicated by a colour gradient from green (negative correlation) to red (positive correlation), as shown in the coloured bar. GEO IDs and number of samples (between brackets) are reported on the left of the matrix. Grey is used for datasets where correlation for the specific pair was impossible to calculate (expression data for at least one of the miRNAs not available). Tissues and diseases (between brackets) are reported on the left of the matrix. Statistically significant p-values are indicated by asterisks; **P-value <0.05, **P-value <0.01, ***P-value <0.001.*

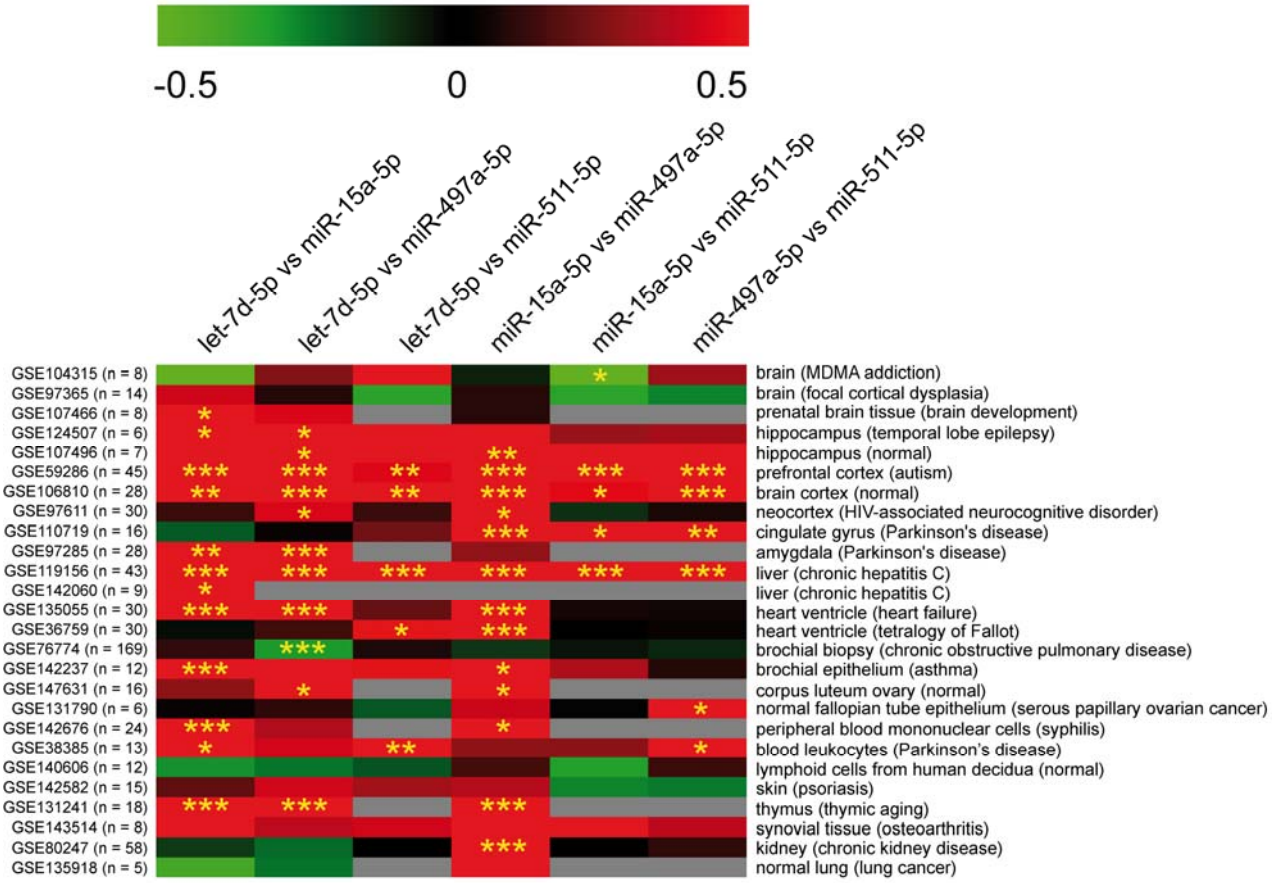

Figure 12. *Correlation matrix of miRNA expression from GEO data in Homo sapiens.* The correlation coefficient is indicated by a colour gradient from green (negative correlation) to red (positive correlation), as shown in the coloured bar. GEO IDs and number of samples (between brackets) are reported on the left of the matrix. Grey is used for datasets where correlation for the specific pair was impossible to calculate (expression data for at least one of the miRNAs not available). Tissues and diseases (between brackets) are reported on the left of the matrix. Statistically significant p-values are indicated by asterisks; **P-value <0.05, **P-value <0.01, ***P-value <0.001.*

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CRediT authorship contribution statement

Oriana M. Maurel: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing-original draft. Sebastiano A. Torrisi: Data curation, Investigation, Methodology, Review original draft. Cristina Barbagallo: Data curation, Formal analysis, Investigation, Writing-original draft. Michele Purrello: Resources, Review original draft. Filippo Drago: Funding acquisition, Project administrator, Resources, Supervision, Review original draft. Salvatore Salomone: Funding acquisition, Project administrator, Resources, Supervision, Review original draft. Marco Ragusa: Conceptualization, Validation, Data curation, Methodology, Supervision, Resources, Writing-original draft. G. M. Leggio: Funding acquisition, Project administrator, Resources, Supervision, Review original draft.

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4. General discussions and conclusions

Although exposure to major psychological trauma is a common factor, the risk to develop PTSD, varies greatly among individuals (Holmes and Singewald 2013). This underlines the importance of individual differences in vulnerability arising from a combination of life history, exposure to stress and trauma, predisposing genetic and biological factors (Auxemery 2012). Rapid progress has been made through the identification of key molecular circuits, epigenetic mechanisms, and gene variants associated with differences in resilience and susceptibility, however several mechanisms are not fully understood (Wu, Feder et al. 2013). Studies in rodent are providing new knowledge into neural system dysfunctions associated with PTSD (Barre-Sinoussi and Montagutelli 2015). To date, even if the most important neural circuitry implicated in PTSD in humans is related to the neural circuitry of fear, it is unquestionable that, a model of PTSD based entirely on Pavlovian fear conditioning may not be sufficient to explain the associated symptoms (Krystal, Abdallah et al. 2017). Thus, the AIS model was developed, to look for a select group of symptoms (Richter-Levin, Stork et al. 2019), based on the difference between susceptible and resilient phenotype. This new traslational PTSD-like model cover multiple aspects listed in DSM-5 for PTSD diagnosis ((2013) DSM-5 Task Force), starting from 24h restraint stress, which in terms of severity went successfully beyond the coping abilities of mice. Employing the ASR, supported by a mathematical model Z-normalization we were able to capture the individual trauma susceptibility/resilience according to long-term change of startle reactivity at different post-trauma time points. The discrimination between susceptible and resilient individuals was relevant in our work, given that only few PTSD preclinical studies involving mice have included this aspect (Olson, Rockett et al. 2011, Sillivan, Joseph et al. 2017). More specifically, the segregation obtained through the AIS model is consistent with PTSD diagnosis based on long-term symptoms (criterion F of DSM-5). Our electrophysiological findings showed an impairment in LTP hippocampal CA1, as well as, the high basal level of serum corticosterone exclusively in susceptible mice. This data further validated the AIS model and support the hypothesis PTSD symptoms could be a "synaptic disconnection syndrome" (Krystal, Abdallah et al. 2017). Moreover the AIS model might represent a new tool to identify new pharmacological strategies for SSRI resistant individuals with PTSD. Here we found that paroxetine tended to worsen the hyperarousal of susceptible mice, as show in clinical data (Krystal, Abdallah et al. 2017), however paroxetine exerted anxiolytic-like effects in susceptible mice (Huang,

Liu et al. 2014) and ameliorated the social memory impairment of susceptible mice assessed in the 5-trial SM test, which is a hippocampal-dependent task (Hitti and Siegelbaum 2014). Life experiences could interact with the genetic background producing long-lasting alterations in coping abilities later in life (Dirven, Homberg et al. 2017). For this purpose, we assessed the expression level of particular transcriptional factor involved in PTSD. In particular, we found alterations of FKBP5, BDNF, SGK1 and NC3R1 mRNA confirming a different molecular profiling between susceptible and resilient mice (Scharf, Liebl et al. 2011, Hartmann, Wagner et al. 2012). Thus, brain adaptation to stress involves coordinated and region-specific changes in gene expression. In brain regions affected by stress, microRNAs are important regulators of neuronal plasticity and synaptic transmission (Schratt, Tuebing et al. 2006, Schouten, Aschrafi et al. 2013, O'Connor, Gururajan et al. 2016). In our study, we investigated also the expression of miR-15a-5p, miR-497a-5p, let-7d-5p, miR-511-5p in relation with their putative targets FKBP5 and BDNF (Andero and Ressler 2012, Young, Inslicht et al. 2018, Duman and Girgenti 2019, Regue, Poilbout et al. 2019). We observed that in hypothalamus and hippocampus their expression increase in resilient mice, which in turn could contribute alone or synergically to downregulate their mRNA targets. These data would suggest that variations both in miRNAs expression and in their targets could potentially promote the resiliency processes by contributing to neuroadaptations in these specific brain regions (Russo, Murrough et al. 2012). However, different molecular factors could be involved in the mRNA modulation at level brain regions as suggested by lack of correlation among BDNF, FKBP5 and miRNAs in mPFC. The data on mPFC suggested that miRNAs were not specifically involved in vulnerability and resiliency phenomena in this brain area but rather in biological events of the trauma. Moreover, in the same area this miRNA was functionally unrelated to BDNF and FKBP5, suggesting that mRNA modulation is controlled by other molecular factors. Unlike the other miRNAs studied, levels of let-7d-5p did not change in the hippocampus but increased in the hypothalamus of traumatized mice. These results may suggest an involvement of let-7d-5p in the stress endured but not in the discrimination of vulnerable or resilient phenotypes. These observations would suggest that in this specific PTSD-like model these miRNAs could also target other mRNAs other than BDNF and FKBP5. The latter could be regulated by diverse miRNAs or other regulators. Moreover, in AIS model miRNAs showed several positive correlations among their expressions in the different brain areas. Both in our model and in other human and mouse neuronal and not neuronal tissues, suggesting their co-regulation due to common transcriptional or epigenetic control. Through comparative

analysis of miRNA promoters by screening of ENCODE chip-seq experiments we were able to detect common transcriptional factor (Cebpa/Cebpb and Spi1) at least for miR- 15a, miR-497 and miR-511 involved in brain processes (Calella, Nerlov et al. 2007, Kfoury and Kapatos 2009, Citron, Saykally et al. 2015) and several common lncRNAs potentially sponging that could impair their function (i.e., Kcnq1ot1) involved in brain processes (Yu, Yu et al. 2019, Li, Liu et al. 2020, Wang, Tang et al. 2020). The inverse correlation between behavioral scores and the expression of some miRNAs suggest their involvement in the modulation of some behavioral trait (arousal, avoidance, social memory). In particular, in hypothalamus, miR-497a-5p expression was inversely related to arousal, social memory and PTSD-like scores, suggesting its functional centrality in PTSD-associated phenotypes. Furthermore, our linear regression model showed a synergic effect of expression of all miRNAs on modulation of behavioral scores in the hypothalamus. These data reveals that specific psychiatric alteration, are the combined result of expression modulation of different miRNAs, rather than the effect of an individual miRNA (Liufu, Zhao et al. 2017). Overall, the AIS model could be a translational and comprehensive tool that may serve for studying PTSD and, more in general, trauma susceptibility/resilience. It might be beneficial for the development of new and more effective pharmacological and psychological interventions for PTSD. Moreover, all molecular findings would suggest that these miRNAs as well as the genes target are part of a complex transcriptional and post-transcriptional signaling that controls fundamental processes in neurons and, consequently, their dysregulation would contribute to molecular cascades induced by neuropsychological stress.

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