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ALTERED GUT MICROBIOTA COMPOSITION AND TRAUMATIC STRESS SUSCEPTIBILITY: NEW INSIGHTS INTO THE PATHOPHYSIOLOGY OF PTSD

PhD Thesis

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LIST OF ABBREVIATIONS

PTSD	Post-traumatic stress disorder
GF	Germ-free
SSRIs	Serotonin reuptake inhibitors
SCFAs	Short-chain fatty acid
MDD	Major depressive disorder
GABA	γ-aminobutyric acid
IBS	Irritable bowel syndrome
5-HT	Serotonin
GI	Gastrointestinal
ABX	Antibiotics
SPF	Specific-pathogen free
HDAC	Histone-deacetylase
GPCRs	G protein-coupled receptors
GB	Gut-brain
ASDs	Autism spectrum disorders
2BAs	Secondary bile acids
Trp	Tryptophan
EECs	Enteroendocrine cells
ECCs	Enterochromaffin cells
GM	Gut-microbiota
CNS	Central-nervous system
FXR	Farnesoid X receptor
FGF	Fibroblast grow factor
GLP-1	Glucagon-like peptide-1
TNF-α	tumor necrosis factor-α
MAMPs	Microbe-associated molecular patterns
MHC II	Major histocompatibility complex II

AHR	Aril hydrocarbon receptor
EAE	Experimental autoimmune encephalomyelitis
BBB	Blood-brain barrier
NE	Norepinephrine
DA	Dopamine

PREFACE

Post-traumatic stress disorder (PTSD) is a psychiatric disorder that occur after the exposure to a traumatic event. The onset of PTSD is about 5 – 31% indicating that the exposure to a trauma is not necessary and sufficient to the development of this pathology (Berger et al., 2009; Girgenti et al., 2017; Torrisi et al., 2019). The animal model used to date for the study of this pathology have provided important results, but several reports have outlined the importance to develop new animal models for the study of PTSD (Berardi et al., 2014; Richter-Levin et al., 2019).

Different factors have been considered and correlated with predisposition to develop PTSD (Yehuda et al., 2015) but less is known about the correlation with microbiota alterations.

The microbiota consists of all microorganisms (bacteria, virus, fungi, archaea and eukaryote) that cohabit with a host, and in the last years, there has been an increased interest in the microbiota composition and its correlation with psychopathology onset (Cenit et al., 2017; Iannone et al., 2019).

The role of microbiota in brain functionality was demonstrated by several works. The lack of microbiota in germ-free (GF) mice causes different physiological and behavioural alterations such as immature microglia and a reduced anxiety-like behaviour (Cryan et al., 2019).

The microbiota bidirectionally communicates with the brain by the gut-brain axis (Mayer et al., 2015) and produces several metabolites that bind receptors on the intestinal epithelium and through the vagus nerve influence the brain. Furthermore, some of these metabolites can cross the blood brain barrier (BBB) and reach the brain (Martin et al., 2018).

The main metabolite produced by microbiota are short chain fatty acids (SCFAs), which the most abundant are butyrate, acetate and propionate,

neuroactive molecules such as dopamine, serotonin and γ-aminobutyric acid (GABA), and metabolites derived from tryptophan (Trp) metabolism.

Several data correlate alterations in the gut microbiota with psychopathologies. It was observed that anxiety, major depressive disorder (MDD), Parkinson's disease, Alzheimer's disease and irritable bowel syndrome (IBS), seems to be correlated with alteration in the microbiota composition (Cryan et al., 2019). However, the role of gut microbiota in PTSD has not yet been studied. Thus, the central hypothesis of my PhD thesis was that microbiota alterations could be involved in the pathophysiology of PTSD. In particular, the present thesis aimed: 1) To develop a new translational animal model for the study of crucial features of PTSD; 2) To evaluate microbiota alterations that occurs in control and trauma-exposed mice; 3) To evaluate metabolomic and molecular changes that could be correlated with PTSD onset.

CHAPTER I

GENERAL INTRODUCTION

1. Post-traumatic stress disorder

PTSD is a condition developed following the exposure to a traumatic event such as natural disaster, violence, or life-threatening accidents. The risk to develop PTSD range from 5 to 31% (Berger et al., 2009; Girgenti et al., 2017; Torrisi et al., 2019). The diagnosis of PTSD is based on criteria of Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5), according to which an individual must respect some criteria (Astill Wright et al., 2019; Torrisi et al., 2019):

- Criterion A: exposure to a traumatic event
- Criterion B: exhibit intrusive symptoms
- Criterion C: avoidance symptoms
- Criterion D: negative alteration in mood and cognitions
- Criterion E: hyperarousal symptoms
- Criterion F: symptoms must last for more than 1 month
- Criterion G: symptoms have to cause functional impairment
- Criterion H: exclude that these symptoms are linked to the use of medications or other psychiatric disorder

PTSD generally co-occur with other pathologies among which the most commons are mood and anxiety disorders and substance-use disorders that are associated with medical illness and increased premature death (Shalev et al., 2017). To date the only two drugs approved for the treatment of PTSD are two selective serotonin reuptake inhibitors (SSRIs), sertraline and paroxetine (Torrisi et al., 2019).

Understand which mechanisms are involved in the pathophysiology of PTSD is important for the correct development of new pharmacological target for the treatment of patients.

For this purpose, animal models are an important tool for the study of this pathology. Those used to date are the stress enhanced fear learning, a model of sensitized responding to threat, in which rodents are exposed to 15-shock stressor non-associatively which enhances subsequent fear conditioning training with a single trial. Another model is the single-prolonged stress which consists of 2h of restraint followed by 20 min of forced swim and ether anaesthesia (Richter-Levin et al., 2019).

Although these animal models have provided important results for the study of PTSD, several reports have outlined the need to develop a higher translational model for the study of this neuropsychopathology (Berardi et al., 2014; Deslauriers et al., 2018; Richter-Levin et al., 2019).

To date, it is known that there are different factors that could be involved in the susceptibility to PTSD, among which could be considered an altered HPA axis activation (Herman et al., 2016), genetic predisposition (Yehuda et al., 2015), sex differences (Tolin & Foa, 2006), and personal history (Hyer et al., 2021).

Among these risk factors the microbiota is the less studied and it is becoming an interesting aspect even because it seems to be involved in different pathologies (Cenit et al., 2017; Iannone et al., 2019).

Microbiota could be considered another “organ” that regulate different host aspects and functions. Host and microbiota are mutually interconnected through the secretion of metabolite, hormones, and neurotransmitters (Cryan et al., 2019).

Different factors could influence microbiota composition, and consequently the metabolite produced. Diet, stress and antibiotic drugs are just some of the factors that can alter bacteria composition in the gut (Cryan et al., 2019). For the impacts of the microbiota on the brain and vice versa it could be interesting investigate if there is a correlation between PTSD-susceptibility/resilience and microbiota composition.

2. THE MICROBIOTA

The microbiota is composed by all microorganisms (bacteria, archaea, virus, and fungi) that cohabit in different district of an organism. The collective genome of the microbiota is referred as microbiome even if both terms are used interchangeably.

Phylogenetic trees of intestinal bacterial microbiota and primates demonstrate a coevolution of microbiota and host with an inter-species transmission of microbes across generations (Dominguez-Bello et al., 2019; Ochman et al., 2010).

Microbiota composition is determined by different factors such as born delivery, antibiotics use and stress. With the birth the infant comes into contact with the mother vaginal microbiota that is rich, for example, in *Lactobacillus* and *Prevotella* spp. (Dominguez-Bello et al., 2010). However, C-section delivered babies seems to be dominated by bacteria typically from skin such as *Staphylococcus* spp. and decreased colonization of *Bifidobacterium, Bacteroides,* and *Lactobacillus.* Comparting C-section born versus vaginal delivered infants it was observed that the first one has a higher risk to develop neonatal infection from *Staphylococcus aureus* and *Clostridium difficile* (Cryan et al., 2019).

The host-microbiota relation is of mutual benefit; indeed, the microbiota receives nutrients by the host's diet and the microbiota lives in a stable environment producing several metabolites/compounds having several benefits for the host. It was demonstrated that gut bacteria can produce several neurotransmitters, such as serotonin (5-HT) and GABA, which can modulate host functions. In mammals, 5-HT plays different roles ranging from host behaviour modulation to gastrointestinal (GI) motility. GABA is the major inhibitory neurotransmitter of the host nervous system, and it was shown that bacteria of the genus *Escherichia* spp. and *Lactobacillus* spp. can synthetize

GABA, although the importance of the bacterial production of GABA in the host intestinal lumen is unclear (Cryan et al., 2019).

The microbiota is involved also in the maturation and the modulation of the immune system as observed in GF mice which exhibit defects in lymphoid tissue development (Al Nabhani & Eberl, 2020; Gensollen et al., 2016).

In addition to the release of metabolite the gut communicates with the brain through the vagus nerve (Bonaz et al., 2018). However, the gut-brain communication is not unidirectional but bidirectional. Factors such as stress can alter the microbiota composition. Indeed, it was observed that acute or repeated stress induce an increased levels of intestinal IgA which impact intestinal homeostasis, inflammatory response, and possibly induce dysbiosis (Moloney et al., 2016).

Different works have been focalized on the gut-brain communication, for this purpose GF mice are a valid tool to study how the microbiota could influence brain development. Indeed, it was observed that GF mice have several deficit concerning microglia maturation (Cryan et al., 2019), exaggerated HPA axis activity (Dinan & Cryan, 2012) and reduced anxiety-like behaviour (Foster et al., 2017).

Similar results were obtained through treatment with antibiotics (ABX). Mice treated with ABX showed impairments in fear extinction. However, these alterations could be restored through the colonization with specific-pathogen free (SPF) microbial communities (Chu et al., 2019).

Dysbiosis is also correlated with the development of an inflammatory state. The SCFAs produced in the gut, (butyrate, acetate, and propionate the main components), are inhibitory of histone-deacetylase (HDACs) and ligands of G protein-coupled receptors (GPCRs). SCFAs inhibiting HDACs promote a tolerogenic and anti-inflammatory state that promote immune homeostasis, supporting the concept that microbiota can regulate epigenetically the host physiology (Cryan et al., 2019; Rooks & Garrett, 2016).

Alterations in the microbiota composition were observed in different neuropsychiatric disorders such as schizophrenia, anxiety, autism spectrum disorder, Parkinson and Alzheimer disease (Cenit et al., 2017; Iannone et al., 2019).

3. GUT-BRAIN AXIS

Bidirectional alterations in the gut-brain (GB) axis are believed to be involved in the pathogenesis of different pathologies such as irritable bowel syndrome (IBS), and recently in other neuropsychiatric disorders including autism spectrum disorders (ASDs), Parkinson's disease and disorders of mood (Mayer et al., 2015).

Gut and brain communication occurs primarily through neuroimmune and neuroendocrine mechanisms, often involving the vagus nerve (Martin et al., 2018). The communication is mediated by different metabolites such as short-chain fatty acid (SCFA), secondary bile acids (2BAs), and tryptophan (Trp) metabolites (Tolhurst et al., 2012; Wikoff et al., 2009; Yano et al., 2015). These molecules propagate signals mainly through the interaction with enteroendocrine cells (EECs), enterochromaffin cells (ECCs), and the mucosal immune system. However, some of this metabolite cross the gut epithelium and through the circulation could reach the brain and cross the blood-brain barrier (BBB) (Haghikia et al., 2015; Samuel et al., 2008; Yano et al., 2015).

In addition to these metabolites, the gut microbiota (GM) produces numerous neuroactive products including GABA, serotonin (5-HT), norepinephrine (NE) and dopamine (DA) (Asano et al., 2012; Barrett et al., 2012; Shishov et al., 2009).

An important pathway by which the GM communicate with the central nervous system (CNS) is through the EECs which contain more than 20 molecules. Chemical or physical stimuli induce a release of these molecules which could enter in the systemic circulation and reach directly the CNS or act indirectly through the vagus nerve activation. Different receptor associated with satiety and hunger have been identified on these cells which are activated by bile acids and SCFAs (Martin et al., 2018).

SCFAs are produced by fermentation of host dietary-resistant starch and nonstarch polysaccharides. These molecules have been implicated as major signalling molecules mediating host-microbe communication via EECs and ECCs. SCFAs stimulate the production of peptide YY and glucagon-like peptide-1 (GLP-1) by L cells located in the ileum inducing satiety and behavioural changes (Topping & Clifton, 2001).

Another well characterized GB interaction is the communication between microbes, ECCs and central nervous system. The 95% of 5-HT is stored in ECCs and only 5% in CNS (Kim & Camilleri, 2000). Considering the central role of serotonin in the gut motility is obvious that natural selection has promoted that microorganism able to regulate serotoninergic system to modulate the environment by influencing transit times and fluid secretions. SCFAs and 2BAs produced by GM regulate the percentage of ECC 5-HT production and release (Yano et al., 2015).

GF mice shown a significant increase plasma level of Trp and hippocampal 5-HT. Colonization with bacteria normalize Trp levels but didn't affect 5-HT levels in hippocampus (Clarke et al., 2013).

Another way of communication between gut and brain is the immune systems. Microglia are the most abundant resident immune cells in the brain that represent 5-20% of glial cells (Fung et al., 2017; Nayak et al., 2014).

The microbiota influence microglia maturation and function. It was demonstrated that GF mice, compared to control group, have increased number of immature microglia. This increase in immature microglia could be reproduced through treatment of conventional mice with antibiotics (Erny et al., 2015). It was also demonstrated that there is an altered gene expression profile comparing newborn from GF mice compared to the one from conventionally, suggesting that metabolites produced by maternal microbiota may contribute to early life programming of microglia development (Matcovitch-Natan et al., 2016).

Microglia from GF adult mice showed also impairment in immune response, displaying altered morphology and reduced production of interleukin (IL)-6, IL-1ß, and tumor necrosis factor-α (TNF-α) (Erny et al., 2015; Matcovitch-Natan et al., 2016), alterations that could be reversed through SCFAs administration (Borre et al., 2014; Erny et al., 2015).

Astrocytes exhibit different functions including the regulation of the BBB integrity, ion gradient balance, neurotransmitter turnover, cerebral blood flow and nutrient transport (Jensen et al., 2013; Khakh & Sofroniew, 2015). Astrocyte integrate information coming from microglia, neurons, vascular and immune cells, regulating neuronal excitability and synapse formation (Rossi, 2015). Even if astrocytes are not considered CNS resident-immune cells they exhibit immune-related functions, expressing receptors for the recognition of microbe-associated molecular patterns (MAMPs) and the major histocompatibility complex II (MHC II) (Dong & Benveniste, 2001). Furthermore, the gut microbiota can modulates astrocyte activity via microbial metabolites that activate astrocyte aril hydrocarbon receptor (AHR) (Rothhammer et al., 2016).

Type I interferon signaling in astrocytes attenuate inflammation and symptoms of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. This effect is mediated in part by activation of AHR. Gut microorganisms metabolize Trp producing natural AHR ligands such as indole-3-aldehyde and indole-3-propionic acid. Depletion of microbiota thorugh antibiotic treatment worsens EAE symptoms that could be reversed through supplementation with Trp metabolites (Rothhammer et al., 2016; Wikoff et al., 2009; Zelante et al., 2013).

Studies investigating the role of specific bacteria in the regulation of the immune system demonstrated that filamentous bacteria, which are associated with the epithelium, promote the development of IL-17A-producing T helper 17 cells (TH17) (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009).

Colonization of GF mice with this type of bacteria stimulate EAE compared to GF control mice (Lee et al., 2011). Other bacteria displayed protective effects. *Bacteroides fragilis* is a species that induce the production of the anti-inflammatory IL-10 via capsular expression of polysaccharide A (PSA) (Round et al., 2011; Round & Mazmanian, 2010). Colonization of mice with wild-type *B. fragilis* reduce EAE severity while the colonization using a PSA-deficient *B. fragilis* restore EAE susceptibility (Ochoa-Reparaz et al., 2010; Ochoa-Reparaz et al., 2009).

In addition to T-cell mediated CNS inflammation, B cells play an important role in the generation of relative CNS antibody and are involved in the pathogenesis of several neuroinflammatory disorders (Matsushita et al., 2008; Pollinger et al., 2009).

Streptococcal infection is often associated with production of antibodies involved in the development of autoimmune neuropsychiatric disorders associated with streptococcal infection (PANDAS). In PANDAS animal model, mice are immunized with group A ß-hemolytic streptococci developing behavioral abnormalities. Transfer of complete not IgG-depleted serum from immunized mice is sufficient to induce behavioral deficits, suggesting that just antibodies produced by immunization are sufficient to develop PANDAS (Yaddanapudi et al., 2010).

Furthermore, antibodies produced by proteins derived from *E. coli,* in particular the heat shock protein ClpB, induce a decreased food intake (Tennoune et al., 2014).

4. GUT-BRAIN AXIS AND DISEASES

Recently, clinical and pre-clinical studies demonstrated that gut-microbiota alteration are implicated in different psychopathologies. The state of evidence is different between disorders. Some are in a preliminary stage, based only on limited correlation in clinical studies, while there is stronger evidence for a casual role of the microbiota in other disease processes (Cryan et al., 2019).

Autism spectrum disorder (ASD) is a heterogenous group of neurodevelopmental disorders characterized by deficit in cognition, sociability, anxiety disturbance and repetitive behavior (Masi et al., 2017).

ASD children reported comorbidity with gastro-intestinal (GI) disturbance (Stephen et al., 2017) and treatment with the broad-spectrum antibiotic vancomycin improve behavioral and gastrointestinal symptoms (Kang et al., 2017; Sandler et al., 2000). Other studies have identified significant changes in the microbiota composition with reduced abundance of beneficial bacteria such as *Bifidobacterium* and increased of potential pathogenic *Desulfovibrio* and *Clostridia* genera (Gora et al., 2018).

Major Depressive Disorder (MDD) is another psychopathology which showed alteration in the microbiota composition. It is known that MDD is correlated with increased pro-inflammatory cytokines (Ticinesi et al., 2017), which in turn activate the HPA axis increasing the hyperactivation associated with depression (Chopra et al., 2011). Most of the study for the correlation between depression and microbiota alteration were conducted in preclinical models (Dinan & Cryan, 2017). GF mice showed a decreased depressive-like behavior (Zheng et al., 2016) and it was observed that some probiotic and prebiotic intervention are able to reduce these symptoms and ameliorate inflammatory responses (Bravo et al., 2011; Burokas et al., 2017; Desbonnet et al., 2010; Tillmann et al., 2019).

Anxiety generally is associated with depression, most of the data about anxiety and microbiota changes come from preclinical studies (Foster & McVey Neufeld, 2013). GF rats showed an exaggerated anxiety response (Crumeyrolle-Arias et al., 2014). A small clinical study on healthy controls demonstrated that a multi-strain probiotic has anxiolytic effects (Colica et al., 2017).

Many preclinical studies have demonstrated that stress impacts gut microbiota composition (Golubeva et al., 2015; Partrick et al., 2018; Tay et al., 2017). Indeed, psychological stressors have been shown to change the gut microbiota (Bailey et al., 2011; Bharwani et al., 2016; De Palma et al., 2015; Hsiao et al., 2013; O'Mahony et al., 2009; Sun et al., 2013). Furthermore, it was observed that the maternal microbiota influences the offspring microbiota and is correlated with hyper-reactivity of the HPA axis (Jasarevic et al., 2018).

AIM OF THE THESIS

According to the data in literature the aim of this PhD project was:

1) To design a long-lasting animal model able to recapitulate a PTSD-like phenotype which was able to include most of the criteria used for the diagnosis of PTSD.

2) To analyse molecular changes occurring in PTSD susceptible and resilient sub-populations of mice to uncover novel transcriptional signatures driven by PTSD-related genes as well as dysfunction of hypothalamic-pituitary-adrenal axis.

3) To estimate LTP in hippocampus in susceptible and resilient mice

4) To investigate pre- and post- microbiota changes in susceptible and resilient mice to study a possible correlation between gut-brain axis alterations and PTSD onset

5) To analyse the possible gut-brain modules and gut-metabolic modules alterations using the KEGG Orthology database

CHAPTER II

“AROUSAL BASED INDIVIDUAL SCREENING REVEALS SUSCEPTIBILITY AND RESILIENCE TO PTSD-LIKE PHENOTYPES IN MICE”

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

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A B S T R A C T

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

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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. 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.

2.1. 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. Experimental design

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. 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).

2.2.3. Experiment 3: assessment of avoidance-like behavior, 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.

2.2.4. 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.

2.2.5. 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).

2.2.6. 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).

2.2.7. 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).

2.3. 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.

2.4. 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.

2.5. 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.

2.5.1. 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.

2.5.2. 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.

2.5.3. 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.

2.5.4. 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.

2.5.5. 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).

2.6. 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).

2.6. 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.

2.7. 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).

2.8. 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.

2.9. 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.

2.10. 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.

3. Results

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, $F(2, 197) = 17.52$, $P < 0.0001$).

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.

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).

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, $r = 0.78$, $P = 0.0006$).

With regard to the PTSD candidate genes mRNA expression in the whole blood, a significant down-regulation of both FKBP5 (Fig. 5B, Stress susceptibility, $F(2, 12) = 10.82$, $P = 0.0021$) and SGK1 (Fig. 5D, Stress susceptibility, $F(2, 12) = 3.945$, $P = 0.048$) was detected in susceptible but not resilient mice. Furthermore, a significant negative correlation between the arousal score and the expression of FKBP5 (Fig. 5C, $r = -0.79$, $P = 0.0066$) but not SGK1 (Fig. 5E) in the whole blood of mice was revealed.

Regarding the HPA axis function, there were no differences in pre-trauma basal corticosterone level between subpopulations. Interestingly, it was detected a significant higher post-trauma basal corticosterone level exclusively in susceptible mice compared to both their pre-trauma basal corticosterone level, and compared to the post-trauma basal corticosterone level of control and resilient mice, (Fig. 5F, Stress susceptibility, $F(2, 21) = 4.984$, $P = 0.0169$; Time $F(1, 21) = 7.306$, $P = 0.0133$; Stress susceptibility x Time $F(2, 21) = 5.474$, $P = 0.0122$). Also, a significant positive correlation between the arousal score and the post-trauma corticosterone level was detected (Fig. 5G, $r = 0.75$, $P = 0.0006$).

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).

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 pharmacological 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.

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.

Credit authorship contribution statement

Sebastiano A. Torrisi: Conceptualization, Formal analysis, Writing - original draft, conceived and designed the study, performed behavioral and pharmacological experiments, analyzed behavioral and pharmacological data, analyzed qPCR data, wrote the manuscript. Gianluca Lavanco: Formal analysis, performed behavioral and pharmacological experiments, analyzed behavioral and pharmacological data. Oriana M. Maurel: Formal analysis,

performed qPCR experiments, analyzed qPCR data. Walter Gulisano: Formal analysis, performed electrophysiological experiments, analyzed electrophysiological data. Samuele Laudani: Formal analysis, performed qPCR experiments, performed ELISA assay, analyzed ELISA data, analyzed qPCR data. Federica Geraci: performed behavioral and pharmacological experiments. Margherita Grasso: performed behavioral and pharmacological experiments. Filippo Caraci: performed behavioral and pharmacological experiments. Claudio Bucolo: Supervision, performed qPCR experiments, supervised qPCR experiments. Marco Ragusa: performed qPCR experiments. Francesco Papaleo: Supervision, supervised behavioral and pharmacological experiments. Patrizia Campolongo: Conceptualization, Supervision, conceived and designed the study, supervised behavioral and pharmacological experiments. Daniela Puzzo: Formal analysis, performed electrophysiological experiments, analyzed electrophysiological data. Filippo Drago: Supervision, supervised behavioral and pharmacological experiments. Salvatore Salomone: Conceptualization, Supervision, conceived and designed the study, supervised qPCR experiments. Gian Marco Leggio: Conceptualization, Supervision, Writing - original draft, conceived and designed the study, supervised behavioral and pharmacological experiments, wrote the manuscript.

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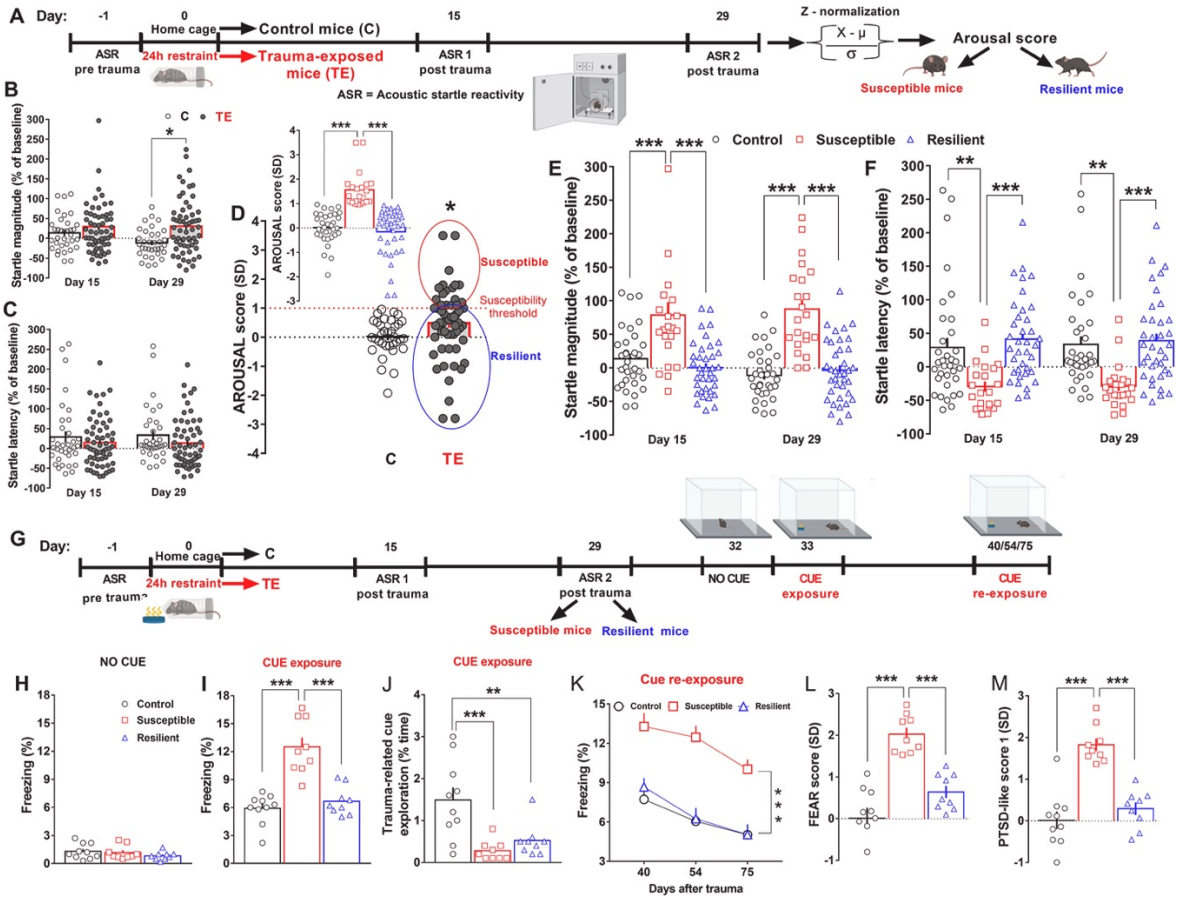

Fig. 1. Susceptible mice exhibited long-lasting hyperarousal and exaggerated fear responses to trauma-related cue, while resilient mice adopted active coping strategies showing normal behavior. (A) Experimental procedure conceived to shape the AIS model, which identified susceptible and resilient subpopulations. (B) Startle magnitude (% of baseline, Stress, $F(1, 91) = 6.244$, $P = 0.0143$) 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 (Stress susceptibility, $F(2, 90) = 35.66$, $P < 0.0001$). (E) Startle magnitude (% of baseline, Stress susceptibility, $F(2, 90) = 32.65$, $P < 0.0001$) and (F) startle latency (% of baseline, Stress susceptibility, $F(2, 90) = 11.35$, $P < 0.0001$) 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 (Stress susceptibility, $F(2, 25) = 27.09$, $P < 0.0001$). (J) Exploration (% time) of the trauma-related cue during the cue exposure session (Stress susceptibility, $F(2, 25) = 10.27$, $P < 0.0001$). (K) Freezing behavior (% time) expressed during the cue re-exposure sessions (Stress susceptibility, $F(2, 25) = 21.27$, $P < 0.0001$; Time, $F(2, 50) = 25.69$, $P < 0.0001$). (L) FEAR score (Stress susceptibility, $F(2, 25) = 28.8$, $P < 0.0001$). (M) PTSD-like score 1 (Stress susceptibility, $F(2, 25) = 30.62$, $P < 0.0001$). 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. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

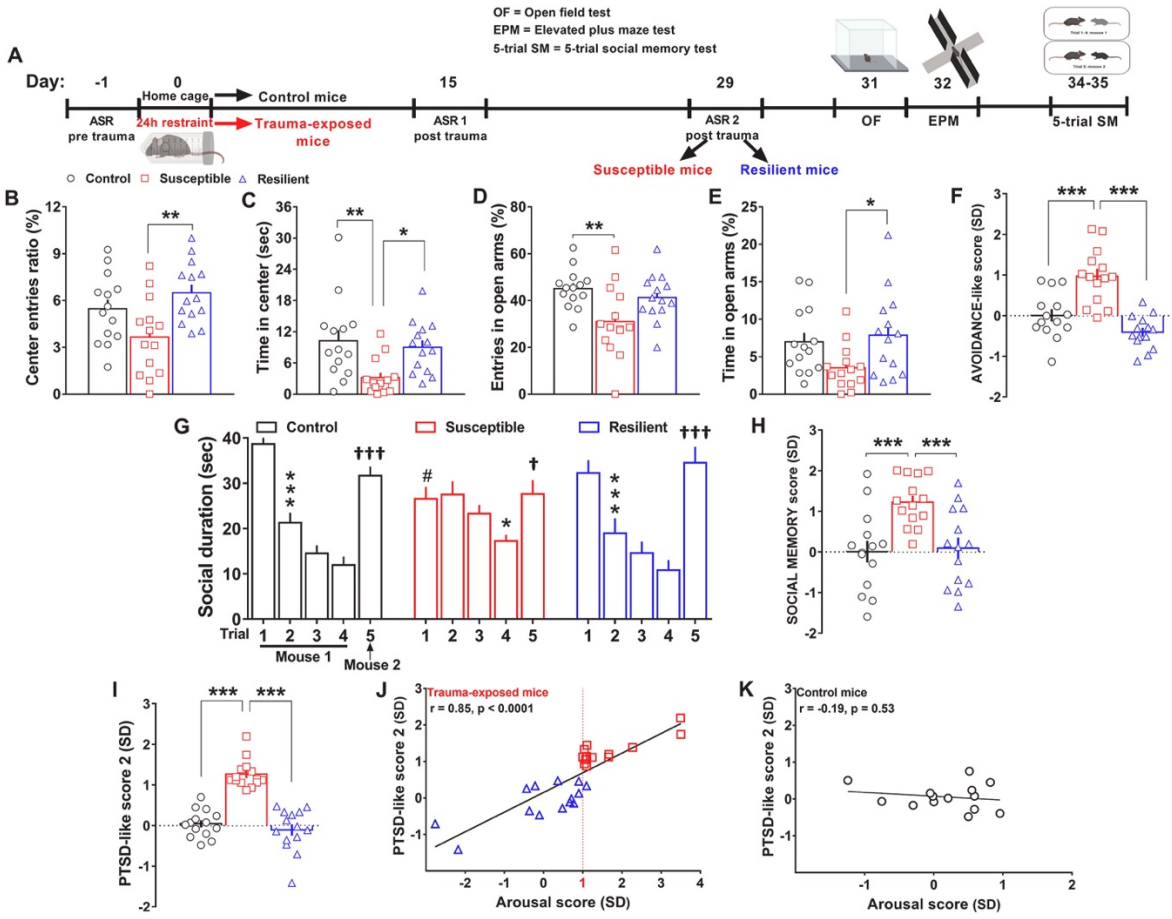

Fig. 2. Susceptible mice identified by the AIS model displayed increased avoidance-like behavior and social memory impairment, while resilient mice behaved similar to control mice. (A) Experimental procedure conceived for the assessment of control (n = 14), susceptible (n = 14) and resilient mice (n = 14) in the OF test, EPM test and 5-trial SM test. (B) Center entries ratio (Stress susceptibility, $F(2, 39) = 5.957$, $P = 0.0055$) and (C) time spent in center of the OF (Stress susceptibility, $F(2, 39) = 6.237$, $P = 0.0045$). (D) Entries in open arms (Stress susceptibility, $F(2, 39) = 5.526$, $P = 0.0077$) and (E) time in open arms of EPM (Stress susceptibility, $F(2, 39) = 3.629$, $P = 0.036$). (F) AVOIDANCE-like score (Stress susceptibility, $F(2, 39) = 22.24$, $P < 0.0001$). (G) Social duration during the 5-trial SM test [Trial, $F(4, 156) = 52.49$, $P < 0.0001$, Stress susceptibility 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 (Stress susceptibility, $F(2, 39) = 8.604$, $P < 0.0001$). (I) PTSD-like score 2 (Stress susceptibility, $F(2, 39) = 47.31$, $P < 0.0001$). (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.

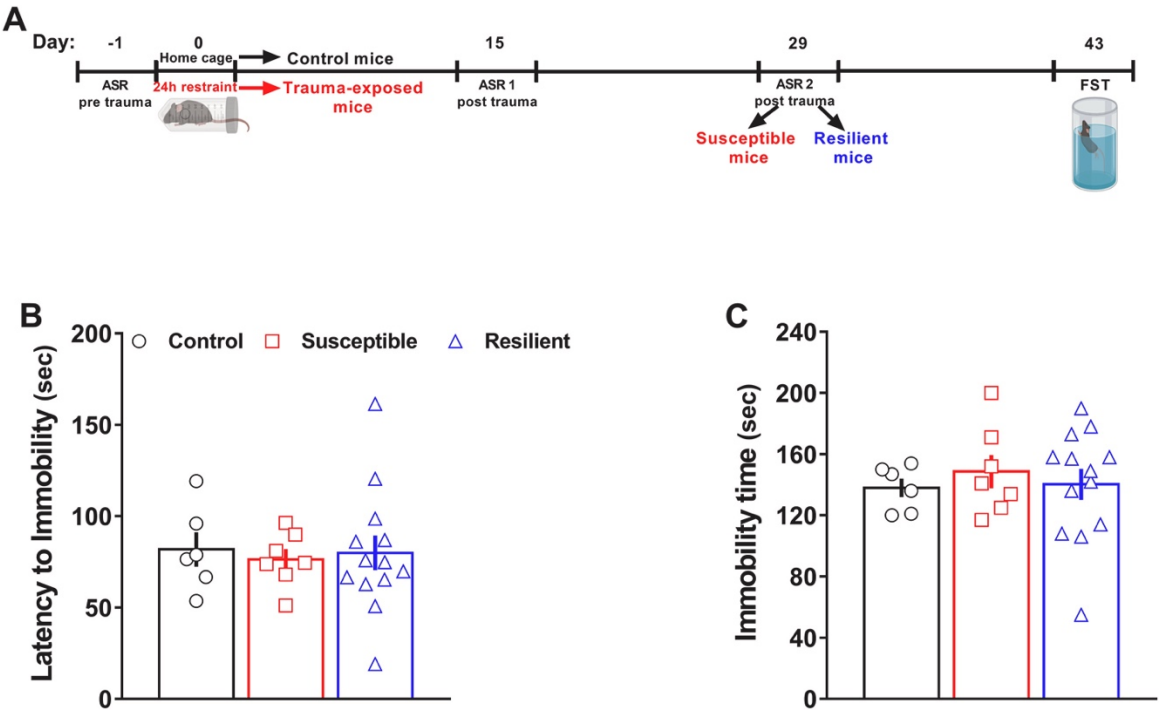

Fig. 3. Both susceptible and resilient mice did not exhibit depressive-like phenotypes. (A) Experimental timeline designed for the long-term assessment of control (n = 6), susceptible (n = 6) and resilient (n = 14) mice tested in the FST. (B) Latency to immobility and (C) total immobility time. One-way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

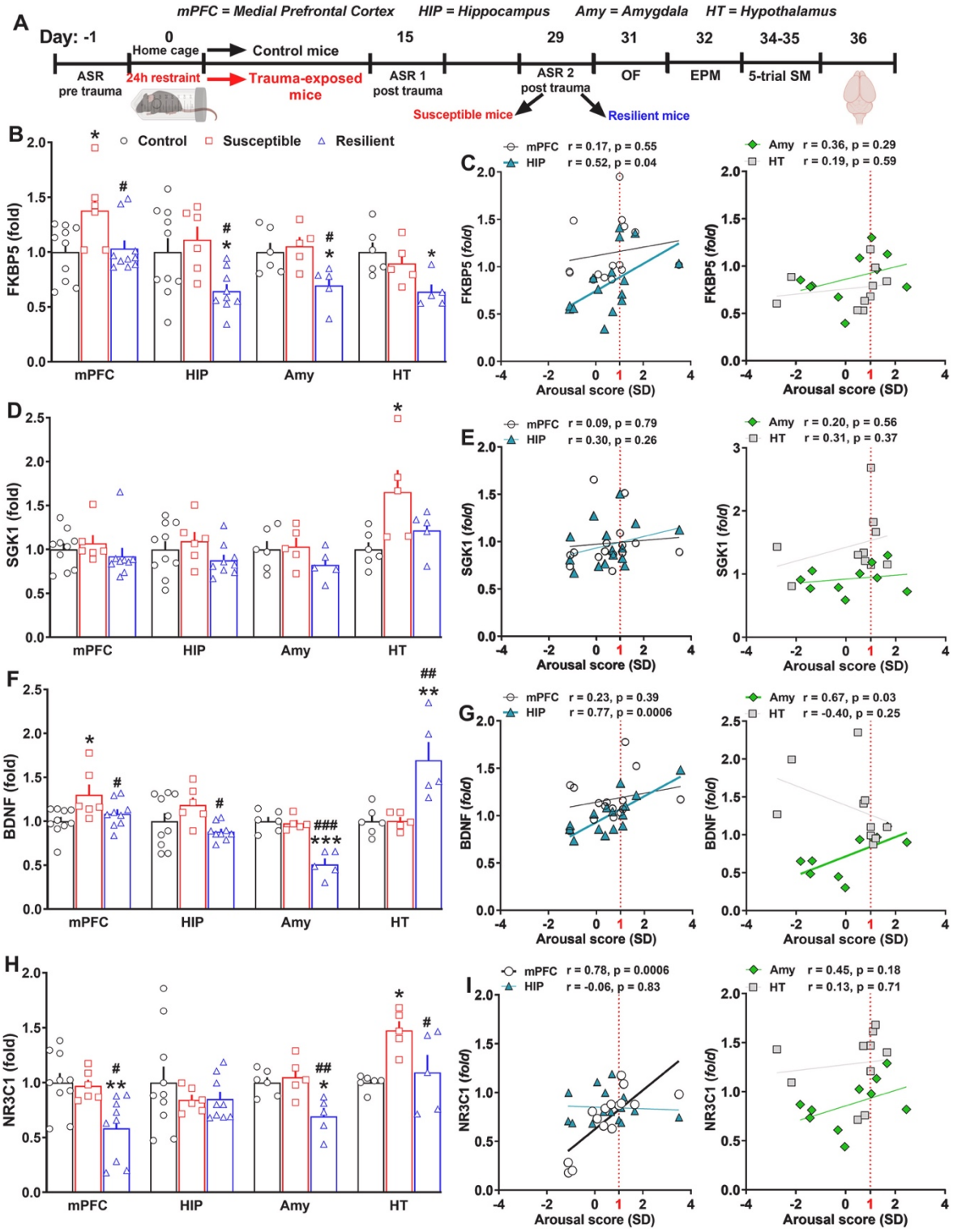
mPFC = Medial Prefrontal Cortex
HIP = Hippocampus
Amy = Amygdala
HT = Hypothalamus
A
Day: -1
0
15
29
31
32
34-35
36
Home cage
Control mice
ASR pre trauma
24h restraint
Trauma-exposed mice
ASR 1 post trauma
ASR 2 post trauma
OF
EPM
5-trial SM
Susceptible mice
Resilient mice
B
Control
Susceptible
Resilient
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

Fig. 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 (Stress susceptibility, $F(2, 22) = 5.055$, $P = 0.0156$), HIP (Stress susceptibility, $F(2, 22) = 4.931$, $P = 0.017$), Amy (Stress susceptibility, $F(2, 13) = 5.132$, $P = 0.022$) and HT (Stress susceptibility, $F(2, 13) = 5.235$, $P = 0.0215$). (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 (Stress susceptibility, $F(2, 13) = 4.318$, $P = 0.0303$). (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 (Stress susceptibility, $F(2, 22) = 4.65$, $P = 0.0206$), HIP (Stress susceptibility, $F(2, 22) = 3.50$, $P = 0.047$), Amy (Stress susceptibility, $F(2, 13) = 27.79$, $P < 0.0001$) and HT (Stress susceptibility, $F(2, 13) = 10.7$, $P = 0.0018$). (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 (Stress susceptibility, $F(2, 22) = 7.429$, $P = 0.0034$), HIP, Amy (Stress susceptibility, $F(2, 13) = 7.958$, $P = 0.0055$) and HT (Stress susceptibility, $F(2, 13) = 6.471$, $P = 0.0112$). (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.

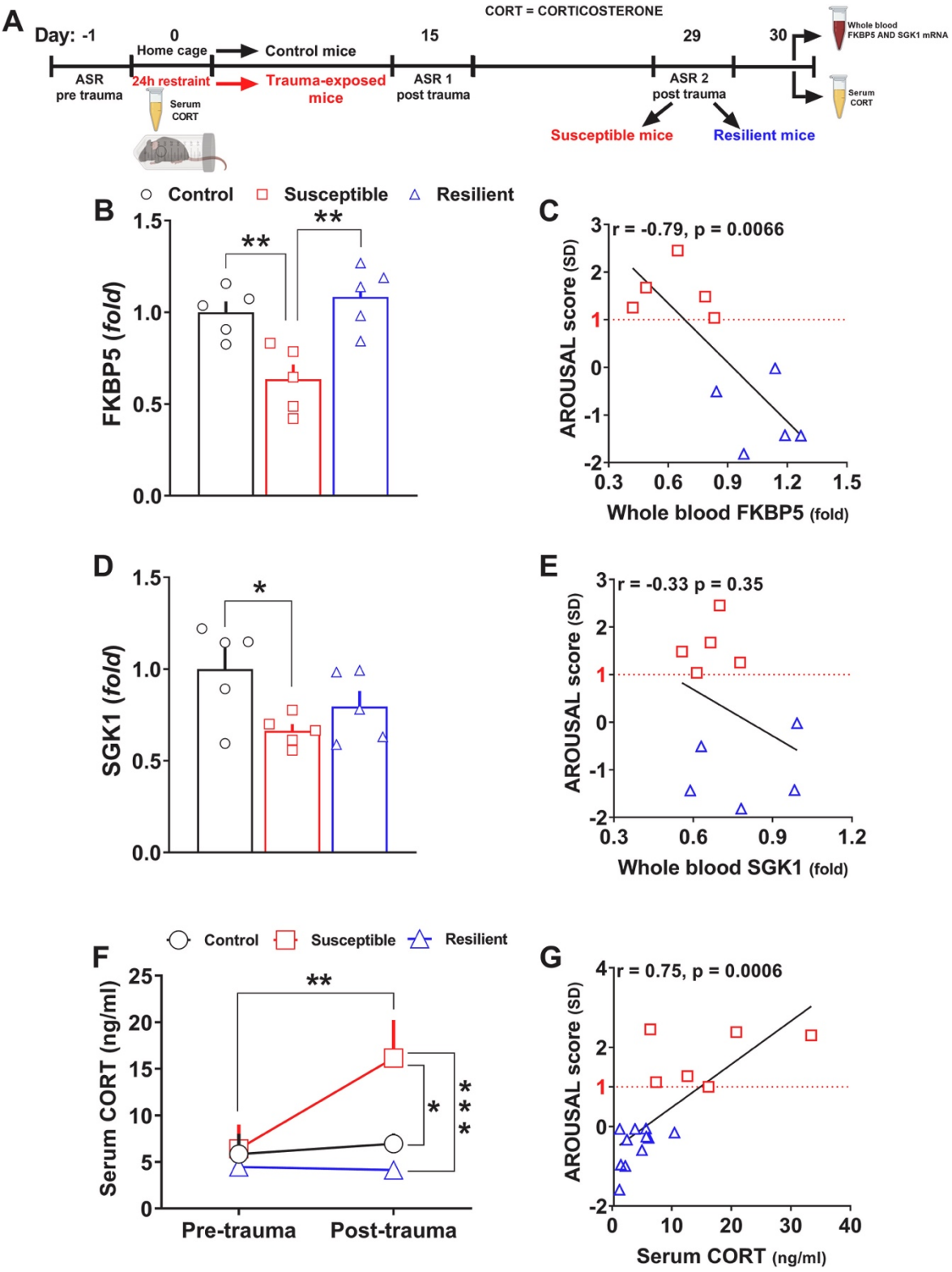
A
CORT = CORTICOSTERONE
Day: -1
0
Home cage
Control mice
15
29
30
Whole blood
FKBP5 AND SGK1 mRNA
ASR
pre trauma
24h restraint
Trauma-exposed
mice
ASR 1
post trauma
ASR 2
post trauma
Serum
CORT
Serum
CORT
Susceptible mice
Resilient mice
B
Control
Susceptible
Resilient
FKBP5 (fold)
C
r = -0.79, p = 0.0066
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)

Fig. 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. In other control (n = 7), susceptible (n = 6) and resilient mice (n = 11) from an independent cohort, serum was isolated from blood collected both 5–6 h before the trauma and the day after the identification in subpopulations. (B) FKBP5 mRNA expression (Stress susceptibility, $F(2, 12) = 10.82$, $P = 0.0021$) 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 (Stress susceptibility, $F(2, 12) = 3.945$, $P = 0.048$) in the whole blood. (E) Linear correlation between the arousal score and the expression of SGK1 in the whole blood. (F) Pre-trauma and post-trauma serum corticosterone levels (Stress susceptibility, $F(2, 21) = 4.984$, $P = 0.0169$; Time $F(1, 21) = 7.306$, $P = 0.0133$; Stress susceptibility x Time $F(2, 21) = 5.474$, $P = 0.0122$). (G) Linear correlation between the arousal score and post-trauma serum corticosterone level ($r = 0.75$, $P = 0.0006$). Fold changes are expressed relative to transcript levels of 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.

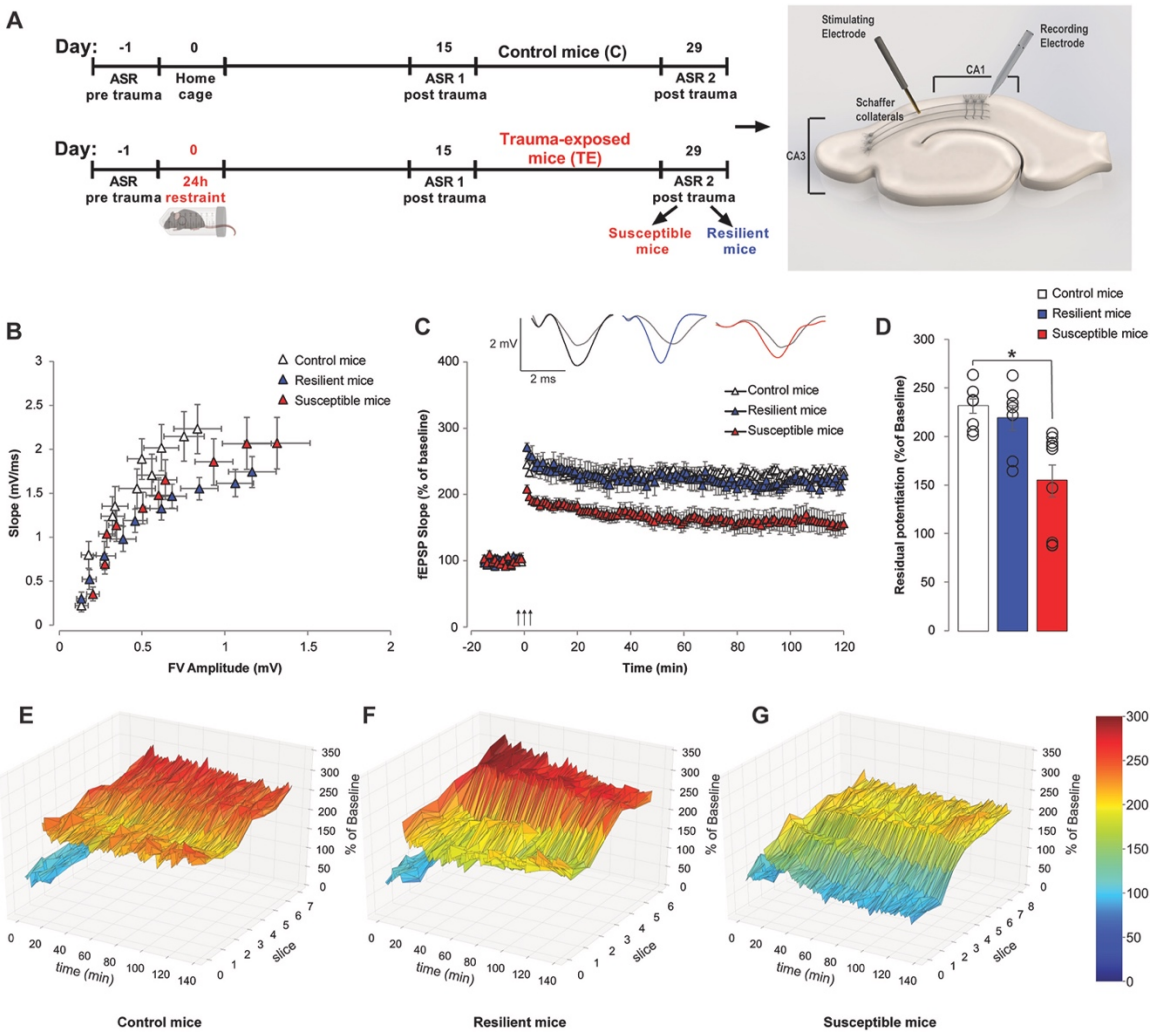

Fig. 6. LTP is impaired only in susceptible mice identified through the AIS model. (A) Experimental design. On the right, cartoon representing electrodes placement within the hippocampal slice. (B) No differences in fEPSP slope or fiber volley (FV) are found when analyzing Basal Synaptic Transmission (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; Stress susceptibility, $F(1,13) = 16.505$, $P = 0.001$, 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 (Stress susceptibility, $F(2,21) = 9.403$, $P = 0.001$ 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.

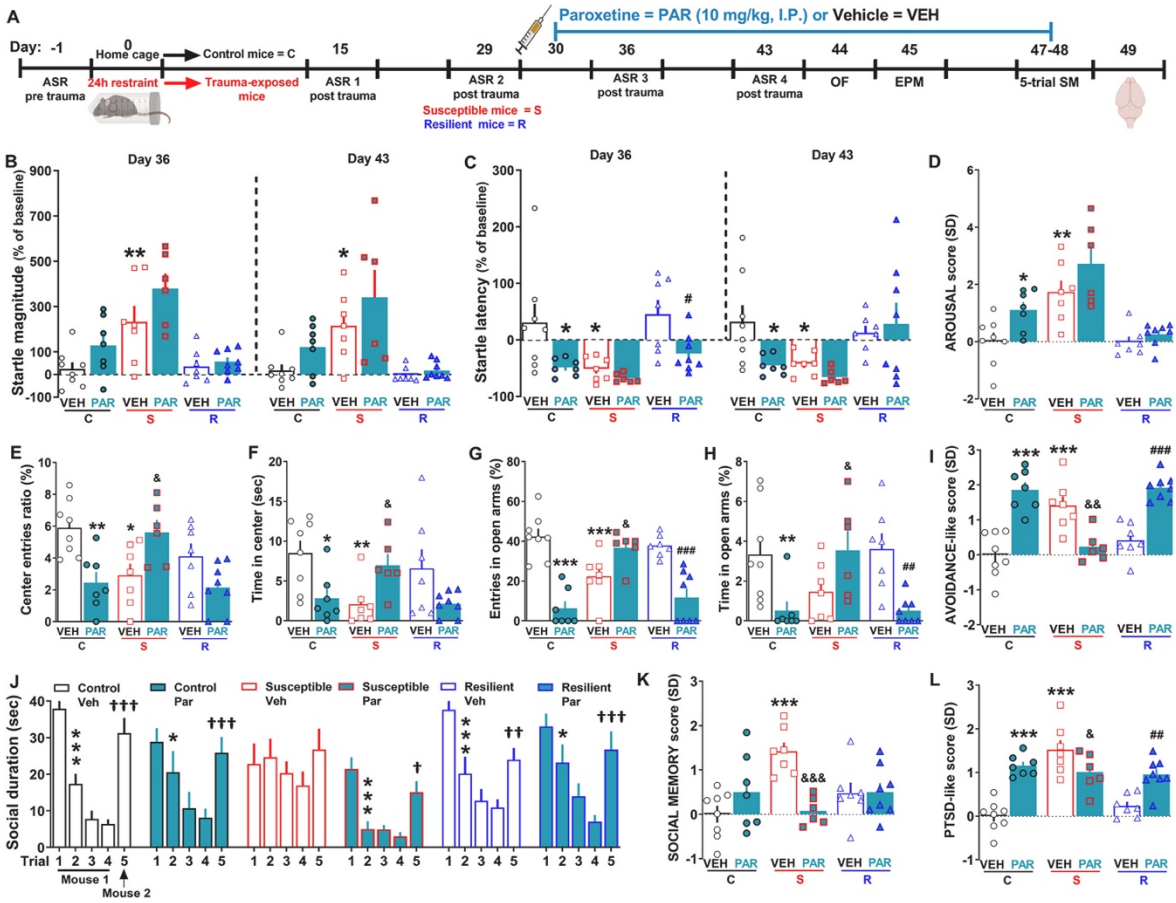

Fig. 7. Chronic treatment with paroxetine was effective in susceptible mice but detrimental in control and resilient mice. (A) Experimental timeline designed for the assessment of the effect of chronic treatment with paroxetine in control (vehicle n = 8, paroxetine n = 7), susceptible (vehicle n = 7, paroxetine n = 6) and resilient mice (vehicle n = 7, paroxetine n = 8). (B) Startle magnitude (% of baseline) (Day 36: Treatment, $F(1, 37) = 6.024$, $P = 0.019$; Stress susceptibility, $F(2, 37) = 18.88$, $P < 0.0001$. Day 43: Treatment, $F(1, 37) = 3.84$, $P = 0.049$; Stress susceptibility, $F(2, 37) = 14.25$, $P < 0.0001$). (C) Startle latency (% of baseline) (Day 36: Treatment, $F(1, 37) = 12.02$, $P = 0.0014$; Stress susceptibility, $F(2, 37) = 6.64$, $P = 0.0034$. Day 43: Stress susceptibility, $F(2, 37) = 5.271$, $P = 0.0097$). (D) AROUSAL score (Treatment, $F(1, 37) = 8.67$, $P = 0.0056$; Stress susceptibility, $F(2, 37) = 22.23$, $P < 0.0001$). (E) Center entries ratio (Treatment x Stress susceptibility $F(2, 37) = 10.45$, $P = 0.0003$) and (F) time spent in center of the OF (Treatment x Stress susceptibility $F(2, 37) = 7.45$, $P = 0.0019$). (G) Entries in open arms (Treatment $F(1, 37) = 25.14$, $P < 0.0001$; Treatment x Stress susceptibility $F(2, 37) = 22.68$, $P < 0.0001$) and (H) time in open arms of EPM (Treatment $F(1, 37) = 5.29$, $P = 0.0272$; Treatment x Stress susceptibility $F(2, 37) = 8.77$, $P = 0.0008$). (I). AVOIDANCE-like score (Treatment $F(1, 37) = 18.3$, $P < 0.0001$; Treatment x Stress susceptibility $F(2, 37) = 30.48$, $P < 0.0001$). (J) Social duration 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 Stress susceptibility $F(2, 37) = 8.69$, $P = 0.0008$). (L) PTSD-like score 2 (Treatment $F(1, 37) = 15.05$, $P = 0.0004$; Stress susceptibility $F(2, 37) = 14.31$, $P < 0.0001$; Treatment x Stress susceptibility $F(2, 37) = 17.45$, $P < 0.0001$). 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 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.

SUPPLEMENTARY INFORMATION

Torrisi et al.

SUPPLEMENTARY MATERIALS AND METHODS

The AIS model (traumatic procedure)

A correct air flowing was allowed both by drilling 2 holes (0.5 mm of diameter) along the sidewall and cutting the end of the tube. A sufficient quantity of paper towel was placed in each tube to fill the space between the mouse and the cap and one hole was also drilled in each cup to keep the tails of the mice out of the tube. About 10 tubes containing mice, randomly chosen, were placed in conventional cages (Tecniplast, 425 x 266 x 185 mm) with a level of illumination of 400 lux. Only for the assessment of fear responses to and avoidance of trauma-related cue, a cap for 50ml falcon tube containing a piece of cotton wool soaked with lemon oil (600 µl at the beginning and other 600 µl 3 hours before the end of restraint) was fixed in the center of each cage next to the noses of mice. During the restraint, mice had no access to food and water. Once the restraint ended, mice were immediately put back to their home cages, with free access to food and water. During the 24h of restraint, C mice remained in their home cages in a different room. TE mice were monitored for the recovery of the pre-trauma body weights as well as for the general physical state. TE mice remained group-housed until the end of experiments because we observed no aggressive behavior post-trauma.

Odor-cued fear conditioning test

During the no cue exposure session (day 32), mice were individually taken from the home-cage and habituated to the open field in absence of the trauma-related cue for 10 min. The cue exposure session was carried out 24h after the end of

the no cue exposure session (day 33). Mice were placed in the open field containing the trauma-related cue, a cap for 50ml falcon tube containing a piece of cotton wool soaked with 600 µl of lemon pure essential oil (citrus limonum), which was fixed centrally 10 cm far from one sidewall. The three cue re-exposure sessions (day 40, 54, 75) were performed respectively 7 days, 14 days and 21 days after, in the same way as the cue exposure session. 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.

Open field (OF) test

Sixteen square plus one central square (the center) were drawn on the floor of the apparatus. Mice were located in the center of the open field and avoidance-like behavior was quantified by counting the numbers of entries and the time spent in the center. To circumvent any weighted effect of locomotion on avoidance-like behavior, the number of entries in the center was expressed as center entries ratio that was calculated by the following formula: (Center crossings / total crossings) x 100.

Elevated plus maze (EPM) test

The apparatus consisted of two opposite open arms (30 x 5 cm) and two arms with walls (30 x 5 x 14 cm) that were attached to a central platform (5 x 5 cm) to form a cross. The maze was elevated 50 cm from the floor. Illumination measured in the center of the maze was 40 lx. Each mouse was placed in the center of the maze with the nose facing one of the closed arms, and allowed to explore the maze for a 5-min period. The following parameters were considered

to measure avoidance-like behavior: number of entries in the open arms (%) and time spent in the open arms (%). To differentiate entries from stretched attend postures, one entry was scored only when half of the body of the mouse was inside the arm (Mosienko et al., 2012).

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

Each test mouse was individually housed in a clean testing cage containing sawdust for 1 h of habituation in the testing room. The test began when a stimulus male mouse (mouse 1) was placed in the testing cage for a 1-min trial. At the end of this first trial, the stimulus mouse was removed from the testing cage and placed into its home cage. The test mouse was left in the testing cage for a 3-min of inter trial interval. The same procedure was repeated for additional three trials (trial 2, 3 and 4). In a fifth trial (trial 5), a novel stimulus mouse (mouse 2) was placed in the testing cage containing the test mouse for 1 min. If the social memory is intact, mice normally decrease the social interaction with the mouse 1 over the course of multiple exposures (trial 1-4, habituation) and then increase their social interaction with the mouse 2 (trial 5, dishabituation). Social interactions were scored for the cumulative duration of the following behavioral responses performed by the test mouse: anogenital sniffing (direct contact with the anogenital area), body sniffing (sniffing or snout contact with the flank area), and nose-to-nose sniffing (sniffing or snout contact with the head/neck/mouth area).

Forced swim test (FST)

Mice were placed in glass cylinders (height 30 cm, diameter 20 cm) containing 25 ± 1 °C water filled up to 15 cm. White opaque screens were used to visually separate cylinders. A single 6-min session standard protocol was performed. The immobility time was analyzed during the last 4-min (240 sec) of the 6-min. The first 2-min of the forced swimming test were not analyzed and were

considered as a habituation period as mice show a high frequency of exploratory and escape-directed behavior that may results in measurement inaccuracy if considered (Gerhard et al., 2020; Pignatelli et al., 2020). Immobility was defined as the animal floating on the water or making only those movements necessary to maintain its head above water. Increased immobility time is indicative of depressive-like behavior. The latency until the first episode of immobility (> 3 sec) was also measured (Li et al., 2007). Shorter latencies reflect depressive-like behavior.

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

Whereas RNA from brain areas was extracted by TRIzol (InvitrogenTM, Carlsbad, CA, U.S.A.), RNA from blood was extracted by Quick-RNA Miniprep Plus Kit (Zymoresearch, Irvine, CA, U.S.A., Item N° R1057) according to manufacturer's instructions. RNA quantification was performed by using Nanodrop ND-1000 Spectrophotometer. cDNA was synthesized with SuperScript IV First-Strand Synthesis System (InvitrogenTM, Carlsbad, CA, U.S.A). Aliquots of cDNA were amplified in parallel reactions with external standards at known amounts, using specific primer pairs for NR3C1, FKBP5, SGK1, BDNF and GAPDH (Reference gene). RT-PCR was performed by using iTaq Universal SYBR Green Supermix (Biorad, Hercules, CA, U.S.A.). Each sample was run in triplicate and quantification was obtained by the 2-DDCt method.

Electrophysiological recordings

Briefly, after cutting procedure, slices were transferred to a recording chamber and perfused (1–2 ml/min) with ACSF containing the following (in mM): 124 NaCl, 4.4 KCl, 1 Na2HPO4, 25 NaHCO3, 2 CaCl2, 2 MgCl2, 10 glucose, and kept at 29°C bubbled with an O2/CO2 mixture at 95% and 5%. After 120 min recovery, field excitatory post-synaptic potentials (fEPSPs) were recorded in

CA1 stratum radiatum by a glass electrode filled with ACSF. Stimuli were induced at Schaffer collateral fibers by a bipolar tungsten electrode. Recordings were performed and analyzed in pClamp 10.

Supplementary references

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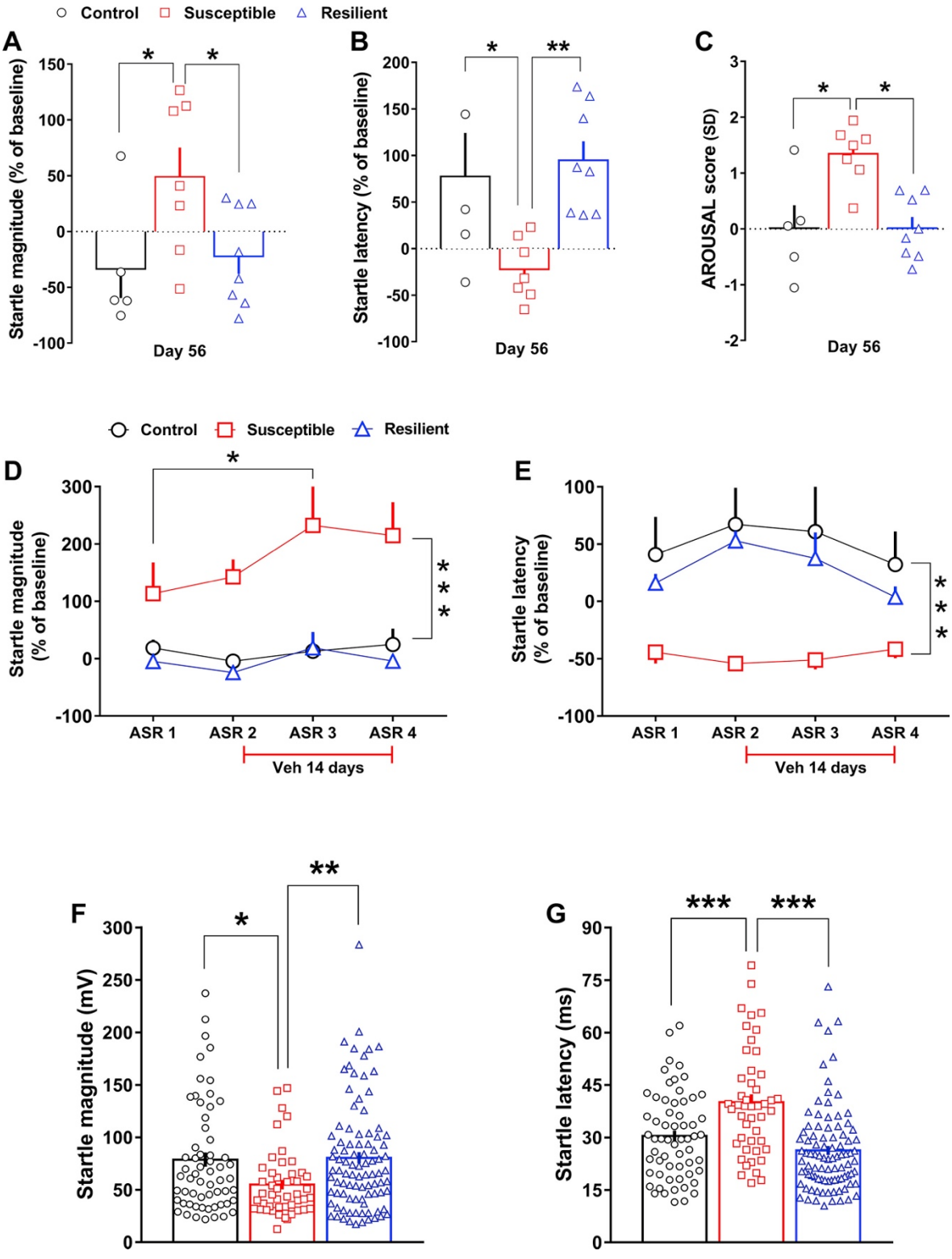
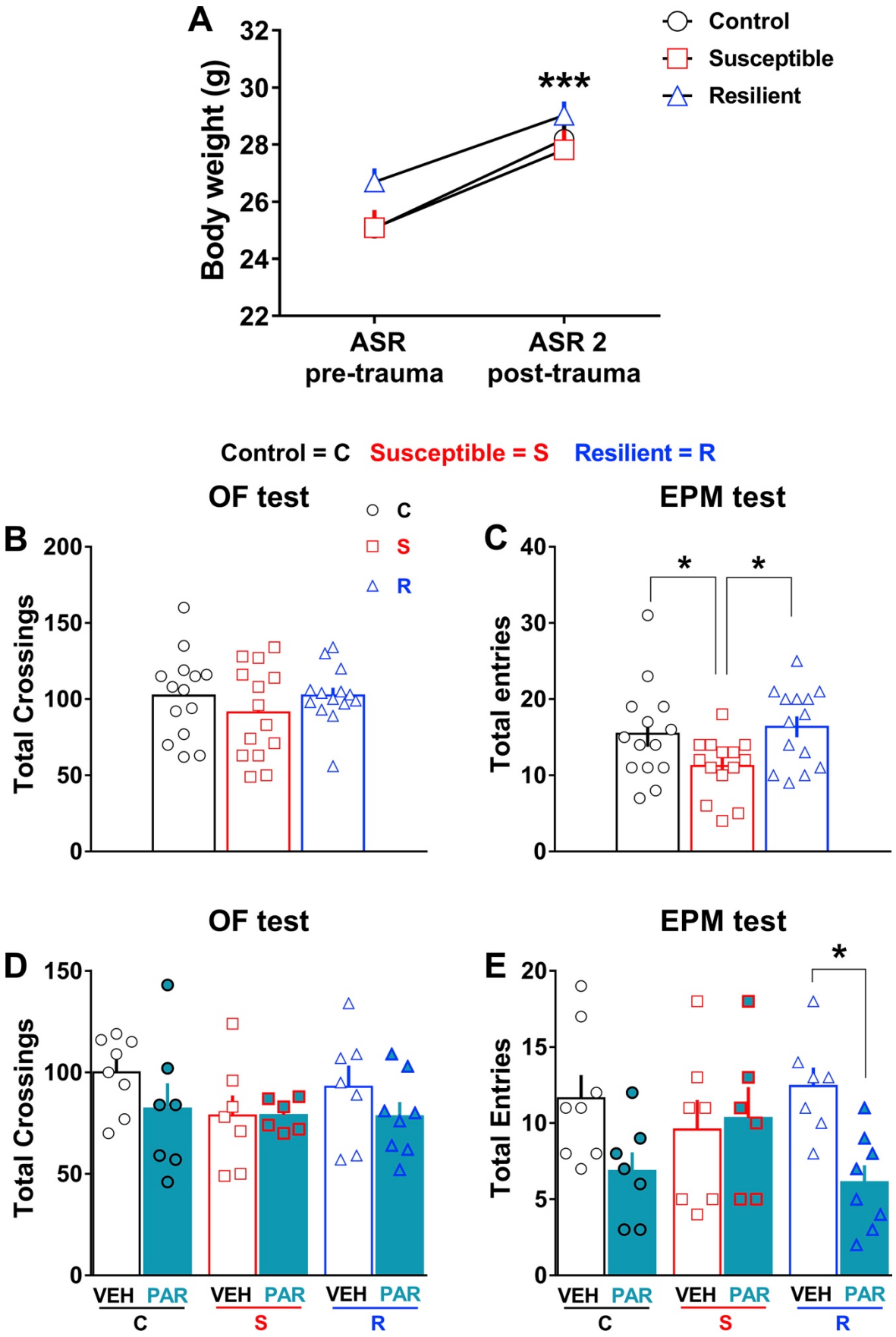

Control
Susceptible
Resilient
A
Startle magnitude (% of baseline)
Day 56
B
Startle latency (% of baseline)
Day 56
C
AROUSAL score (SD)
Day 56
D
Startle magnitude (% of baseline)
ASR 1
ASR 2
ASR 3
ASR 4
Veh 14 days
E
Startle latency (% of baseline)
ASR 1
ASR 2
ASR 3
ASR 4
Veh 14 days
F
Startle magnitude (mV)
G
Startle latency (ms)

Figure S1: Susceptible mice showed pre-trauma low startle reactivity and a persistent post trauma hyperarousal that became more pronounced over time.

(A) Startle magnitude (% of baseline) (Stress susceptibility, $F(2, 17) = 4.03$, $P = 0.037$), (B) Startle latency (% of baseline) (Stress susceptibility, $F(2, 17) = 6.53$, $P = 0.0079$) and (C) AROUSAL score (Stress susceptibility, $F(2, 17) = 9.543$, $P = 0.0017$) of some control mice (n = 5), susceptible mice (n = 7) and resilient mice (n = 8) identified through the AIS model and further tested two months post-trauma. (D) Startle magnitude (% of baseline) (Stress susceptibility, $F(2, 19) = 13.46$, $P = 0.0002$; Treatment $F(3, 57) = 2.81$, $P = 0.048$) and (E) Startle latency (% of baseline) (Stress susceptibility, $F(2, 19) = 6.76$, $P = 0.0060$) of control mice (n = 8), susceptible mice (n = 7) and resilient mice (n = 7), which received 14 days of I.P. vehicle administration for pharmacological experiments. (F) Pre-trauma startle magnitude (Stress susceptibility, $F(2, 197) = 4.863$, $P = 0.0087$) and (G) startle latency (Stress susceptibility, $F(2, 197) = 17.52$, $P < 0.0001$) of all control mice (n = 60), susceptible (n = 47) and resilient mice (n = 93) identified. One-way ANOVA or two-way RM 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
Body weight (g)
32
30
28
26
24
22
***
ASR
pre-trauma
ASR 2
post-trauma
Control
Susceptible
Resilient
Control = C
Susceptible = S
Resilient = R
OF test
EPM test
B
Total Crossings
200
150
100
50
0
C
S
R
C
Total entries
40
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150
100
50
0
VEH
PAR
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Total Entries
20
15
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Figure S2: Body weight and locomotor activity of control, susceptible and resilient mice treated or not with paroxetine. (A) Body weight (Time, $F(1, 39) = 219$, $P < 0.0001$; Bonferroni post hoc test: ***$P < 0.001$ vs ASR pre trauma), (B) total crossings performed during the OF test and (C) total entries (Stress susceptibility, $F(2, 39) = 3.888$, $P = 0.029$) performed during the EPM test by control (n = 14), susceptible (n = 14) and resilient mice (n = 14). (D) Total crossings performed during the OF test and (E) total entries (Treatment, $F(1, 37) = 7.648$, $P = 0.0088$) performed during the EPM test by control mice (vehicle n = 8, paroxetine n = 7), susceptible mice (vehicle n = 7, paroxetine n = 6) and resilient mice (vehicle n = 7, paroxetine n = 8). One-way ANOVA or two-way RM ANOVA followed by Bonferroni post hoc test: *$P < 0.05$. Values are expressed as means ± s.e.m.

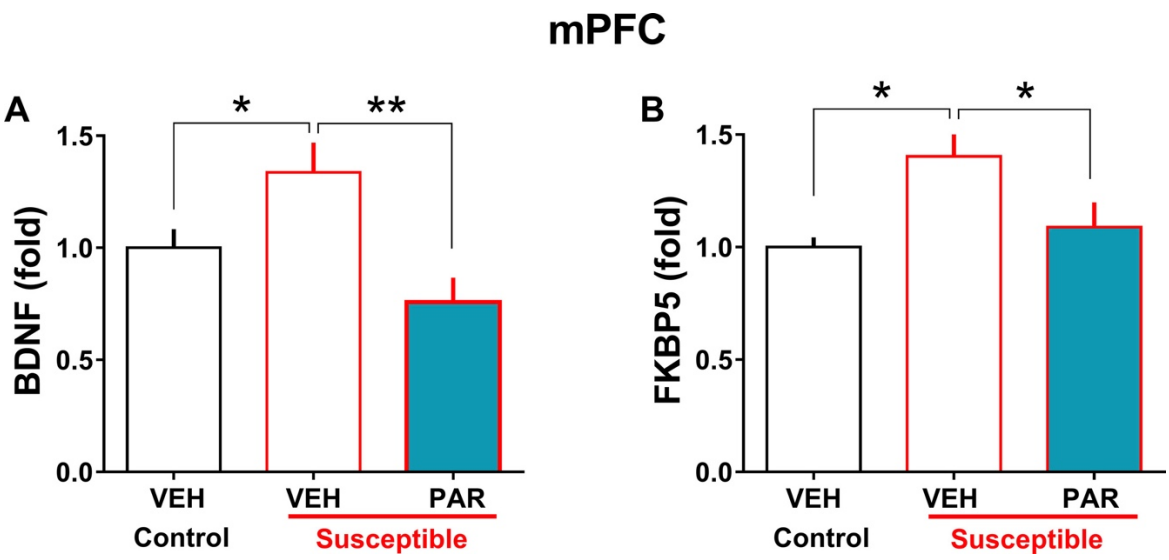

Figure S3: Paroxetine rescued the increased mRNA expression of BDNF and FKBP5 in the mPFC of susceptible mice The day after the end of the 5-trial SM test (day 49), control mice (vehicle n = 6), susceptible mice (vehicle n = 6, paroxetine n = 6) were sacrificed to microdissect mPFC. Abundance of transcripts was assessed by qPCR. (A) BDNF mRNA expression (Treatment, $F(2, 15) = 6.93$, $P = 0.0074$) and (B) FKBP5 mRNA expression (Treatment, $F(2, 15) = 4.3$, $P = 0.033$) in the mPFC. One-way ANOVA followed by Bonferroni post hoc test: *$P < 0.05$, **$P < 0.01$. Values are expressed as means ± s.e.m.

CHAPTER III

“ALTERED GUT MICROBIOTA COMPOSITION AND TRAUMATIC STRESS SUSCEPTIBILITY: NEW INSIGHTS INTO THE PATHOPHYSIOLOGY OF PTSD”

1. INTRODUCTION

Mammal and microorganisms co-evolved together creating a symbiotic relationship. The whole of microorganisms that live in the different mammal districts is called microbiota while the collective genome is defined as the microbiome, even if both terms are used interchangeably (Dominguez-Bello et al., 2019).

In the last years it has been an increased attention for the mutual influence of the gut microbiota and the brain.

They both communicate through different ways among which the most known are the vagus nerve, hormones, and metabolites (Dalile et al., 2019; Morais et al., 2021). All of these affect different aspect of the equilibrium of an organism that concern, among many, the immune system and the inflammation state (Schluter et al., 2020), the stress response and the volatility of the microbiota (Bastiaanssen et al., 2021; Tetel et al., 2018).

Maladaptive stress response is involved in different psychopathology and it was observed that glucocorticoids are able to influence microbiota composition as well in the same way microbiota influence systemic response to the stress through, for example, the modulation of the immune system and the production of metabolites that could increase permeability of the blood-brain barrier (BBB) which all together influence negatively the stress response (Braniste et al., 2014; Foster et al., 2017; Pearson-Leary et al., 2020; Tetel et al., 2018).

Further researches have demonstrated that lack of a microbiota in germ-free (GF) mice induce an exaggerated hypothalamic-pituitary axis response to the stress that could be reversed through the colonization with SPF microbiota (Cryan, 2016).

Furthermore, it was shown that antibiotic treated (ABX) mice, and GF mice have impairment in fear extinction and microglia maturation (Chu et al., 2019).

To date, different neuropsychopathology seems to be correlated with an alteration in the microbiota composition (Cryan et al., 2019). Schizophrenia (Xu et al., 2020; Zhu et al., 2020), autism spectrum disorder (Kang et al., 2019; Sharon et al., 2019), major depressive disorder (Kazemi et al., 2019; Slyepchenko et al., 2017; Zhuang et al., 2020) and anxiety (Lach et al., 2018) are some of the pathology that are linked with a dysbiosis condition.

Post-traumatic stress disorder (PTSD) is a psychopathology developed after the exposure to a traumatic event with an incidence between 20-30% characterized by an altered response to the stress. Symptoms include intrusive memories, avoidance to trauma-related stimuli, mood and cognitive alteration and hypervigilance. (Torrisi et al., 2019).

PTSD often co-occur with other condition that get worse the pathology which can include substance abuse, chronic pain and inflammation, mood and anxiety disorders (Yehuda et al., 2015).

Considering that microbiota seems to be involved in the regulation of the aforementioned aspects that are in common with PTSD and also that some clinical works reported a dysbiosis condition in PTSD patients (Farooqui et al., 2017; Watson, 2019), we would investigate if there is a correlation between stress response and microbiota alteration.

For the purpose of this work, we will use the AIS model already published by our group (Torrisi et al., 2021). The aim will be to investigate the changes that occur in the microbiota and the metabolite produced in susceptible and resilient mice sub-populations to understand how the microbiota contribute to trauma coping in this disorder.

2. MATERIALS AND METHODS

Animals

Male mice C57BL6/J (8 weeks, weight 28 ± 2g) were purchased from Charles River Laboratory (Milano, 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 weighted one a week until the end of the experimental protocol. The experimenter handled animals on alternate days during the week preceding the stress procedure. 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).

Arousal-based individual screening (AIS)

Animal's segregations were carried out according to our previous work (Torrisi et al., 2021). Briefly, mice acoustic startle reactivity (ASR) was measured the day before the trauma (day -1) and mice were divided into two groups with a similar average (control mice and trauma-exposed (TE) mice). The day after (day 0) animals were gently put into polyethylene Falcon 50mL centrifuge tubes and exposed to 24h of restraint, from 3 pm (3h before the dark phase) to 3pm of the next day. After the stress mice return into the home cages, with food and water *ad libitum*. Two other sessions of ASR were performed after 14 (ASR1) and 28 days (ASR2) on control and trauma exposed mice in order to assess changes in the arousal (% of arousal baseline). TE mice exhibiting an arousal score ≥ 1 were classified as susceptible, while TE mice with an arousal score < 1 were considered resilient.

Fecal collection, DNA extraction and sequencing

Fecal samples were collected in order to analyze the microbiota composition before the trauma, during ASR baseline at day -1 and during ASR segregation at day 28. Briefly, after ASR sessions, mice were single housed in a sterile cage and fecal pellets were rapidly collected in sterile Eppendorf using sterile tweezers, snap frozen in liquid nitrogen and stored at -80°C until processing (Pascual Cuadrado et al., 2021).

DNA from feces was extracted using Stool DNA Isolation kit (Norgen Biotek Corp. – Cat. 27600) following manufacturer's instructions. Concentration and purity were measured using Nanodrop ND-1000.

DNA samples were amplified as previously described by (Takahashi et al., 2014). Amplicons were purified using Thermolabile Exonuclease I (NEB), diluted 1:2 and amplified with Index Nextera XT. Subsequently, amplicons were normalized with SequalPrep (Thermo Fisher) and multiplexed. The pool was purified using magnetic beads Agencourt XP 1X and sequenced with Miseq 300PE.

Sample collection

After segregation, mice were sacrificed through decapitation. Brain was collected into a polyethylene Falcon 15mL and snap frozen in nitrogen. Blood was collected in a sterile Eppendorf and kept at room temperature for 3h. Afterwards, it was centrifuged a 1000 x g for 15 min to isolate serum which was collected in a new sterile Eppendorf and stored at -80°C until use (Torrisi et al., 2021).

Bioinformatic and Statistical analysis

Data analysis was carried out using Qiime2 and RStudio (Version 1.4.1106). Qiime2 was used to eliminate primers using Cutadapt v. 2021.11.0, denoised with the package DADA2, and chimeras were eliminated using vsearch package. Taxonomy was assigned applying Silva v. 138 and exported for Rstudio analysis. Custom R script is available at https://github.com/thomazbastiaanssen/Tjazi (Bastiaanssen, 2018). Genera that were never detected at a 1% relative abundance or higher were aggregated and defined as 'rare taxa' for the purpose of the barplot. Principal component analysis was performed on centered log-ratio transformed (clr) values using ALDEx2 library (Fernandes et al., 2014). An overall PCA was generated by principal component analysis for visualization and quality check purposes using the ggplot2 package (Wickham, 2016) and was calculated as the Aitchison distance between the two timepoint (Aitchison et al., 2000; Bastiaanssen, 2018). Metagenome data were used to infer KEGG Orthologues. Next, KEGG orthologues were processed using the omixer library in R (Darzi et al., 2016) in order to calculate the gut-brain modules (GBMs) (Valles-Colomer et al., 2019) and gut-metabolic modules (GMMs) (Vieira-Silva et al., 2016). Then, the same implementation of ALDEx2 was used to calculate the differential abundance.

3. RESULTS

Alpha-diversity

To investigate which change occurred in susceptible and resilient mice we analyzed data obtained from sequencing of 16S rRNA. Alpha-diversity didn't show any change for Chao1 and Shannon indexes while Simpson index showed

a significant difference in the timepoint among groups (Pr(>F) = 0.0276) (Figure 1A).

Beta-diversity

Beta-diversity showed a significant difference in the timepoint comparing each group (Pr(>F) = 0.0009). A significant difference was observed in susceptible after the exposure to the trauma (p-value = 0.047) (Figure 1B). Furthermore, comparing the baseline of susceptible mice versus the baseline of control and resilient groups it was observed a significant difference between groups (susceptible vs control: p-value = 0.048; susceptible vs resilient: p-value = 0.049) (Figure 1B).

Differential abundance and gut modules

Analyzing microbial genera, it reveals significant changes in differential abundance. The most significant changes occur in susceptible mice which showed a reduction of *Proteobacteria* and *Bacteroidetes* after the exposure to the stress. Most variable are the changes of the *Firmicutes* phylum which showed a more variability in response to the stress (Figure 3A).

Among *Firmicutes*, there is an increase of bacteria belonged to the *Ruminococcaceae* and some bacteria of the *Lachnospiraceae* family (Figure 3A).

It is possible to see a reduction *Prevotellaceae* family in susceptible mice after the exposure to the stress (Figure 3A).

Interesting is also the increase of the genus *Bilophila*, this belongs to the sulfate reducing bacteria (SRB) which consist of bacteria that inhibit butyrate ß-oxidation and degrade butyrate (Ye et al., 2018), furthermore, it is possible to see a reduction of *Bacteroides* genus in susceptible mice after stress exposure (Figure 3A).

Gut-Brain and gut-metabolic modules alterations

The analysis of gut-brain modules showed the possible neuroactive potential change that occur following microbiota changes. In both susceptible and resilient it is possible to see changes in the tryptophan pathways, particularly what concern tryptophan, quinolinic acid and kynurenine synthesis and degradation. Furthermore, it is possible to see changes in p-cresol degradation which is strongly reduced in susceptible mice after the exposure to the trauma. Other alterations concern neurotransmitters such as glutamate, GABA, and dopamine. In resilient but more highlighted in susceptible is possible to see an increase in 17-ß-estraiol degradation (Figure 4A).

The abundance of bacteria was used also to construct gut-metabolic modules in which it is possible to see different changes that occurs in both groups. Interesting is that some of those occurred in susceptible didn't in resilient group and vice versa. This indicates a different coping of resilient compared to susceptible (Figure 4B).

4. DISCUSSION

This study demonstrated that PTSD susceptible and resilient sub-populations mice showed distinctive changes in the microbiota composition.

It is interesting the difference in the baseline ß-diversity between susceptible compared to control and resilient groups. Differential abundance showed changes in bacteria involved in inflammatory processes, mainly the increase of *Ruminococcaceae* and *Lachnospiraceae*, bacteria involved in protein degradation and fermentation. These processes lead to the production of harmful metabolites such as ammonium, indole, and p-cresol (Amaretti et al., 2019).

The family of *Prevotellaceae* consists of bacteria that possess several enzymes and gene clusters essential for fermentation and utilization of complex polysaccharides (Kovatcheva-Datchary et al., 2015).

Another bacterium of interest is the *Bilophila* genus, this is a SRB bacteria that seems to be correlated with inflammatory processes through the inhibition of butyrate ß-oxidation and degradation of butyrate (Ye et al., 2018).

Bacteroides include bacteria involved in the digestion of carbohydrates for host nutrient use and that exhibit anti-inflammatory effects (Lee et al., 2013; Mazmanian et al., 2008).

All these alteration in the microbiota and the prediction obtained through the analysis of the gut-brain modules and gut-metabolic modules seem to indicate a shift of the susceptible gut-microbiota versus a pro-inflammatory state. The increase of p-cresol could be correlated with a reduction in myelination processes (Ntranos & Casaccia, 2018) and could increase anxiety-like behavior and alter dopamine metabolism in different brain areas (Pascucci et al., 2020).

The imbalance in the tryptophan pathway may induce a reduction bioavailability of this amino acid and alteration in the equilibrium between kynurenic acid and quinolinic acid that, as discussed elsewhere (Schwarcz et al., 2012) is linked with different psychopathology.

Neurotransmitter's imbalance, if confirmed by metabolomic analysis, could explain the different phenotype of susceptible compared to resilient and control groups.

Furthermore, of interest is the increase in 17-ß-estradiol degradation. It is known that estradiol is a steroid hormone with neuroprotective capacity, involved in memory consolidation, hippocampal long-term potentiation and acquisition and extinction of fear memory (Taxier et al., 2020). The possible reduction of this hormone in the brain could be involved, together with the increase of neurotoxic metabolite, in the maladaptive changes specific of susceptible.

5. CONCLUSION

Acute stress induced changes in microbiota composition in susceptible and resilient mice. A pre-existing difference in ß-diversity composition in susceptible mice comparing with resilient and control groups could be considered as a predisposition factor which could be exacerbated after the stress exposure. Microbiota changes that occur after 28 days post-trauma are different if comparing susceptible and resilient mice, indicating that both populations cope in a different way to the stress. Dysbiosis of susceptible mice seems to shift the microbiota towards a fermentation metabolism due to increased *Ruminococcaceae* and *Lachnospiraceae* bacteria. The change towards a fermentation-like metabolism is highlight by predicted gut-brain modules which showed a significant size effect in p-cresol degradation of susceptible mice.

Furthermore, the predicted imbalance of tryptophan metabolism could be also linked with alteration in neuronal functions (Schwarcz et al., 2012), that could be exacerbated by increased 17-ß-estradiol degradation in susceptible mice.

This study may be used as a start point to investigate deeply the aspect that correlate microbiota alteration with susceptibility to develop PTSD.

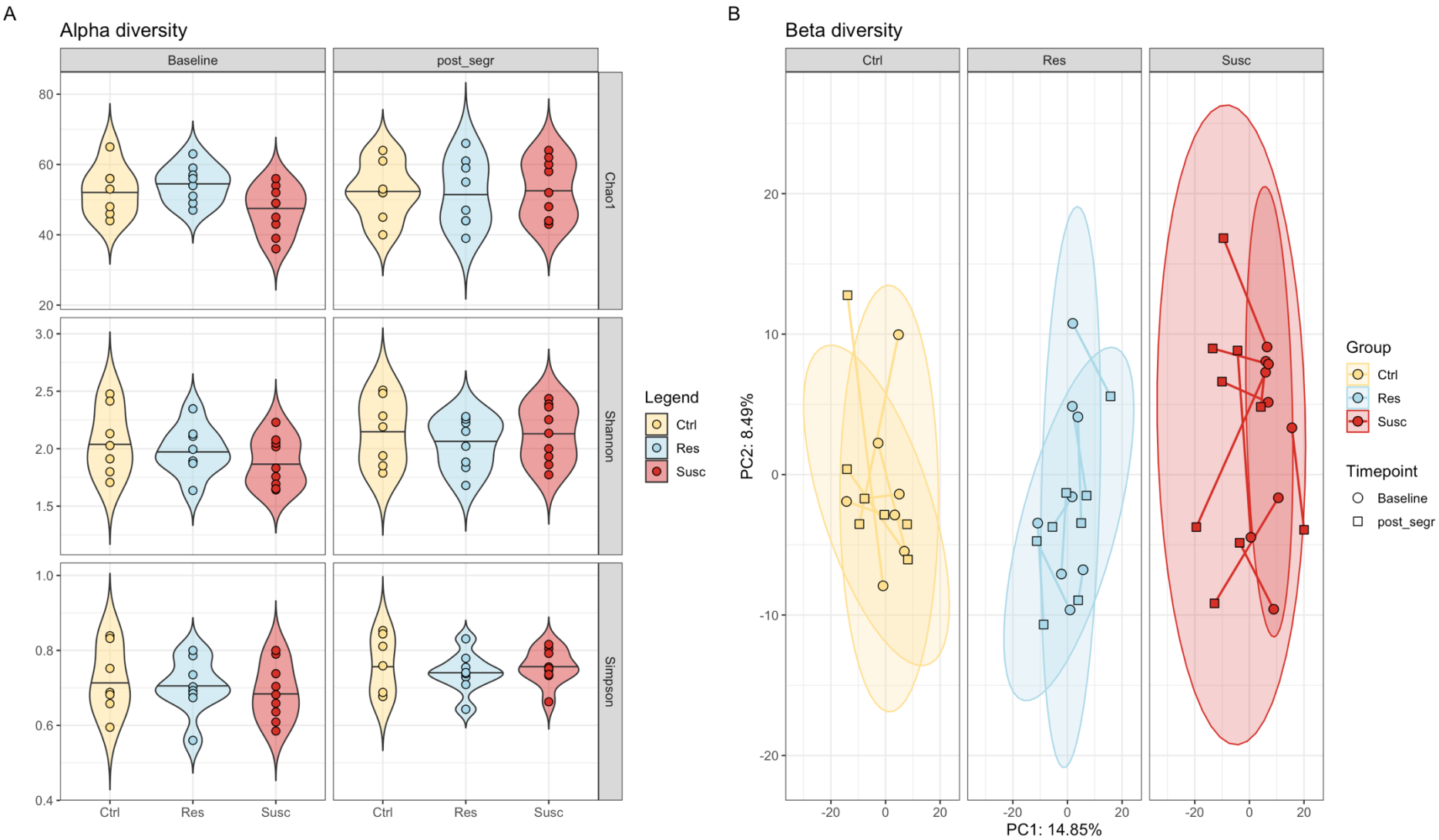
A
Alpha diversity
Baseline
post_segr
Chao1
Shannon
Simpson
Ctrl
Res
Susc
Legend
B
Beta diversity
PC2: 8.49%
PC1: 14.85%
Group
Timepoint
Baseline
post_segr

Fig. 1: **A**: α-diversity in baseline and post-segregation. Simpson index (ANOVA – Timepoint Pr(>F) = 0.0276); **B:** Aitchison distance (PERMANOVA analysis – baseline susceptible vs post_segr susceptible, p = 0.0479; baseline ctrl vs baseline susceptible, p = 0.047; baseline susceptible vs baseline resilient, p = 0.049)

A

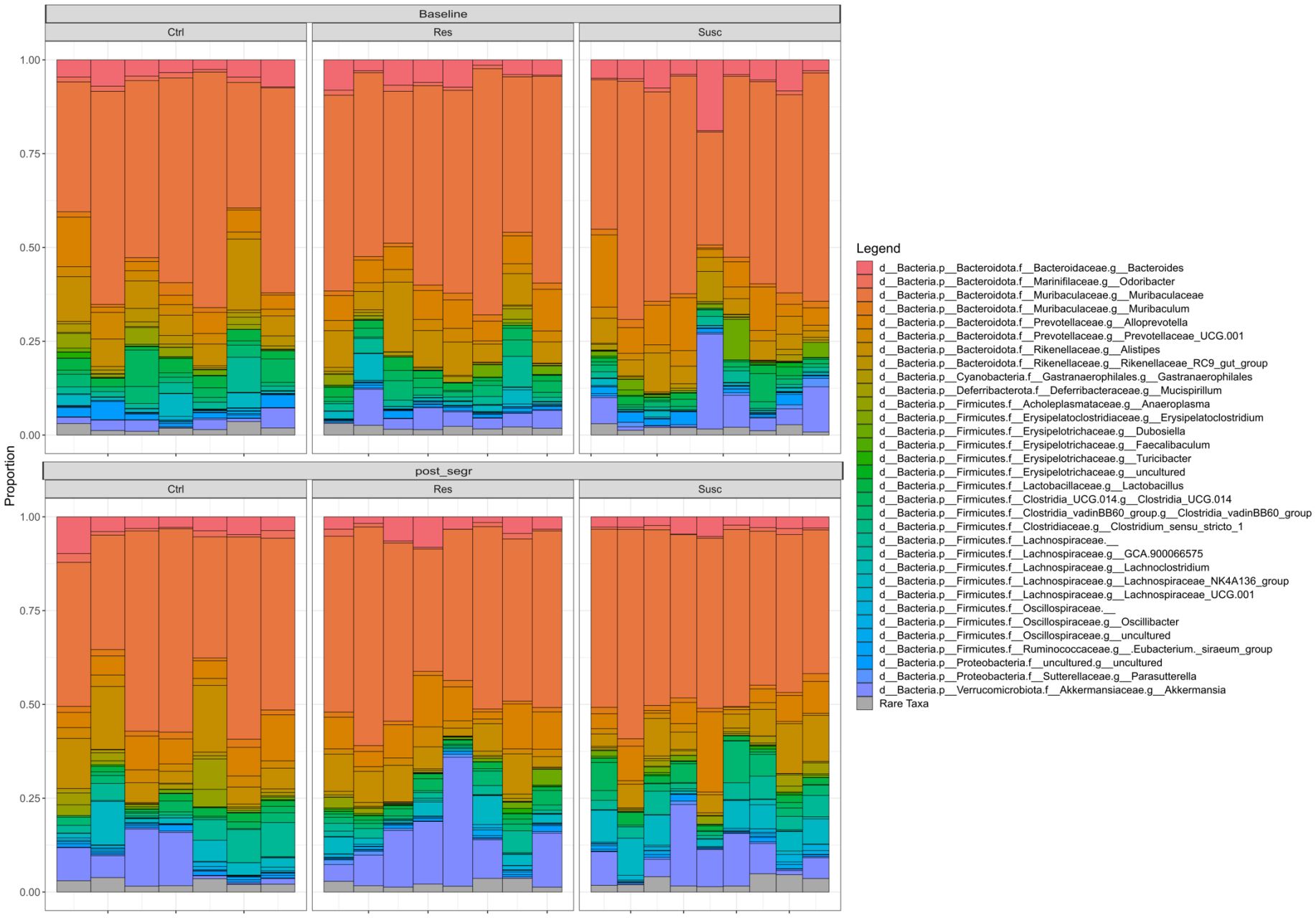
Baseline
Ctrl
Res
Susc
post_segr
Ctrl
Res
Susc
Proportion
1.00
0.75
0.50
0.25
0.00
Legend
d__Bacteria.p__Bacteroidota.f__Bacteroidaceae.g__Bacteroides
d__Bacteria.p__Bacteroidota.f__Marinifilaceae.g__Odoribacter
d__Bacteria.p__Bacteroidota.f__Muribaculaceae.g__Muribaculaceae
d__Bacteria.p__Bacteroidota.f__Muribaculaceae.g__Muribaculum
d__Bacteria.p__Bacteroidota.f__Prevotellaceae.g__Alloprevotella
d__Bacteria.p__Bacteroidota.f__Prevotellaceae.g__Prevotellaceae_UCG.001
d__Bacteria.p__Bacteroidota.f__Rikenellaceae.g__Alistipes
d__Bacteria.p__Bacteroidota.f__Rikenellaceae.g__Rikenellaceae_RC9_gut_group
d__Bacteria.p__Cyanobacteria.f__Gastranaerophilales.g__Gastranaerophilales
d__Bacteria.p__Deferribacterota.f__Deferribacteraceae.g__Mucispirillum
d__Bacteria.p__Firmicutes.f__Acholeplasmataceae.g__Anaeroplasma
d__Bacteria.p__Firmicutes.f__Erysipelatoclostridiaceae.g__Erysipelatoclostridium
d__Bacteria.p__Firmicutes.f__Erysipelotrichaceae.g__Dubosiella
d__Bacteria.p__Firmicutes.f__Erysipelotrichaceae.g__Faecalibaculum
d__Bacteria.p__Firmicutes.f__Erysipelotrichaceae.g__Turicibacter
d__Bacteria.p__Firmicutes.f__Erysipelotrichaceae.g__uncultured
d__Bacteria.p__Firmicutes.f__Lactobacillaceae.g__Lactobacillus
d__Bacteria.p__Firmicutes.f__Clostridia_UCG.014.g__Clostridia_UCG.014
d__Bacteria.p__Firmicutes.f__Clostridia_vadinBB60_group.g__Clostridia_vadinBB60_group
d__Bacteria.p__Firmicutes.f__Clostridiaceae.g__Clostridium_sensu_stricto_1
d__Bacteria.p__Firmicutes.f__Lachnospiraceae.__
d__Bacteria.p__Firmicutes.f__Lachnospiraceae.g__GCA.900066575
d__Bacteria.p__Firmicutes.f__Lachnospiraceae.g__Lachnoclostridium
d__Bacteria.p__Firmicutes.f__Lachnospiraceae.g__Lachnospiraceae_NK4A136_group
d__Bacteria.p__Firmicutes.f__Lachnospiraceae.g__Lachnospiraceae_UCG.001
d__Bacteria.p__Firmicutes.f__Oscillospiraceae.__
d__Bacteria.p__Firmicutes.f__Oscillospiraceae.g__Oscillibacter
d__Bacteria.p__Firmicutes.f__Oscillospiraceae.g__uncultured
d__Bacteria.p__Firmicutes.f__Ruminococcaceae.g__.Eubacterium._siraeum_group
d__Bacteria.p__Proteobacteria.f__uncultured.g__uncultured
d__Bacteria.p__Proteobacteria.f__Sutterellaceae.g__Parasutterella
d__Bacteria.p__Verrucomicrobiota.f__Akkermansiaceae.g__Akkermansia
Rare Taxa

Fig. 2: A**:** Bar plot representation of microbiota composition in response to the stress


A
Genera
d__Eukaryota;__;__;__;__;__
d__Bacteria;p__Verrucomicrobiota;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Rubritaleaceae;g__Rubritalea
d__Bacteria;p__Verrucomicrobiota;c__Chlamydiae;o__Chlamydiales;f__Chlamydiaceae;__
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Stenotrophomonas
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Piscirickettsiales;f__Piscirickettsiaceae;g__Candidatus_Endoecteinascidia
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Nitrincolaceae;g__Neptuniibacter
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Massilia
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Delftia
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Comamonas
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales;f__Alcaligenaceae;g__Achromobacter
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Colwelliaceae;g__Colwellia
d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;__;__
d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__uncultured;g__uncultured
d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;__
d__Bacteria;p__Patescibacteria;c__Saccharimonadia;o__Saccharimonadales;f__Saccharimonadaceae;g__Candidatus_Saccharimonas
d__Bacteria;p__Fusobacteriota;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae;g__Fusobacterium
d__Bacteria;p__Firmicutes;c__Clostridia;o__Peptostreptococcales-Tissierellales;f__Anaerovoracaceae;g__[Eubacterium]_nodatum_group
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__UCG-010;g__UCG-010
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;g__uncultured
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;g__Harryflintia
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;g__Anaerotruncus
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Ruminococcaceae;__
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Oscillospiraceae;g__uncultured
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Oscillospiraceae;__
d__Bacteria;p__Firmicutes;c__Clostridia;o__Oscillospirales;f__Butyricicoccaceae;g__UCG-009
d__Bacteria;p__Firmicutes;c__Clostridia;o__Monoglobales;f__Monoglobaceae;g__Monoglobus
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Tyzzerella
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Tuzzerella
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Lachnospiraceae_UCG-010
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Lachnospiraceae_UCG-006
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Lachnospiraceae_NK4A136_group
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;g__Lachnospiraceae_FCS020_group
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Lachnospiraceae;__
d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales;f__Defluviitaleaceae;g__Defluviitaleaceae_UCG-011
d__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Candidatus_Arthromitus
d__Bacteria;p__Firmicutes;c__Clostridia;o__Christensenellales;f__Christensenellaceae;g__Christensenellaceae_R-7_group
d__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus
d__Bacteria;p__Firmicutes;c__Bacilli;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Erysipelotrichaceae
d__Bacteria;p__Firmicutes;c__Bacilli;o__Erysipelotrichales;f__Erysipelatoclostridiaceae;g__Candidatus_Stoquefichus
d__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus
d__Bacteria;p__Desulfobacterota;c__Desulfovibrionia;o__Desulfovibrionales;f__Desulfovibrionaceae;g__Bilophila
d__Bacteria;p__Campilobacterota;c__Campylobacteria;o__Campylobacterales;f__Sulfurimonadaceae;g__Sulfurimonas
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Flavobacteriales;f__Flavobacteriaceae;g__Ulvibacter
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Flavobacteriales;f__Flavobacteriaceae;g__Flavobacteriaceae
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotellaceae_UCG-001
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides
d__Bacteria;p__Bacteroidota;c__Bacteroidia;__;__;__
d__Bacteria;p__Actinobacteriota;c__Coriobacteriia;o__Coriobacteriales;f__Eggerthellaceae;g__Parvibacter
d__Bacteria;p__Actinobacteriota;c__Coriobacteriia;o__Coriobacteriales;f__Eggerthellaceae;g__Gordonibacter
d__Bacteria;p__Actinobacteriota;c__Coriobacteriia;o__Coriobacteriales;f__Atopobiaceae;__
d__Archaea;p__Euryarchaeota;c__Methanobacteria;o__Methanobacteriales;f__Methanothermobacteriaceae;g__Methanothermobacter
d__Archaea;__;__;__;__;__
Delta Ctrl
Delta Susc
Delta Res
Effect Size
3
2
1
0
-1
-2
-3

Fig. 3: A: Heatmap of genera composition. Color depicts effect size, with blue (negative) indicating higher abundance before the trauma and red (positive) indicating higher abundance after the trauma. *q<0.1 Benjamini-Hochberg adjusted q < 0.1 for all cases.

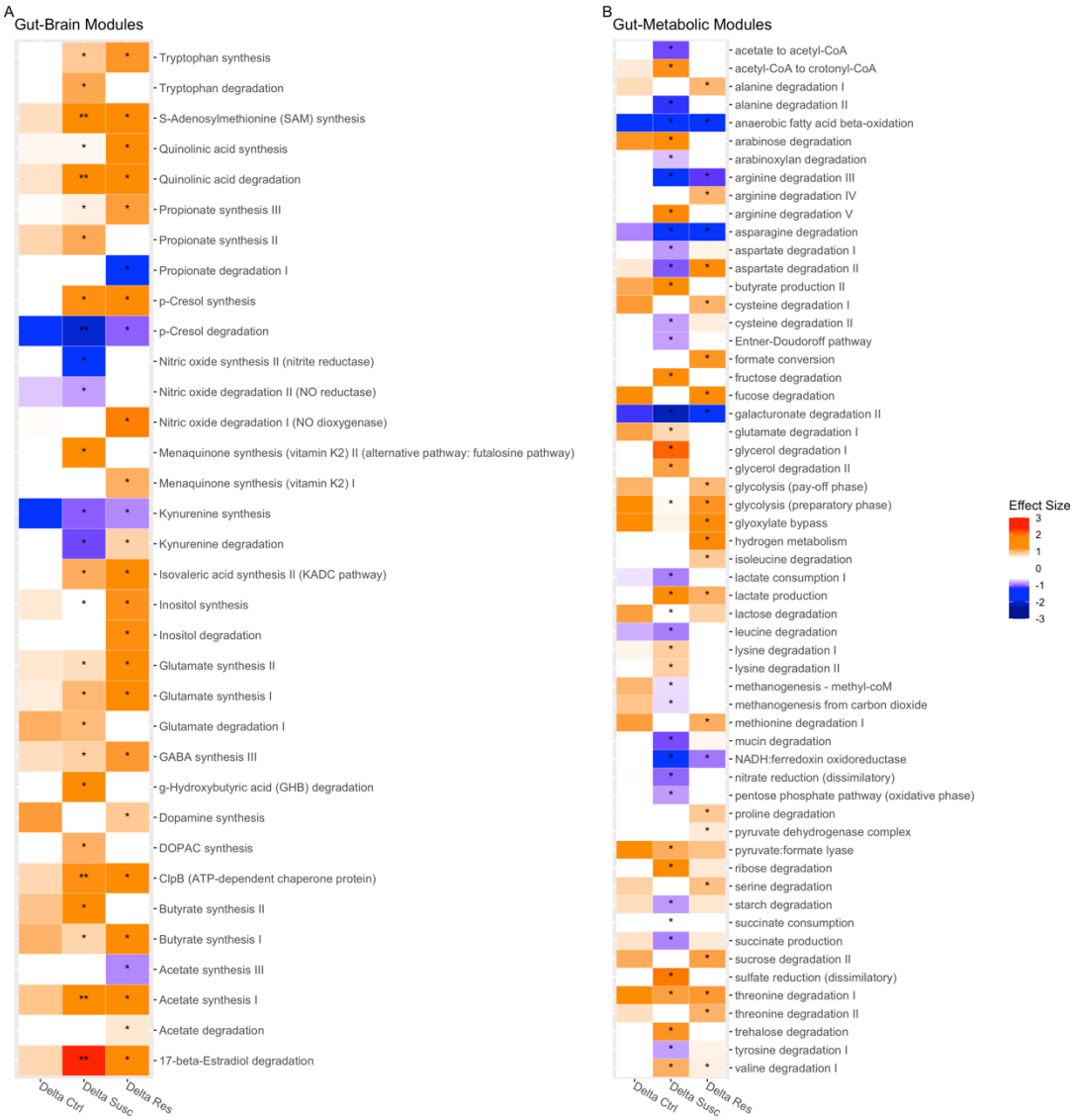
A
Gut-Brain Modules
Tryptophan synthesis
Tryptophan degradation
S-Adenosylmethionine (SAM) synthesis
Quinolinic acid synthesis
Quinolinic acid degradation
Propionate synthesis III
Propionate synthesis II
Propionate degradation I
p-Cresol synthesis
p-Cresol degradation
Nitric oxide synthesis II (nitrite reductase)
Nitric oxide degradation II (NO reductase)
Nitric oxide degradation I (NO dioxygenase)
Menaquinone synthesis (vitamin K2) II (alternative pathway: futalosine pathway)
Menaquinone synthesis (vitamin K2) I
Kynurenine synthesis
Kynurenine degradation
Isovaleric acid synthesis II (KADC pathway)
Inositol synthesis
Inositol degradation
Glutamate synthesis II
Glutamate synthesis I
Glutamate degradation I
GABA synthesis III
g-Hydroxybutyric acid (GHB) degradation
Dopamine synthesis
DOPAC synthesis
ClpB (ATP-dependent chaperone protein)
Butyrate synthesis II
Butyrate synthesis I
Acetate synthesis III
Acetate synthesis I
Acetate degradation
17-beta-Estradiol degradation
ΔDelta Ctrl
ΔDelta Susc
ΔDelta Res
B
Gut-Metabolic Modules
acetate to acetyl-CoA
acetyl-CoA to crotonyl-CoA
alanine degradation I
alanine degradation II
anaerobic fatty acid beta-oxidation
arabinose degradation
arabinoxylan degradation
arginine degradation III
arginine degradation IV
arginine degradation V
asparagine degradation
aspartate degradation I
aspartate degradation II
butyrate production II
cysteine degradation I
cysteine degradation II
Entner-Doudoroff pathway
formate conversion
fructose degradation
fucose degradation
galacturonate degradation II
glutamate degradation I
glycerol degradation I
glycerol degradation II
glycolysis (pay-off phase)
glycolysis (preparatory phase)
glyoxylate bypass
hydrogen metabolism
isoleucine degradation
lactate consumption I
lactate production
lactose degradation
leucine degradation
lysine degradation I
lysine degradation II
methanogenesis - methyl-coM
methanogenesis from carbon dioxide
methionine degradation I
mucin degradation
NADH:ferredoxin oxidoreductase
nitrate reduction (dissimilatory)
pentose phosphate pathway (oxidative phase)
proline degradation
pyruvate dehydrogenase complex
pyruvate:formate lyase
ribose degradation
serine degradation
starch degradation
succinate consumption
succinate production
sucrose degradation II
sulfate reduction (dissimilatory)
threonine degradation I
threonine degradation II
trehalose degradation
tyrosine degradation I
valine degradation I
ΔDelta Ctrl
ΔDelta Susc
ΔDelta Res
Effect Size
3
2
1
0
-1
-2
-3

Fig. 4: Effect of the stress on the gut-brain modules and gut-molecular modules after the exposure to stress. (A) Gut Brain Modules and (B) Gut Metabolic Modules differentially altered after the exposure to the stress. Color depicts effect size, with blue (negative) indicating higher metabolic activity before the trauma and red (positive) indicating higher metabolic activity after the trauma. *$q<0.1$ Benjamini-Hochberg adjusted $q < 0.1$ for all cases.

CHAPTER IV
GENERAL DISCUSSIONS

4.1 AROUSAL BASED INDIVIDUAL SCREENING REVEALS SUSCEPTIBILITY AND RESILIENCE TO PTSD-LIKE PHENOTYPES IN MICE

In the first part of my PhD thesis, we demonstrate that the AIS model include many key features for the study of PTSD. The trauma protocol used for this study have an elevated ecological validity in that a similar threatening trapping situation can happen naturally of both rodents and human (Goswami et al., 2013; Kondrakiewicz et al., 2019). Concerning the duration and severity of the traumatic procedure, a shorter procedure couldn't be sufficient to induce the phenotype because C57BL/6J are mice generally resilient to the stress (Flint & Tinkle, 2001; Mozhui et al., 2010).

Here we show for the first time that a single severe stress is able to reproduce a long-lasting PTSD-like phenotype.

This trauma was coupled with the z-normalization that allowed us to distinguish susceptible and resilient according to the startle reactivity. Startle circuits are highly conserved across species (Bale et al., 2019). Animals and humans are tested in a similar way, so that is higher the probability of gaining translational information focusing on startle reactivity.

The discrimination between susceptible and resilient is particularly relevant because only few studies considered this aspect (Olson et al., 2011; Sillivan et al., 2017). In fact, in numerous other studies this discrimination is not considered but all comparisons are made between naïve vs. trauma-exposed animals (Cohen et al., 2004; Flandreau & Toth, 2017).

Molecular analysis showed an upregulation and downregulation of FKBP5 respectively in mPFC and whole blood of susceptible mice in according to human results (Yehuda et al., 2009; Young et al., 2015).

Changes in BDNF levels were higher in mPFC and HIP of susceptible mice. These results are in line with studies that associate elevated levels of BDNF with fear consolidation (Notaras & van den Buuse, 2020; Revest et al., 2014). This data with other signatures and correlations we found, indicate that the AIS model is a tool that allow the identification of molecular adaptation of susceptibility/resilience.

Furthermore, we found that susceptible mice have a higher basal level of corticosterone compared to control and resilient according to recent data obtained in an animal model for the study of PTSD (Sillivan et al., 2017) and supported by clinical studies (Song et al., 2008).

These results explain also the long-term impairment in the hippocampal CA1 LTP found only in susceptible mice. Indeed, it was reported that higher levels of circulating stress hormone induce impairment of hippocampal plasticity (Popoli et al., 2012).

We further showed the efficacy of paroxetine on susceptible mice, for this reason the AIS model might represent a novel tool to identify novel pharmacological strategies for SSRI-resistant individuals with PTSD.

Paroxetine was also able to restore mRNA levels of BDNF and FKBP5 in the mPFC of susceptible mice to the levels of control. This data may explain the beneficial effect of paroxetine in susceptible mice.

4.2 ALTERED GUT MICROBIOTA COMPOSITION AND TRAUMATIC STRESS SUSCEPTIBILITY: NEW INSIGHTS INTO THE PATHOPHYSIOLOGY OF PTSD

In the second part of my PhD thesis, we used the AIS model to analyze which microbiota changes occur in susceptible and resilient after the trauma.

In our knowledge, we discovered, for the first time, a significant difference in the baseline ß-diversity between susceptible and control group. These results may be considered a predisposition factor for the development of PTSD.

Furthermore, our analysis demonstrates huge alteration in microbiota composition in susceptible mice with an increase of bacteria that are involved in inflammation and in production of metabolite that influence negatively myelination processes, such as *Rumminococcaceae*, *Lachnospiraceae* and *Bilophila* (Amaretti et al., 2019; Ye et al., 2018) and a reduction of bacteria that exhibit anti-inflammatory functions, such as *Prevotella* and *Bacteroides* (Kovatcheva-Datchary et al., 2015; Lee et al., 2013).

Analysis for the gut-brain modules and gut-metabolic modules suggest changes in the gut brain communication.

Indeed, in susceptible mice is possible to see an increase in the synthesis of harmful metabolite such as p-cresol (Ntranos & Casaccia, 2018) and alterations in tryptophan metabolism. These data are in according with previous published data in which was observed that imbalance in tryptophan metabolism is correlated with psychopathologies onset (Schwarcz et al., 2012).

Another predicted change that could be interesting for future studies is the significant reduction of 17ß-estradiol degradation. This hormone improves hippocampal CA3-CA1 LTP in preclinical studies (Taxier et al., 2020) and could explain the impairment of LTP that we have seen in susceptible mice.

These results demonstrate that the pathophysiology of PTSD is related with pre-existing and post-trauma microbiota changes which may impact CNS function and fear response as previously demonstrate (Chu et al., 2019).

Because it was previously demonstrated that transferring the microbiota from anxious animals to GF mice induce anxiety-like behavior (Cryan et al., 2019), in future it could be interesting investigate the effect of microbiota transplantation from resilient-donor mice to susceptible-recipes mice in order to evaluate if the microbiota from resilient mice could ameliorate or revert the susceptible phenotype.

Furthermore, another possibility is to research if treatment with pro- and pre-biotic soon after the trauma or after the segregation, together with pharmacological treatment, could help to revert the phenotype.

In future, it would be needed to study deeply the metabolomic alteration that occurs in susceptible mice in serum and brain areas, such as hippocampus, cortex and amygdala, that are the main areas involved in the pathophysiology of PTSD (Sheynin & Liberzon, 2017) to confirm the predictive analysis. If results would confirm bioinformatic prediction, it could be useful for the development of new pharmacological targets for the treatment of PTSD.

In conclusion this study demonstrates how the AIS model is a preclinical model with a higher translational value that could be used for the study of PTSD and, more in general, trauma susceptibility and resilience. Furthermore, the microbiota changes that occurs in susceptible could be a useful tool to develop new pharmacological target for the microbiota and metabolites produced. However, further studies are needed to understand better which mechanisms are involved in the gut-brain axis communication and PTSD onset.

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Annexes

Torrisi SA, **Laudani S**, Contarini G, De Luca A, Geraci F, Managò F, Papaleo F, Salomone S, Drago F, Leggio GM.

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