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BEHAVIORAL AND MOLECULAR PROFILES OF A HEURISTIC GENETIC MODEL OF VULNERABILITY TO STRESS-INDUCED DEPRESSION AND SCHIZOPHRENIA-RELATED SYMPTOMS: THE ROMAN LOW- VS HIGH-AVOIDANCE RAT LINES/STRAINS

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Thesis Abstract

State of The Art:

There are many different mental disorders, with different presentations. They are generally characterized by a combination of abnormal thoughts, perceptions, emotions, behavior and relationships with others. Mental disorders include, among many others, depression and schizophrenia, which are major contributors to the enormous burden of mental illnesses that impacts significantly on health and also on major social, human rights and economic issues.

Depression (i.e., Major depressive disorder, MDD) is a common and debilitating chronic psychiatric condition associated with functional impairments and a variety of socio-economic difficulties. Affecting an estimated 284 million people worldwide, MDD is the leading cause of disability in terms of total years lost due to disability. Schizophrenia (SCZ) is a chronic and severe mental disorder affecting 20 million people worldwide that is characterized by distortions in thinking, perception, emotions, language, sense of self and behavior. Common experiences include hallucinations (hearing voices or seeing things that are not there) and delusions (fixed, false beliefs). Globally, SCZ is associated with considerable disability and may affect educational and occupational performance. People with SCZ are 2-3 times more likely to die early than the general population. This is often due to preventable physical diseases, such as cardiovascular disease, metabolic disease and infections.

The etiology and pathogenesis of MDD and SCZ are poorly understood, but a large body of experimental evidence indicates that the combination of genetics and environmental factors determine the individual vulnerability to these disorders. Current pharmacotherapies help many MDD patients, but high rates of a partial response or no response, and the delayed onset of the effects of antidepressant therapies, leave many patients inadequately treated and at risk of suicide. A similar situation is observed in schizophrenic patients under treatment with antipsychotics which is frequently associated with partial remissions.

The development of novel and more efficacious pharmacotherapies necessitates new insights into the neurobiology of stress, human mood, and psychotic disorders that shed light on the mechanisms underlying the vulnerability of individuals to MDD and SCZ. The generation and characterization of valid animal models is therefore an essential factor for the identification of such mechanisms.

The Roman high- (RHA) and low-avoidance (RLA) lines of rats represent a valid genetic model to investigate the neurobehavioral underpinnings of both MDD and SCZ. The RHA and RLA rats are psychogenetically selected for rapid versus extremely poor acquisition of active avoidance in a

shuttle box, respectively. Selective breeding for more than 50 years has generated two well characterized phenotypes displaying many differential specific behavioral and neurochemical traits. While RLAs are reactive copers displaying depression-like and anxiety-related behaviors when exposed to stressors, RHA rats show innate impulsivity and behavioral traits that are reminiscent of the positive and cognitive symptoms of SCZ.

The studies of the neurobiology of depression focus largely on the association between stress and depression, with the hope that an understanding of the biological pathways that link stress to depression would inform on the pathophysiology of the disorder. Different forms of stress can induce depressive symptoms in humans and in experimental animals, and accumulating experimental evidence indicates that stressors may hinder the synthesis, release and binding to their specific receptors of different brain neurotrophins. The most abundant of these proteins is the Brain Derived Neurotrophic Factor (BDNF), which modulates the growth and differentiation of neural networks. The disruption of BDNF function by stressors may induce the alterations of synaptic plasticity processes underlying MDD.

As previously mentioned, the environment is considered to play an important role in the onset of mental disorders. Accordingly, it has been hypothesized that a peaceful and quiet environment during the first years of life, which are critically important for brain development, may lead to an improvement in the symptoms of mental illness in susceptible adults. Notably, the long lasting behavioral effects elicited on experimental animals by early postnatal treatments like neonatal handling (NH) are reminiscent of the protection provided by healthy families to the vulnerable children they grow against psychiatric disorders.

Interestingly, NH has long-lasting anxiolytic-like effects on RLA rats, as indicated by a decrease in the frequency of grooming bouts, whereas on the other hand it reduces traits related to SCZ symptoms such as pre-pulse inhibition (PPI) impairment and latent inhibition (LI) deficits in RHA rats. Moreover, in RHA rats NH improves spatial working memory and cognitive flexibility.

Aims:

The behavioral and neurochemical traits that distinguish the Roman lines from each other suggest that RLA and RHA rats may represent a model of vulnerability or resistance to stress-induced depression, respectively. Furthermore, experimental evidence accumulated in recent years consistently indicates that RHA rats may represent a model of SCZ-related traits because they exhibit behavioral and neurochemical traits reminiscent of positive and cognitive symptoms of SCZ. However, it remained to be established whether RHA rats also exhibit traits resembling negative symptoms of SCZ such as asociality, and whether these traits can be ameliorated by NH.

The above hypotheses were tested in two separate studies:

In the first study (STUDY I) we evaluated the behavioral and neurochemical responses of RLA and RHA rats acutely exposed to Tail Pinch (TP), a mild stressor, or Forced Swimming (FS), a stressor of relatively higher intensity. The expression of the neurotrophic factor BDNF and its receptor trkB was measured by means of Western blots to assess the neural plasticity activity elicited by TP and FS in two areas of the limbic system, the Prefrontal Cortex, involved in the process of decision-making, and the Hippocampus, which plays a major role in the control of emotions and in the consolidation of new memories.

In the second study (STUDY II) we evaluated social interaction (SI) in RHA and RLA rats under basal conditions. Furthermore, in view of the long-term consequences elicited by early-life events on mental health, we studied whether NH may impact, later in adulthood, on social interaction and anxiety/fear related behaviors.

Results:

In STUDY I, we demonstrated that, when exposed to TP and FS, RLA rats display a depression-like behavior (e.g., a longer immobility time in the FS test) while their RHA counterparts exhibit a proactive coping style characterized by active behaviors aimed at gaining control over the stressors. Moreover, line- and brain region-dependent differences were observed in the expression of BDNF and trkB upon exposure to either stressor.

In STUDY II we found that, in RHA rats, which show a low level of SI under basal conditions, NH significantly increased sociality in the SI test, as reflected by a longer social time and higher social preference in the first 5 minutes of the test while, in RLA rats, which are strongly emotional under basal conditions, NH reduced anxiety as indicated by a significant decrease in Grooming time.

Conclusions:

The results of these studies provide further experimental support to the view that the Roman rats may represent a valid experimental approach to investigate the neural substrates and molecular mechanisms that impact on the individual vulnerability or resistance to stress-induced depression and to identify the mechanisms whereby early-life positive events interact with the genetically determined vulnerability to develop psychotic disorders. Therefore, investigation of the Roman lines may provide useful leads for the development of innovative drugs for the treatment of MDD and SCZ.

1. Introduction

Mental disorders are generally characterized by a combination of abnormal thoughts, perceptions, emotions, behavior and social interactions. Of particular importance among many other mental disorders, depression and schizophrenia are major contributors to the enormous burden of mental illnesses that impacts significantly on health and also on major social, human rights and economic issues.

1.1 Depression: diagnosis and social impact

Depression is a common and debilitating chronic psychiatric condition associated with functional impairments and a variety of socio-economic difficulties. Thus, depression has a lifetime prevalence of approximately 10–15% and is more frequent in women than in men (Kessler, 2003; McGrath et al., 2016). In Europe, with a 12- month prevalence around 6.9% (corresponding to approximately 30 million people), depression is the leading cause of disability in terms of total years lost due to disability (World Health Organization, 2018).

Since the 1960s, depression has been diagnosed as "major depression" (i.e., major depressive disorder, MDD) based on symptomatic criteria set forth in the 5th edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-V, American Psychiatric Association, 2013). Milder cases are classified as "dysthymia," although there is no clear distinction between the two. It is obvious from the criteria summarized in Table I that the diagnosis of depression, as opposed to most diseases of other organ systems like type II diabetes, cancer, chronic obstructive pulmonary disease, to name a few, is not based on objective diagnostic tests (serum chemistry, organ imaging, or biopsies), but rather on a highly variable set of symptoms.

Table I. Diagnostic Criteria for Major Depression according to DSM-V
Depressed mood
Irritability
Low self-esteem
Feelings of hopelessness, worthlessness, and guilt
Decreased ability to concentrate and think
Decreased or increased appetite
Weight loss or weight gain

Insomnia or hypersomnia
Low energy, fatigue, or increased agitation
Anhedonia (decreased interest in pleasurable stimuli, e.g., sex, food, social interactions)
Recurrent thoughts of death and suicide
A diagnosis of major depression is made when a certain number of the above symptoms are reported for longer than a 2 week period of time, and when the symptoms disrupt normal social and occupational functioning (see DSM-V, 2013, American Psychiatric Association).

Therefore, depression may be viewed as a heterogeneous syndrome comprised of numerous diseases of distinct causes and pathophysiologies. Attempts have been made to establish subtypes of depression defined by certain sets of symptoms; however, these subtypes are based solely on symptomatic differences and there is as yet no evidence that they reflect different underlying disease states (Akiskal, 2000; Blazer, 2000). Each of these depressive forms includes different subtypes that differ in the frequency, duration and severity of the symptoms in which, in addition to mood, cognitive, psychomotor and neurovegetative functions are compromised (Table II).

Table II. Examples of Proposed Subtypes of Depression according to DSM-V

Depression Subtype	Main Features
Melancholic depression[a]	Severe symptoms; prominent neurovegetative abnormalities
Reactive depression[b]	Moderate symptoms; apparently in response to external factors
Psychotic depression	Severe symptoms; associated with psychosis: e.g., believing depression is a punishment for past errors (a delusion) or hearing voices that depression is deserved (a hallucination)
Atypical depression	Associated with labile mood, hypersomnia, increased appetite, and weight gain
Dysthymia	Milder symptoms, but with a more protracted course (> 2 years)

These subtypes are based on symptoms only and may not describe biologically distinct entities. The subtypes also cannot generally be distinguished by different responses to various subclasses of antidepressant medications.

[a] Melancholic depression is similar to a syndrome classified as "endogenous depression," based on the speculation that it is caused by innate factors.

[b] Reactive depression is similar to a syndrome classified as "exogenous depression," based on the speculation that it is caused by external factors.

Epidemiologic data support the view that although about 40%-50% of the risk for MDD is genetically determined, the individual vulnerability to develop depression is strongly influenced by environmental factors. Notably, the polygenic pattern of inheritance is very complex and, thus far, no specific associations between genes or gene markers and any of the mood disorders have been identified with certainty. Thus, despite substantial research efforts the etiology of MDD remains poorly understood; however, many effective treatments for MDD are currently available, as indicated by the high percentage (around 70%) of people with depression showing some improvement with any of several antidepressant medications or electroconvulsive seizures (ECS).

On the other hand, about 30% of patients with major depression fail to achieve remission despite treatment with multiple monoaminergic antidepressants and are considered to have treatment-resistant depression (TRD). Most important, in patients who respond to antidepressants, the time to onset of effect is typically several weeks, during which time patients remain symptomatic and at risk of suicidal behavior. Hence, there is a need to develop better treatments for MDD, in particular those that provide rapid relief of depressive symptoms (i.e., rapid-acting antidepressants, RAADs). The development of novel and more efficacious pharmacotherapies necessitates new insights into the neurobiology of stress, human mood, and psychotic disorders that shed light on the mechanisms underlying the vulnerability of individuals to MDD. The generation and characterization of valid animal models is therefore an essential factor for the identification of such mechanisms. However, very few genetic animal models of mood disorders with face, construct, and predictive validity have been characterized to date (see section 1.5.2).

1.2 The neurobiology of Depression

1.2.1 The monoamine hypothesis

In 1965, Joseph J. Schildkraut formulated the monoaminergic hypothesis to explain the pathogenesis and etiology of depression. According to this theory, the depressive states are associated with a reduction of monoamines in specific brain areas; the manic ones, on the contrary, with an excessive increase (Schildkraut, 1965).

This hypothesis derives from the observation that reserpine, a drug used in the past as an antihypertensive, induces severe depressive symptoms. The antihypertensive effect of reserpine is due to its ability to prevent the storage of norepinephrine (NE), dopamine (DA) and serotonin (5-HT) into the synaptic vesicles thereby leading to their conversion in the cytosol into inactive metabolites by the monoamine oxidases (MAOs).

The serendipitous discoveries that iproniazid, a potential antitubercular (Crane, 1956; Kline, 1970) and imipramine, a potential antipsychotic (Kuhn, 1958)**,** could relieve depressive symptoms further supported the monoaminergic hypothesis. Their action is carried out in two different ways: imipramine by blocking the reuptake of monoamines into the presynaptic neuronal terminals; iproniazid, on the other hand, is a MAO inhibitor (Shelton et al., 1991). Therefore, these two drugs are considered the progenitors of tricyclic antidepressants (TCA) and MAO inhibitors (MAO-I), respectively.

The main limitation of these classes of antidepressants was their non-selectivity, which caused several side effects due to their interaction with various neurotransmitter systems other than the monoaminergic ones. For this reason, over the years, the focus has been on the development of selective reuptake inhibitors for 5-HT (SSRI, selective 5-HT reuptake inhibitors) and NE (SNRI, selective NE reuptake inhibitors), which have become the drugs of choice in the treatment of depressive syndromes.

1.2.2 The role of Dopamine

Besides NE and 5-HT, DA is another monoaminergic neurotransmitter extremely important in the pathophysiology of depression. The dopaminergic system is involved in multiple neural functions controlling the state of mood, as indicated by the following experimental evidence:

- Drugs that increase DA in the mesolimbic pathway, such as psychostimulants, or inhibitors of DA reuptake (e.g., bupropion) have antidepressant action (Jacobs and Silversone, 1986; Little, 1988; Willner, 1995); on the contrary, drugs that antagonize dopaminergic transmission cause the appearance of depression-like symptoms (Belmaker and Wald, 1977);
- Depressed patients show reduced concentrations of homovanillic acid (HVA), a major DA metabolite, in the cerebrospinal fluid (Post et al., 1973; Roy et al., 1985, 1992; Reddy et al., 1992; Brown and Gershon, 1993).
- In Parkinson's disease, where the nigrostriatal dopaminergic projections undergo progressive degeneration, depressive episodes can occur both before and during the disease (Van Praag et al., 1975; Guze and Barrio, 1991).

The behavioral relevance of dopaminergic neurotransmission is also underlined by the key role played by the mesolimbic and mesocortical pathways in the mechanisms of motivation, gratification

and aversion. The pathways that control these processes are the mesolimbic and mesocortical, which originate in the ventral tegmental area (VTA) and project specifically to the nucleus accumbens (Acb), the amygdala (AMYG) and the prefrontal cortex (PFC).
The function of the limbic system is to allow the recognition of rewarding stimuli, both natural such as sexual activity and food, and "artificial", like addictive drugs that induce stronger gratification than the natural stimuli. Given these premises, it has been hypothesized that the lack of motivation in carrying out rewarding activities and anhedonia, two of the cardinal symptoms of depression, are due to a reduction in the activity of this neuronal system (Willner, 1995).

Dopaminergic systems are also involved in the control of aversive stimuli; in fact, prolonged and intensive stress factors lead to the activation of the mesolimbic projections (Bradberry et al., 1991) while the mesocortical projections are activated by milder aversive stimuli (Abercrombie et al., 1989; Deutch and Roth, 1990; Giorgi et al., 2003).
The mesocortical DA pathway plays also a key role in the control of cognitive processes; thus, reduced memorization, attention and concentration capacities found in depressed patients may be attributed to alterations of this system (American Psychiatric Association, 2013).
Furthermore, given the role of DA in the control of motor activity, it has been proposed that psychomotor retardation, another depressive symptom, may depend on a reduced dopaminergic function (Willner, 1995).
Although monoaminergic transmissions represent the main target of the antidepressant drugs currently used, the complexity of depressive syndromes implies that interactions among different neurotransmitters and also between neurotransmitters and endocrine systems are involved in their pathophysiology.

1.2.3 The role of Glutamate

As previously mentioned, about 30% of patients with a diagnosis of MDD fail to achieve remission despite treatment with multiple monoaminergic antidepressants and are considered to have treatment-resistant depression (TRD). Moreover, in patients who respond to monoaminergic antidepressants, the time to onset of effect is typically several weeks, during which time patients remain symptomatic and at risk of suicidal behavior. Therefore, the serendipitous finding that rapid antidepressant effects can be elicited in treatment-resistant patients by a single intravenous infusion of the NMDA receptor (NMDA-R) antagonist ketamine was of considerable interest and the subsequent confirmation of this effect in a randomized-controlled trial (Zarate et al., 2006) led to an

exponential increase in the number of studies on ketamine's antidepressant effects and mechanisms of action.

The rapid-acting antidepressant effects of ketamine are believed to be the result of a cascade of events, which include (1) blockade of NMDA-Rs located in cortical inhibitory GABAergic interneurons, (2) disinhibition of cortical pyramidal cells leading to a glutamate surge, (3) activation of the pro-synaptogenic AMPA receptors, (4) blockade of the excitotoxic extrasynaptic NMDA-Rs, and (5) activation of synaptogenic intracellular signaling, including mTORC1 and BDNF pathways (Abdallah et al., 2016). Moreover, accumulating evidence indicates that ketamine's short-latency antidepressant effects are probably due to rapid neural plastic changes in the prefrontal cortex (PFC) which are very similar to those induced by chronic treatments with monoaminergic antidepressants (Abdallah et al., 2016). Importantly, these studies have established that glutamatergic neurotransmission plays a key role in the pathophysiology of MDD and, therefore, represents a key target for the development of novel and more efficacious therapies for this disorder.

1.2.4 The glucocorticoid hypothesis

Besides the hypotheses concerning the role of different neurotransmitters in the pathophysiology of depression, there are other hypotheses that take into account the role of the neuroendocrine systems. Thus, many neurotransmitters, such as NE, 5-HT, acetylcholine (Ach) and γ-aminobutyric acid (GABA), modulate the secretion of CRF by the neurons of the hypothalamic paraventricular nucleus (PVN) which integrate information relevant to stress that is conveyed by direct excitatory afferents from the AMYG and inhibitory (polysynaptic) afferents from the hippocampus (HC). Under stressful conditions, CRF is released by the projections of the PVN neurons into the hypophyseal portal system and acts on the corticotrophs of the anterior pituitary to release ACTH. ACTH reaches the adrenal cortex via the blood stream, where it stimulates the release of glucocorticoids (cortisol in humans and corticosterone in rats). In addition to its many functions, glucocorticoids (including synthetic forms such as dexamethasone) repress CRF and ACTH synthesis and release. In this manner, glucocorticoids inhibit their own synthesis. Glucocorticoids have profound effects on both, general metabolism and behavior, like the consumption of energy and the promotion of certain cognitive abilities, that are necessary to tackle adverse situations (Nestler et al., 2002).

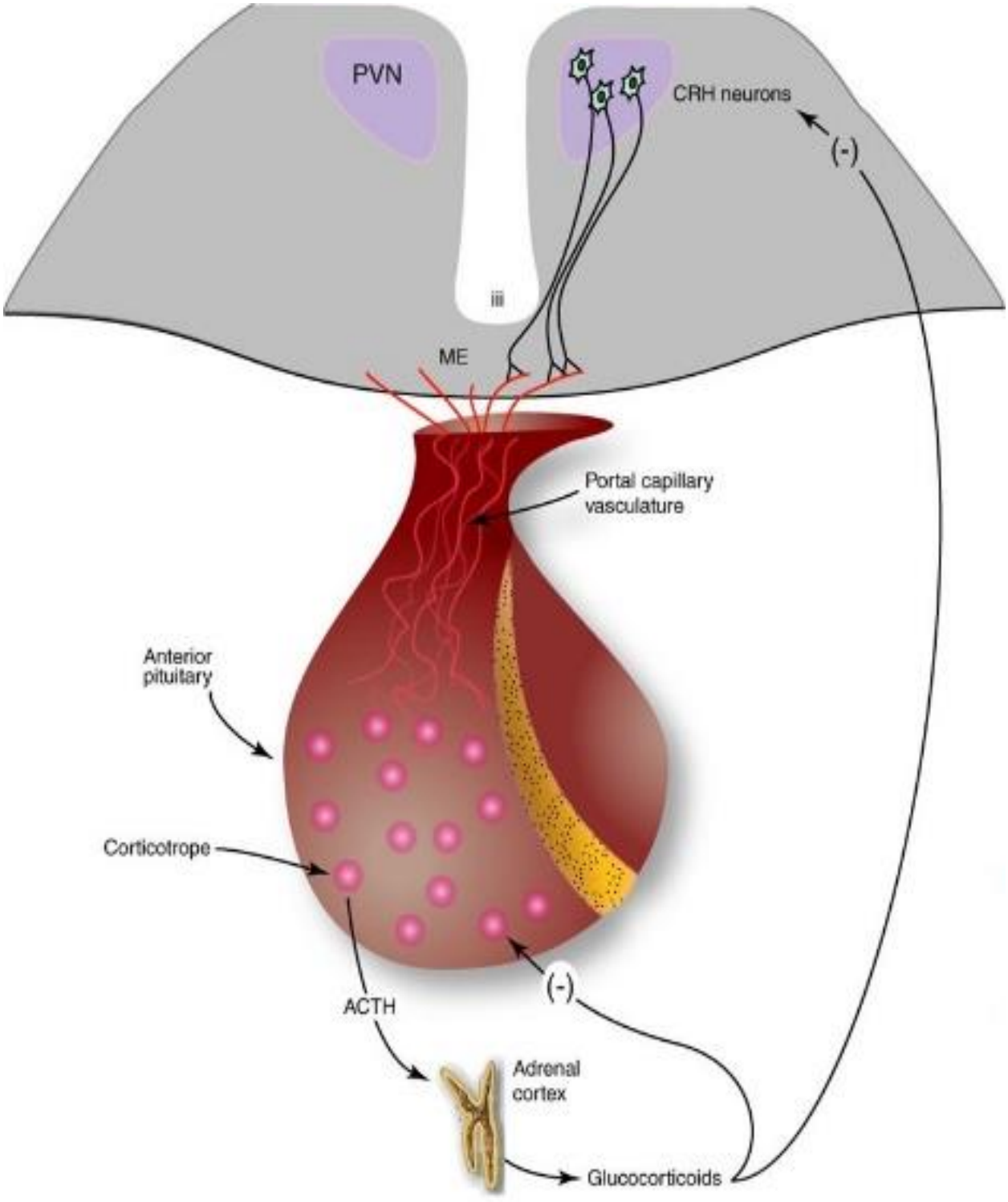

Figure 1. Schematic representation of the brain-pituitary-adrenal axis showing CRF neurons in the paraventricular nucleus projecting their neuroterminals to the external zone of the median eminence and the negative feedback loop whereby cortisol inhibits CRF release in the medial eminence.

Normally, glucocorticoids exert an inhibitory feedback on the HPA axis (mediated by the activation of hippocampal neurons that control the activity of hypothalamic CRF neurons), so that the activation of this signaling cascade is short-lived (Figure 1). However, in conditions of severe and/or prolonged stress, the levels of glucocorticoids remain high and the hippocampal neurons are not able to inhibit their release (Meyer and Quenzer, 2009).
Several studies have demonstrated that in a large percentage of symptomatic depressed patients the plasmatic concentration of cortisol is persistently higher with respect to healthy controls. Moreover, in these individuals the administration of dexamethasone does not suppress the release of cortisol induced by CRF, suggesting that the regulation of the HPA axis activity through the negative feedback is altered. Interestingly, the intracerebral administration of CRF in experimental animals produces effects that are similar to those observed in individuals affected by MDD, such as a reduction in locomotor activity, loss of appetite, insomnia and anxiety. Collectively, these preclinical and clinical findings suggest a correlation between the hyperactivity of the HPA axis and depression (Holsboer, 2001).

The mechanisms by which sustained high levels of glucocorticoids are able to alter the inhibitory feedback on the HPA axis are not yet completely understood but *in vitro* studies have revealed glucocorticoid-induced damage of hippocampal neurons which involves a reduction in dendritic branching and a loss of highly specialized dendritic spines that make synaptic contacts with afferent glutamatergic fibers (McEwen, 2000; Sapolsky, 2000). Moreover, the damage triggers positive feedback because the reduction in the inhibitory control exerted by the HC on the HPA axis leads to a further increase in circulating glucocorticoid levels and subsequent hippocampal damage (Nestler et al., 2002).

The identification of the deleterious effects of stress on the HC has led to the hypothesis that neurotrophic factors play a key role in the pathophysiology of depression (Altar, 1999; Duman et al., 1997).

1.2.5 The neurotrophic factor hypothesis and the role of BDNF

The neurotrophic hypothesis of depression posits that a deficiency in neurotrophic support may contribute to hippocampal pathology during the development of depression, and that reversal of this deficiency by antidepressant treatments may contribute to the resolution of depressive symptoms.

Neurotrophic factors are a group of brain proteins initially considered as regulators of neural growth and differentiation during development, but are now known to be potent regulators of plasticity and survival of neurons and glia along adult life (Nestler et al., 2002). The neurotrophin family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin 4/5. Although all of these neurotrophins are expressed throughout the central nervous system, the most widely distributed and most abundant in the brain is BDNF.

BDNF is synthesized as a precursor protein known as prepro-BDNF that is cleaved into pro-BDNF, which can then be further cleaved into mature BDNF (Lessmann et al., 2003)**.** Recent data suggest that pro-BDNF and mature BDNF activate different intracellular signaling pathways (Woo et al., 2005; Matsumoto et al., 2008; Yang et al., 2009). Pro-BDNF binds to the low-affinity neurotrophin receptor p75 that is believed to be involved in apoptosis (Lessmann et al., 2003; Roux and Barker, 2002), whereas mature BDNF binds to the high-affinity tropomyosin related kinase B (trkB) receptor (Autry and Monteggia, 2012). Upon binding to trkB, BDNF induces the dimerization and autophosphorylation of its receptor thereby triggering the activation of the intracellular signaling cascades listed below (Levine et al., 1998):

(i) the phospholipase Cγ (PLCγ) pathway, which leads to activation of protein kinase C;
(ii) the phosphatidylinositol 3-kinase (PI3K) pathway, which activates serine/threonine kinase AKT;
(iii) the mitogen-activated protein kinase [MAPK, or extracellular signal-related kinase (ERK)] pathway, which activates several downstream effectors.

Each of these signaling pathways mediates the unique functions of BDNF on cells (Mattson, 2008; Yoshii and Constantine-Paton, 2010). Specifically, the rapid synaptic and ion channel effects are thought to depend on PLCγ-mediated calcium release from intracellular stores while the longer-lasting genomic effects are considered to be mediated by the PI3K and MAPK pathways. In addition, BDNF may directly activate voltage-gated sodium channels to mediate rapid depolarization of target neurons (Blum et al., 2002).

There are several lines of preclinical and clinical evidence supporting a role for BDNF in depression and in the mechanisms of action of antidepressants.
Numerous studies have been performed in a variety of animal models of depression, in which the depression-like behavior may be the result of a genetic selection or it can be induced by environmental or pharmacological manipulations during the perinatal period or in adulthood (Rezvani et al., 2002).

In particular, the results of preclinical studies show that (1) chronic unpredictable stress decreases hippocampal mRNA and protein levels of BDNF in mice and rats (Duman and Monteggia, 2006), (2) the long-term administration of corticosterone decreases BDNF expression in the rat HC (Jacobsen and Mork, 2006), (3) in contrast, subacute and chronic treatment with antidepressant drugs increases the expression of BDNF in the HC (Nibuya et al., 1995; Altar et al., 2004), and electroconvulsive seizures increase the concentrations of BDNF in the HC, striatum, and occipital cortex (Angelucci et al., 2002), (4) infusion of BDNF into the midbrain, ventricles, or hippocampal regions results in antidepressant-like behavior (Siuciak et al., 1996, 1997; Shirayama et al., 2002; Koponen et al., 2005; Monteggia et al., 2004). Collectively, these preclinical findings indicate that the appearance of depression-like behaviors is associated with a decreased hippocampal concentration of BDNF that normalizes when such behaviors are ameliorated by antidepressant treatments (Figure 2).

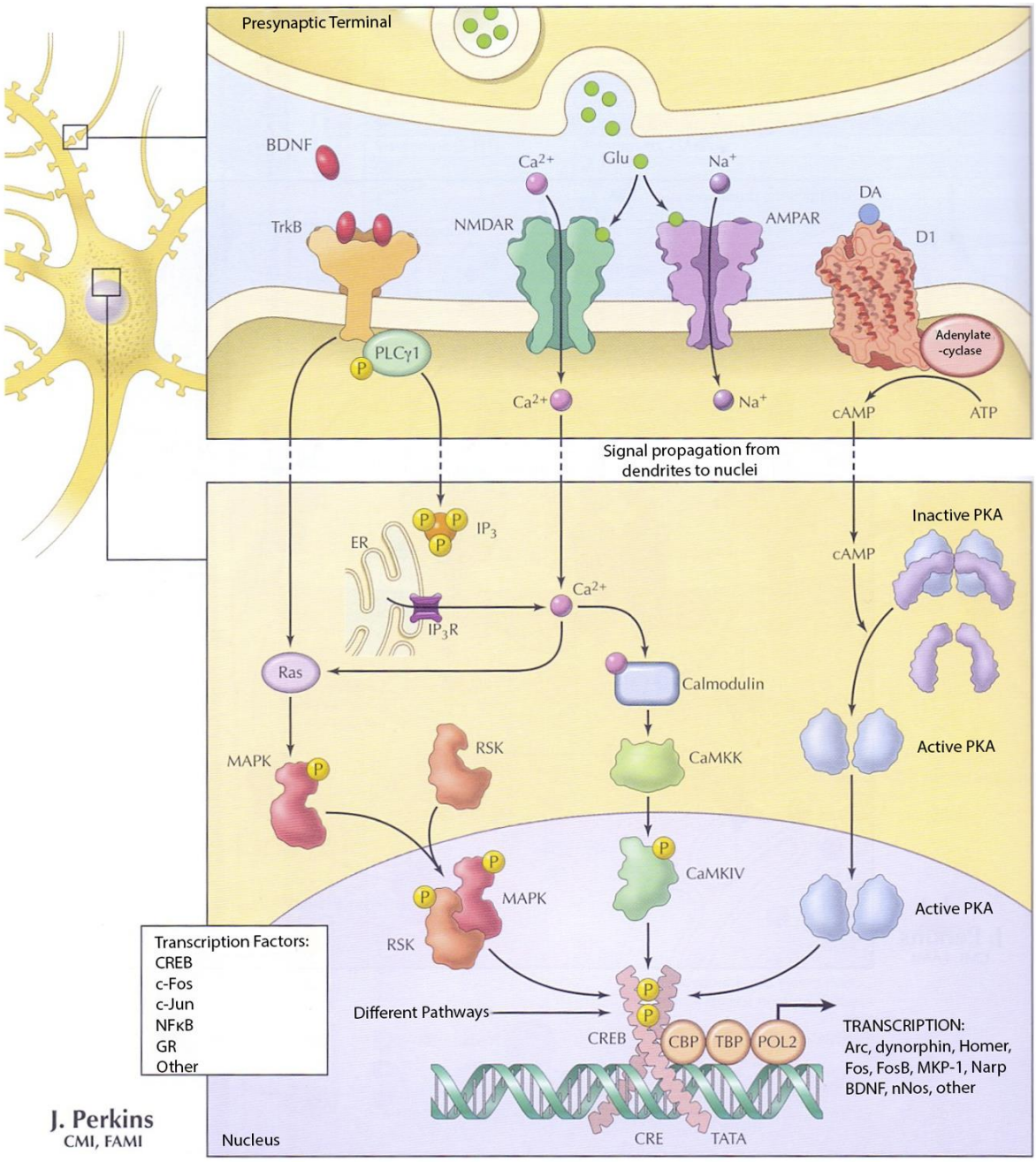

Figure 2. Schematic representation of the intracellular signaling cascades mediating the adaptive plastic effects of BDNF, glutamate and dopamine on different central neuronal populations.

In keeping with the aforementioned experimental findings, clinical studies in depressed patients have revealed decreased plasma levels of BDNF, a condition that is normalized by antidepressant treatments (Sen et al., 2008; Monteleone et al., 2008) although it is not clear if the plasma levels of BDNF are indicative of its cerebral concentration. The ability of antidepressant treatments to increase BDNF concentrations in the brain of depressed patients was confirmed by post-mortem investigations comparing brain samples from depressed patients without treatment with those

obtained from depressed patients that were good responders to antidepressants. Those studies demonstrated that in not-treated patients the concentrations of BDNF and trkB in the HC and prefrontal cortex (PFC) are reduced (Castren and Rantamaki, 2010; Thompson et al., 2011) and the hippocampal volume is reduced (Autry and Monteggia, 2012). Conversely, patients successfully treated with antidepressants show an increase in the hippocampal and cortical concentrations of BDNF and trkB (Duman and Monteggia, 2006).

Altogether, these results support the hypothesis that the concentrations of BDNF in the hippocampus may be correlated with both, the appearance of depressive symptoms and their resolution with antidepressant treatments. It is noteworthy, however, that other neurotrophic factors and brain areas may also be involved.

1.2.6 Structural and functional alterations in Depression

Neuroimaging studies in patients with an MDD diagnosis have identified structural and functional abnormalities in several brain areas. These abnormalities, which can be permanent or reversible, include changes in the volume, in the cerebral blood flow (CBF) and in glucose metabolism of the involved brain area. Accordingly, postmortem studies demonstrate reductions in the volume of the orbital and medial PFC in the brain of patients who died during symptom remission. Conversely, changes in physiological activity (assessed by measurements of CBF and glucose metabolism) are mood state-dependent and generally normalize with antidepressant drug treatment (Drevets, 2000). In particular, a decreased CBF and glucose metabolism have been observed during depressive episodes in dorsal PFC areas implicated in language, selective attention, visuospatial or mnemonic processing (Drevets, 2000). Conversely, in the ventrolateral, orbital and pregenual anterior cingulate portions of the PFC abnormal elevations of CBF and metabolism have been recorded (Baxter et al., 1987; Drevets and Botteron, 1997; Drevets et al., 1997). The activation of the posterior orbital cortex is thought to reflect endogenous attempts to break persevering patterns of negative thoughts and emotions which may be driven by pathological limbic activity involving the AMYG (Drevets and Botteron, 1997). Regarding the AMYG, regional CBF and glucose metabolism consistently correlate positively with depression severity (Abercrombie et al., 1998; Drevets et al., 1992, 1995), but this abnormally elevated activity decreases toward normal levels during antidepressant drug treatment (Drevets et al., 1996). The AMYG participates in the assignment of emotional significance to experimental stimuli and in the organization of behavioral, autonomic, and neuroendocrine manifestations of emotional expression (LeDoux, 1987). In particular, during depressive episodes, the metabolic rate of the AMYG is positively correlated with

plasma cortisol concentrations, suggesting that pathological amygdala activity may increase HPA axis activity in depression (McEwen, 1995).
Collectively, these results support a neural model of depression in which dysfunctions involving brain regions that modulate or inhibit emotional behavior may lead to the emotional, motivational, cognitive, and behavioral manifestations of MDD.

1.3 Schizophrenia: diagnosis and social impact

Schizophrenia is a severe and chronic psychiatric pathology, characterized by complex symptomatology that can be classified into 3 categories: positive, negative and cognitive symptoms. Positive symptoms include disorganized speech, hallucinations and delusions and may be normalized by treatment with antipsychotics (NIMH, 2011; Owen et al., 2016). In contrast, the negative symptoms tend to become chronic and include apathy, social withdrawal, reduced expression of emotions, anhedonia and lack of initiative (NIMH, 2011; Owen et al., 2016). Cognitive symptoms are also markedly disabling and include impairments in visual-verbal learning, poor ability to understand the information learned, and to make decisions (Volk and Lewis, 2015; Gold, 2004; NIMH, 2011; Kalkstein et al., 2010, Ellenbroek, 2010).

The key criteria for making the diagnosis of schizophrenia are listed in the DSM-V (American Psychiatric Association, 2013; see Table III). First, at least two of the following symptoms must be present and preponderant for at least one month: (1) delusions, (2) hallucinations, (3) disorganized speech (e.g. frequent derailment or incoherence), (4) grossly disorganized or catatonic behavior, and (5) negative symptoms (i.e. diminished emotional expression or avolition). Also, for a significant portion of the time since the onset of the disorder, the level of functioning in one or more of the main areas, such as work, interpersonal relationships, or self-care must be reduced when compared to the pre-onset phase. Finally, continuous signs of the disorder must persist for at least 6 months, in which at least 1 month of symptoms of the active phase is included (NIMH, 2011; Ellenbroek, 2010).

The comprehension of the onset of schizophrenia is complex, due to the very different classes of symptoms and, above all, their not equal manifestations in different patients (Volk and Lewis, 2015). It has been proposed that several factors, which interact with each other, may explain the etiology:

• Genetics, as indicated by the studies on twins, adopted children and genealogy (Ellenbroek, 2010) and the identification of copy number variants (CNVs) and single-nucleotide polymorphisms (SNPs) that predispose to the onset of the disease (Owen et al., 2016).

• Environmental, which occur mainly during the gestation period [stress, malnutrition, labor complications, infections (Owen et al., 2016; Ellenbroek, 2010; Holder and Wayhs, 2014)] or in puberty [cannabis assumption, living in disadvantaged social contexts (Volk and Lewis, 2015)].

• Neurodevelopmental disorders (Volk and Lewis, 2015).

In addition to the problems that patients with schizophrenia have to face during the disease, they also have to bear inequalities and social discrimination. Although information campaigns regarding psychiatric diseases have improved in the last twenty years, this has not led to a consequent greater social acceptance of the mentally ill. On the contrary, schizophrenics have been further ghettoized; thus, individuals affected by this pathology are more marginalized than other psychiatric patients (Angermeyer and Dietrich, 2006).

Table III. Diagnostic criteria for Schizophrenia according to DSM-V

A. ***Characteristic symptoms***: Two (or more) of the following, each present for a significant portion of time during a 1-month period (or less if successfully treated):

(l) delusions
(2) hallucinations
(3) disorganized speech (e.g., frequent derailment or incoherence)
(4) grossly disorganized or catatonic behavior
(5) negative symptoms, i.e., affective flattening, alogia or avolition

Note: Only one Criterion A symptom is required if delusions are bizarre or hallucinations consist of a voice keeping up a running commentary on the person's behavior or thoughts, or two or more voices conversing with each other.

B. ***Social/occupational dysfunction****:* For a significant portion of the time since the onset of the disturbance, one or more major areas of functioning such as work, interpersonal relations, or self-care are markedly below the level achieved prior to the onset (or when the onset is in childhood or adolescence, failure to achieve expected level of interpersonal, academic, or occupational achievement).

C. ***Duration****:* Continuous signs of the disturbance persist for at least 6 months. This 6-month period must include at least 1 month of symptoms (or less if successfully treated) that meet Criterion A (i.e., active-phase symptoms) and may include periods of prodromal or residual symptoms. During these prodromal or residual periods, the signs of the disturbance may be manifested by only negative symptoms or two or more symptoms listed in Criterion A present in an attenuated form (e.g., odd beliefs, unusual perceptual experiences).

D. ***Schizoaffective and Mood Disorder exclusion****:* Schizoaffective Disorder and Mood Disorder With Psychotic Features have been ruled out because either (1) no Major Depressive, Manic, or Mixed Episodes have occurred concurrently with the active-phase symptoms; or (2) if mood episodes have occurred during active-phase symptoms, their total duration has been brief relative to the duration of the active and residual periods.
E. ***Substance/general medical condition exclusion****:* The disturbance is not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition.
F. ***Relationship to a Pervasive Developmental Disorder****:* **If** there is a history of Autistic Disorder or another Pervasive Developmental Disorder, the additional diagnosis of Schizophrenia is made only if prominent delusions or hallucinations are also present for at least a month (or less if successfully treated).

Discrimination takes place in different situations: in job search (Schulze and Angermeyer, 2003); in access to health care and the treatment of diseases of different nature, such as cardiovascular diseases and diabetes (Levinson et al., 2003; Druss et al., 2000; Desai et al., 2002); in the description made by media, in which schizophrenics (and, more generally, psychiatric patients) are defined as violent and dangerous for society (Corrigan et al., 2004).

In light of the several problems exposed so far, it is clear that it is necessary to continue the study of the neurobiological bases of schizophrenia, in order to be able to develop new and more efficacious treatment strategies for this pathology.

1.4 The neurobiology of Schizophrenia

1.4.1 The role of Dopamine

Three dopaminergic pathways, mesolimbic, mesocortical and nigrostriatal, play a key role in the pathophysiology and treatment of schizophrenia. The mesolimbic and mesocortical pathways are formed by the projections of the dopaminergic neurons of the ventral tegmental area (VTA) to the nucleus accumbens (Acb) and medial prefrontal cortex (PFC), respectively, whereas the nigrostriatal pathway is formed by dopaminergic neurons located in the substantia nigra pars compacta that project to the striatum. DA signaling is mediated by G protein-coupled receptors that are subdivided into two families: D_1 (which includes subtypes D_1 and D_5) and D_2 (which includes subtypes D_2, D_3 and D_4) (reviewed in Brisch, 2014).

The first experimental evidence on the possible role of dopamine in the pathogenesis of schizophrenia is attributed to the discovery of the D_2 subtype receptor (Seeman et al., 1975, 1976) which is the binding site for typical antipsychotics (Madras, 2013; Seeman et al., 2006). It has been hypothesized that the excessive activation of D_2 receptors in the accumbal projection field of the mesolimbic system may explain psychotic crises or, more generally, positive symptoms (Brisch, 2014; Swerdlow et al., 1996; Lewis and González-Burgos, 2006; Desbonnet, 2016); on the other hand, a reduced dopaminergic signaling in the PFC can be at the basis of the negative and cognitive symptoms (Desbonnet, 2016). Further pharmacological evidence demonstrates the involvement of dopaminergic transmission in the pathophysiology of schizophrenia (Swerdlow et al., 1996). Thus, psychostimulant drugs like amphetamines and cocaine, which are potent indirect DA mimetic drugs, induce psychotic symptoms in healthy subjects and exacerbate them in schizophrenic patients (Bramness et al., 2012); moreover, the higher the binding affinity for D_2 receptors by a competitive antagonist the greater its antipsychotic activity (i.e., the lower the therapeutic dose) (Seeman et al., 1976).

Several postmortem studies confirm the presence of dysfunctions in the pre- and postsynaptic dopaminergic pathways, although contrasting results have also been reported (Howes et al., 2015). Thus, the density of D_1 receptors in individuals with schizophrenia appears to be increased in the parieto-temporal cortex and decreased in the PFC (Brisch, 2014); in contrast, it has been reported that the density of D_2 receptors is increased in the striatum (Seeman, 2013). It should be taken into account, however, that it remains to be clarified whether the alterations in DA receptor density in postmortem brain samples represent intrinsic features of schizophrenia or are caused by antipsychotic treatment.

The synthesis of atypical antipsychotics, which bind with high affinity to other receptors in addition to D_2 receptors, has led to the formulation of further hypotheses on the etiology of schizophrenia.

1.4.2 The role of Serotonin

Serotonin (5-HT) is broadly distributed in different body areas. About 90% of the total amount of 5-HT in the human body is located in the enterochromaffin cells of the gastrointestinal mucosa while the remaining 10% is located in platelets and in the serotonergic neurons of the central nervous system where it regulates the release or inhibition of other neurotransmitters, such as DA, GABA and glutamate (Nichols and Nichols, 2008). Its action is expressed through ionotropic 5-HT_3 receptors and several subtypes of G-protein coupled 5-$HT_{1,2,4\text{-}8}$ membrane receptors (Hannon and Hoyer, 2008).

The finding that lysergic acid diethylamide (LSD), a 5-HT_{2A} receptor (5-HT_{2A}-R) agonist, has hallucinogenic effects that resemble positive symptoms of schizophrenia provides strong support to the hypothesis that alterations of the serotonergic transmission are involved in the pathogenesis of the disorder (Aghajanian and Marek, 2000). Further support to this argument derives from the ability of the atypical antipsychotics aripiprazole, risperidone and clozapine to block 5-HT_{2A}-Rs (Celada et al., 2013; Miyamoto et al., 2012; Geyer and Vollenweider, 2008; Geyer et al., 2012).

Importantly, the 5-HT_{2A}-R interacts through specific transmembrane helix domains with the type 2 metabotropic glutamate receptor ($mGlu_2$-R) to form functional heterodimers in the brain cortex; moreover, the interaction of hallucinogenic 5-HT_{2A}-R agonists with the 5-HT_{2A}-R/$mGlu_2$-R complex triggers unique cellular responses while the activation of $mGlu_2$-Rs abolishes hallucinogen-specific signaling and behavioral responses (González-Maeso et al., 2008). Of note in this context, in postmortem human brain samples from untreated schizophrenic subjects, the 5-HT_{2A}-R is upregulated and the $mGlu_2$-R is downregulated, a pattern that could predispose to psychosis. These regulatory changes indicate that the 5-HT_{2A}-R/$mGlu_2$-R complex may be involved in the altered cortical processes of schizophrenia, and this complex is, therefore, a promising new target for the treatment of psychotic disorders (González-Maeso et al., 2008).

1.4.3 The role of Glutamate

Glutamate is the main excitatory neurotransmitter in the central nervous system and plays a role in different functional activities including brain development, memory and learning (Harvard Health, 2009; see section 1.2.3).

The hypothesis that a glutamatergic dysfunction plays a role in schizophrenia was driven by the observation that phencyclidine (PCP), a noncompetitive antagonist of the NMDA glutamate receptors induces hallucinations and delusions in healthy subjects and aggravates these symptoms in schizophrenic patients (Cohen et al., 1962; Javitt and Zukin, 1991; Krystal et al., 1994; Tamminga, 1998; Neill et al., 2010; Moghaddam and Javitt, 2011). Moreover, in addition to psychosis, PCP may cause behavioral alterations reminiscent of cognitive and negative symptoms of schizophrenia, which are reduced by interruption of the intake of the drug (Javitt and Zukin, 1991; Luby et al., 1959; Cosgrove and Newell, 1991). There are now numerous postmortem studies in schizophrenic patients supporting this proposal. For example, in the temporal lobe, glutamatergic markers are decreased (Tsai et al., 1995), with reduced expression of non-NMDA glutamate receptors (Harrison et al., 1991). In the PFC, the alterations affect NMDA receptor subunit mRNAs (Akbarian et al., 1996) as well as $mGlu_2$-R, indicating a regional and receptor subtype selectivity of these

neurochemical alterations. (Tamminga, 1998; Meador-Woodruff et al., 2000; González-Maeso et al., 2008).

1.4.4 The role of BDNF

As previously mentioned, BDNF is a neurotrophic factor implicated in a wide variety of functions, including synaptic plasticity (Marosi and Mattson, 2014), the modulation of numerous neural circuits and their survival (Krebs et al., 2000; Martinowich and Lu, 2008; Baker et al., 2005), and the regulation of brain development. Much effort has been therefore dedicated to investigate its role in the etiology of schizophrenia.

It has been found that a single nucleotide polymorphism (SNP) in the prodomain of BDNF (Methionine instead of Valine in position 66) is associated with a reduction in the activity-induced secretion of BDNF (Chen et al., 2004). Notably, this polymorphism is also associated with changes in the volume of the lateral ventricles and the gray matter of the frontal lobes in individuals with schizophrenia (Beng-Choon Ho et al., 2007). In addition, the expression of the NTRK2 gene, implicated in the synthesis of the BDNF receptor, trkB, is significantly reduced in schizophrenics (Hashimoto et al., 2005).

Epigenetic mechanisms in BDNF transmission, such as DNA methylation controlled both through genetic and environmental mechanisms, may also be involved in schizophrenia (Ibi and González-Maeso, 2015). Thus, a postmortem study identified differences in DNA CpG methylation between healthy individuals and patients with schizophrenia (Jaffe et al., 2016). Moreover, further postmortem investigations revealed a reduction in BDNF expression in PFC and HC of schizophrenics compared to controls, although these findings were not confirmed by other studies (Hashimoto et al., 2005; Takahashi et al., 2000; Weickert et al., 2003; Iritani et al., 2003).

Brain BDNF levels are also altered in a neurodevelopmental animal model of schizophrenia. Thus, in rats submitted to bilateral neurotoxic lesions of the ventral HC in the early neonatal period, a significant reduction in BDNF mRNA is observed in the PFC, cingulate cortex and HC in the postpubertal period (Ashe et al., 2002; Lipska et al., 2001; Molteni et al., 2001). Interestingly, the hippocampal levels of BDNF mRNA can be modified by the chronic administration of antipsychotics: atypical antipsychotics increase BDNF expression in rodents while typical antipsychotics reduce it (Chlan-Fourney et al., 2002).

In conclusion, although numerous preclinical and clinical studies suggest a possible relationship between BDNF-mediated signaling and schizophrenia, further research is warranted to establish the role of BDNF in the pathophysiology and therapy of this disorder.

1.4.5 Structural and functional alterations in Schizophrenia

Although for over a century much effort was dedicated to postmortem studies in the search for the structural and biochemical underpinnings of schizophrenia, little progress was made until the emergence, beginning in the 1970s', of brain imaging techniques that led to the identification of several structural abnormalities intrinsic to the disorder that are summarized in Table IV (Harrison, 2000).

Table IV. Macroscopic Brain Changes in Schizophrenia
Enlargement of lateral and third ventricles (+25% / +40%)
Smaller brain volume (-3%)
Smaller cortical volume (-4%)
Smaller gray matter volume (-6%)
Relatively smaller medial temporal lobe volume (-5%)
Relatively smaller thalamic volume (-4%)
Larger basal ganglia (especially the globus pallidus) *
Percent change is shown in parentheses
* Due to antipsychotic medication.

The imaging data have also allowed other important conclusions to be drawn, which inform and support postmortem research (Table V) (Suddath et al., 1990; Harrison et al., 1999; Garver et al., 1999; Elkis et al., 1995). In particular, since the structural changes are present at the time of disease onset and are not progressive thereafter (Garver et al., 1999; Lieberman, 1999), it is reasonable to assume that the corresponding histological abnormalities share this property.

Table V. Characteristics of Structural Imaging Findings in Schizophrenia
In monozygotic twins discordant for schizophrenia, anatomical abnormalities are present only in the brain of the schizophrenic twin
Differences are present in first-episode, untreated patients, and in high-risk individuals
No convincing evidence of heterogeneity (eg, subtypes or gender differences), or clinicopathological correlations (eg, progression of changes)
The alterations are not seen in bipolar disorder to the same extent, if at all

The two most robust and important histological findings concerning the neuropathology of schizophrenia are both negative: there is no excess of gliosis, or Alzheimer's disease or other neurodegenerative pathology. The neuropathological process involves a change in the normal cytoarchitecture of the brain, probably originating in development. A summary of the most established and the most often cited findings is given in Table VI (Harrison, 2000).

Table VI. Histological findings in schizophrenia	Weight of evidence
Lack of neurodegenerative lesions (eg, Alzheimer changes)	+ + + + +
Lack of gliosis	+ + + +
Smaller cortical and hippocampal pyramidal neurons	+ + +
Decreased cortical and hippocampal synaptic markers	+ + +
Decreased dendritic spine density	+ +
Loss of neurons from dorsal thalamus	+ +
Entorhinal cortex dysplasia	+
Loss of hippocampal or cortical neurons	**0**
0: no good evidence; + to + + + + +: increasing amounts of supportive data	

1.5 Animal models of psychiatric disorders

1.5.1 General aspects

Animal models of complex heterogeneous psychiatric disorders are clearly essential preclinical tools with which to investigate the neurobiological basis of the disorder. They offer a heuristic platform to monitor disease progression more rapidly than in humans, and the possibility to perform invasive assessments of the structural and molecular underpinnings of the disease and test novel therapies that cannot be administered to patients. However, an unresolved problem is how to assess some of the core symptoms of psychiatric disorders which are uniquely human traits and, therefore, cannot be modeled in animals (e.g., feelings of worthlessness, excessive or unmotivated guilt and recurring thoughts of suicide in the case of depression and delusions, hallucinations and disorganized speech in the case of schizophrenia).

It is extremely important to bear in mind that the use of animals in biomedical research must be based on ethical principles that can be summarized by the "3 R rules":

- **Reduction**: the number of animals used should be as low as possible but maintaining statistical power. The improvement of experimental techniques and the sharing of information between researchers is essential to pursue this aim;
- **Refinement**: the suffering of animals must be reduced through the improvement of the experimental protocols or how animals are treated;
- **Replacement**: alternative techniques instead of animals should be utilized whenever possible.

Animal models should meet specific criteria to be translational and relevant. There are three requirements that an ideal model should satisfy: 1) face validity (symptomatic manifestation similar to the clinical condition); 2) construct validity (similar biological variables between the model and the pathology); 3) predictive validity (response to clinically effective treatments) (McKinney et al., 1969). These criteria, however, are not always present simultaneously in a single model, especially in the case of psychiatric pathologies, because, as mentioned above, some uniquely human traits of the disorder cannot be modeled in animals. To reduce the complexity of these disorders to more easily assessable aspects, it is preferred to use an approach based on endophenotypes, based on biological markers, specific symptoms or risk factors and modeling them on animals.

1.5.2 Animal models of Depression

The development of animal models of depression that satisfy the three validity criteria is a difficult endeavor. However, since depression often occurs following an abnormal reaction to stressful situations, most models are based on the exposure to stressors of different nature and duration to induce a depression-like phenotype.

One of the models based on the stress response is the chronic unpredictable stress paradigm (CUS) in which the animal is exposed to a series of different unpredictable stressful conditions, such as food and water deprivation, a reversal of light/dark periods, low-intensity unavoidable foot shocks, exposure to noise and intense light, and changes in housing conditions. After two or three weeks of exposure to these stressful situations, rats will show a series of changes in behavior such as anhedonia, evidenced by the reduction in the consumption of sucrose solution compared to tap water, or in the psychomotor behavior, as shown by reduced motility in the open field test (Willner et al., 1992); these dysfunctional behaviors persist for months and are reminiscent of depressive symptoms. The depression-like behavior induced by the CUS can be ameliorated by long-term treatment with antidepressants, which return sucrose intake to normal conditions: a clear demonstration of the strong predictive validity of the model (Holsboer, 1999; Vitale et al., 2009).

The induction of behavior similar to depression can also occur through lesions of brain areas involved in the pathogenesis of the disease. One of these is the olfactory bulbectomy, based on the observation that the olfactory system of the rat is part of the limbic system, in which the HC and the AMYG are implicated in the mnemonic and emotional components of behavior and appears to be dysfunctional in depressed patients. Bilateral olfactory bulbectomy, therefore, damaging the cortical-hippocampal-amygdala circuit, leads to pathological changes in neurotransmitter, immune and endocrine systems that are reminiscent of those of depressed patients and are normalized by chronic administration of antidepressant drugs (Song and Leonard, 2005).
However, none of these models accounts for the genetic vulnerability in the development of depression. Genetic factors strongly influence the pathogenesis of this illness, although the gene(s) involved have not yet been identified. The research of specific genes is difficult for different reasons. Firstly, depression is a complex disorder that involves a large number of genes. In addition, epidemiological data underline the existence of a complex relationship between genotype and environment. Thus, genetic factors partially influence the overall risk of illness but also influence the sensitivity of individuals to the depression-inducing effects of environmental adversities (Kendler et al., 1995). The combination of genetics, early life stress, and ongoing stress may

ultimately determine individual responsiveness to stress and the vulnerability to psychiatric disorders, such as depression. It is likely that genetic factors and life stress contribute not only to the neurochemical alterations, but also to the impairments of cellular plasticity and resilience observed in depression (Charney and Manji, 2004).

Considering the pivotal role of genetic factors in the pathogenesis of mood disorders, several genetic animal models of depression have been developed. Some of these models use genetically engineered animals, such as DAT, SERT and NET (DA, 5-HT and NE transporters, respectively) knock out (KO) mice, to assess the contribution of specific genes, in particular those encoding for monoamine transporters, which are the targets of common antidepressant drugs (Perona et al., 2008). Other models are based on the use of lines/strains of mice or rats selectively bred for their differences in the phenotypic characteristics associated with depression.

One of these models is represented by the Flinders sensitive (FSL) and resistant (FRL) rats, that were selectively bred for respectively high vs. low sensitivity to the effects of di-isopropyl-fluoro-phosphate (DFP), an organophosphate cholinesterase inhibitor (Overstreet et al., 1979, 2005; Russell et al., 1982). Since depressed individuals are more sensitive to cholinergic agonists than normal controls, FSL rats were proposed as a genetic model of depression (Janowsky et al., 1980, 1994; Risch et al., 1981). Thus, FSL rats display several behavioral phenotypes reminiscent of symptoms of depression, such as increased REM sleep, reduced locomotor activity, cognitive deficits and reduced body weight (Overstreet, 1993), and have been very helpful in testing the effects of chronic antidepressants (Overstreet et al., 1995).

1.5.3 Animal models of Schizophrenia

The development of animal models related to schizophrenia presents the same difficulties already mentioned in the general aspects section. Nevertheless, several models have been developed showing behavioral and neurochemical alterations that resemble different aspects of the schizophrenic symptomatology (Jones et al., 2011; Del Río et al., 2014; Carpenter and Koenig, 2008).

All available animal models of schizophrenia fit into four different induction categories: developmental, drug-induced, lesion or genetic manipulation. Human epidemiology provides compelling evidence that exposure of individuals to adverse environmental insults, either during gestation or the early postnatal life, increases the risk of developing schizophrenia. Thus, maternal stress, malnutrition, infection or immune activation as well as obstetric complications (such as

hypoxia) during the perinatal period are just some of the diverse perturbations that increase the risk of developing schizophrenia, consistent with it having a neurodevelopmental origin (Lewis and Levitt, 2002). A favored neurodevelopmental working hypothesis posits that exposure of individuals with a genetic predisposition to an early-life adverse event may trigger an altered pattern of neuronal development and connectivity that remains asymptomatic throughout childhood and subsequently results in the expression of a schizophrenic phenotype in the postpubertal period. While the precise nature of the early-life adverse event may not be critical, the time that this occurs is important. Accordingly, a few animal models have been established that replicate the time course of the clinical pattern of schizophrenia. These models utilize manipulations of environment, or drug administration during the sensitive perinatal period, to produce irreversible changes in CNS development. Among the most frequently used procedures, it is worthwhile mentioning methylazoxymethanol (MAM) administration to rat dams (Moore et al., 2006; Lodge and Grace, 2009) and neonatal bilateral neurotoxic lesions of the ventral HC (Corda et al., 2006; Lipska and Weinberger, 2000).

All useful animal models of schizophrenia should satisfy the triad of face, construct and predictive validity. For schizophrenia, a suitable constellation of behavioral and neurochemical abnormalities should include postpubertal onset, replicating the chronology of symptomatology seen in schizophrenia, loss of hippocampal and cortical connectivity and function, limbic dopamine dysregulation, cortical glutamatergic hypofunction, vulnerability to stress, abnormal response to reward, social withdrawal and cognitive impairment.

The post-weaning social isolation is a broadly used environmental manipulation in which the lack of a littermate causes in puppies behavioral deficits and cognitive, neuroanatomical and neurochemical pathological changes that become apparent in the post-pubertal period and are closely related to schizophrenia features (Rapoport et al., 2012; Jones et al., 2011; Powell, 2010; Silva-Gomez et al., 2003; Weiss et al., 2004; Day-Wilson et al., 2006; Möller et al., 2011; Heidbreder et al., 2000).

Other developmental models include the prenatal exposure to *polyriboinosinic-polyribocytidilic* acid (PolyI: C) (Ibi et al., 2009) or influenza virus (Fatemi et al., 2002; Shi et al., 2003), or the intracerebral infusion of rats with ibotenic acid on postnatal day 7 to cause a bilateral lesion of the ventral HC (Jones et al., 2011).

A broadly used pharmacological model involves the administration to rodents of phencyclidine (PCP), a non-competitive antagonist of the NMDA glutamate receptor (see section 1.4.3 The role of

Glutamate) which induces hallucinations and delusions in healthy subjects and aggravates these symptoms in schizophrenic patients (Cohen et al., 1962; Krystal et al., 1994; Javitt and Zukin, 1991). In animal models it has been observed that acute and chronic administration of PCP causes reduced sociability, deficits in cognition and Prepulse Inhibition (PPI) and locomotor hyperactivity (Sams-Dodd, 1995, 1996; Egerton et al., 2005; Mansbach and Geyer, 1989; Kalinichev et al., 2007). Importantly, these effects are prevented by acute doses of both clozapine and haloperidol (Sams-Dodd, 1998; Qiao et al., 2001; Lee et al., 2005), but see contrasting results by Jenkins et al. (2008).

Tween studies unequivocally demonstrate that schizophrenia is predominantly a genetic disorder with heritability estimated to be around 80% (Jones, 2011). The first attempts to develop valid models to assess the influence of the genetic make up on the vulnerability to schizophrenia were based on the selective breeding of animals for differences in the response to a specific test or drug, allowing the differentiation into distinct groups (Del Río et al., 2014; Ellenbroek and Karl, 2016). Thus, several lines displaying phenotypic traits reminiscent of schizophrenia symptoms have been selectively bred. Among these lines, it is worth mentioning (1) the Brattleboro Rats (BRAT), derived from Long Evans rats carrying a mutation that compromises the secretion of vasopressin and showing an innate deficit in PPI (Feifel and Priebe, 2001), (2) the Low and High-PPI lines, selected for low/high sensorimotor gating of the acoustic startle response (Schwabe et al., 2007), and (3) the APO-SUS and UNSUS rats respectively sensitive and not sensitive to the effects of apomorphine (Ellenbroek et al., 1995).

In the last few years, thanks to advanced genetic engineering technologies, mice and rats have been developed in which specific genes have been inserted, deleted or their expression has been reduced. Some good examples are the KO rats for the serotonin transporter (SERT), the heterozygous mice for Neuregulin 1 (NGR1); the KO mice for DISC1 (Disrupted-in-schizophrenia 1) and calcineurin (CN) (Jones et al., 2011; Del Río et al., 2014; Ellenbroek and Karl, 2016).

1.6 The Roman high- and low-avoidance rats

1.6.1 Background

Another genetic model that may provide a valid approach to investigate the contribution of the genotype, and its interactions with environmental factors on the neural substrates of depression and schizophrenia, is represented by the Roman High- and Low-Avoidance rats.

The Roman rat lines were established in Rome in the early 1960s through bidirectional selection and outbreeding of Wistar rats showing very high (RHA) vs. extremely low (RLA) rates of acquisition of the two-way active avoidance response (Bignami, 1965). In the following years, several subcolonies were established in different locations, including Birmingham, UK (Broadhurst and Bignami, 1964), London, UK (Durcan et al., 1984), and Zurich, Switzerland (Driscoll and Battig, 1982). Subsequently, breeding nuclei from the Swiss colony were used to establish two other colonies, one at the Autonomous University of Barcelona in 1993 and the other, in 1998, at the University of Cagliari. Unlike the Italian colony where the initial outbreeding process was maintained (Giorgi et al., 2005), two inbred strains, RHA-I and RLA-I, were generated in the Autonomous University of Barcelona through brother/sister mating of the respective Swiss sublines (Escorihuela et al., 1999). The experiments described herein were carried out using animals bred in the colonies of the Autonomous University of Barcelona and the University of Cagliari.

1.6.2 The selection criterion: Two-way active avoidance test

The selection criterion of Roman rats is based on their behavioral response in the two-way active avoidance test. This test is carried out in a shuttle box with two compartments, which communicate by an opening, and a floor consisting of a metal grid connected to an instrument that provides a conditioned stimulus (CS), represented by a light and a tone, and an unconditioned stimulus (US) that follows the CS, consisting of an electric shock to the paws. Both stimuli take place in the compartment occupied by the rat: consequently, the animal must necessarily flee to the opposite chamber during the conditioned stimulus, in order to avoid the electric shock that occurs immediately afterward. This specific response is called **avoidance**; if the animal flees to the other compartment during the US the response is called an **escape** whereas if the rat remains immobile in the same compartment throughout the shock time the response is defined as a **failure**. There are strikingly large differences in the responses displayed by the two lines: compared with their RHA counterparts, RLA rats show fewer avoidances and escapes and a dramatically larger number of failures.

Growing evidence demonstrates that diverse mental processes can affect the ability to acquire the avoidance response, in addition to those related to learning and memory. Thus, the learning ability of avoidant behavior seems to be influenced by a conflict situation in the animal, which is generated in the initial phase of the test. This conflict leads the animal to an emotional response called conditioned fear, which is responsible for freezing behavior, a state of complete immobility. This response does not allow the acquisition of active avoidance (Fernández-Teruel et al., 1991). In

support of these studies, pharmacological treatments (Fernández-Teruel et al., 1991) and behavioral manipulations (Levine and Wetzel, 1963; Escorihuela et al., 1995) that reduce anxiety and fear facilitate the acquisition of avoidance, while the opposite is observed with environmental conditions that increase anxiety and fear (Weiss et al., 1968) or through the administration of anxiety-inducing drugs (Fernández-Teruel et al., 1991). Therefore, although the performance in the two-way active avoidance test is widely considered to depend on learning and memory, the emotional state of the animal (i.e., anxiety and fear) plays a predominant role in the ability to acquire the avoidant response.

1.6.3 Phenotypic characteristics of RHA and RLA rats

As stated above, the poor acquisition of the avoidance response of RLA rats and other Low-avoidance lines is not due to learning deficits, but to their high emotionality (Fernández-Teruel et al., 2002a; Brush, 1991). In fact, in several behavioral learning tests that are only marginally influenced by stressors such as the Hebb-Williams labyrinth test (Nil and Bättig, 1981; Escorihuela et al., 1995; Brush, 1991; Moreno et al., 2010), RLAs perform significantly better than RHAs, further confirming that RLA rats do not suffer from any cognitive impairment.

The opposite performances in the active avoidance test of the two Roman lines are due to their distinct coping style, that is, the ability to react to stressful stimuli. In fact, in a variety of tests used to assess coping styles and emotionality in rodents, hypoemotional RHA rats exhibit proactive coping, which allows to face and control the stress generated by an aversive situation; on the other hand, hyperemotional RLA rats display reactive coping, characterized by peculiar behaviors such as self-grooming and freezing, which impede them from taking control over the stressor (Giorgi et al., 2003; Steimer and Driscoll, 2003; Escorihuela et al., 1995; Fernández-Teruel et al., 2002b; Driscoll et al., 1998; Koolhaas et al., 1999; Steimer et al., 1997).

The Roman rats also differ in the neuroendocrine responses to stressors: after exposure to mild stress, RLAs show a more robust secretion of CRF, ACTH and corticosterone vs. RHAs, indicating a more reactive hypothalamus-pituitary-adrenal (HPA) axis of RLAs (Steimer and Driscoll, 2003; Carrasco et al., 2008; Gentsch et al., 1982; Walker et al., 1989). Moreover, the combination of the dexamethasone suppression test with a CRF challenge has shown that RLA rats, like many symptomatic depressed patients (Holsboer, 2001), are more responsive to CRF administration than RHA rats (Steimer et al., 2007).

On the basis of the aforementioned distinctive behavioral and neuroendocrine traits, it has been proposed that RLA rats are more prone than RHAs to develop depression-like behavior in response to aversive conditions (Steimer et al., 2007); on the other hand, RHA rats are resilient to stress-induced depression (Piras et al., 2010), in keeping with their low emotionality and reactivity of the HPA axis (Giorgi et al., 2003, Steimer and Driscoll, 2003; Driscoll et al., 1998). Accordingly, in the forced swim test (FST) which is widely used to assess depression-like behavior and to evaluate the effectiveness of antidepressant drugs in rats and mice, RLAs display more immobility and fewer active behaviors than RHA rats under basal conditions; furthermore, subacute and chronic treatment with antidepressant drugs do not affect the performance of RHAs but improve that of RLAs, reducing immobility and increasing active behaviors (Piras et al., 2010, 2014).

Therefore, there is considerable converging neurobehavioral and pharmacological evidence suggesting that RLA rats may be considered as a genetic model of vulnerability to stress-induced depression with face, construct and predictive validity.

A disinhibited behavioral phenotype, like the proactive coping style of RHA rats, is often associated with traits that resemble core symptoms of schizophrenia spectrum disorders like intense impulsivity, sensation/novelty seeking behavior, and impairments in attention and cognitive flexibility (Coppens et al., 2010; Brown et al., 2018; Ho et al., 2018). Accordingly, compared with RLAs and other rat strains/stocks, the RHA phenotype is characterized by (1) increased impulsive behavior in the 5-choice serial reaction time task (5-CSRTT) and the DRL-20 operant task (Zeier et al., 1978; Moreno et al., 2010; Klein et al., 2014), (2) intense sensation/novelty seeking, as evidenced by increased preference for novelty in a variety of situations (Cuenya et al., 2016; Escorihuela et al., 1999; Fernández-Teruel et al., 1992, 2002a; Manzo et al., 2014; Pisula, 2003; Río-Álamos et al., 2015; Tournier et al., 2013), and also by an ”augmenting” pattern of cortical visual-evoked potentials, a psychophysiological endophenotype that has been specifically associated to sensation/novelty seeking in humans and cats (Saxton et al., 1987a,b; Siegel, 1997; Siegel and Driscoll, 1996; Siegel et al., 1996; Zuckerman, 1996), and (3) impaired spatial reference learning (i.e. place learning) and cognitive flexibility (i.e. reversal place learning) in the Morris water maze (Driscoll et al., 1995; Escorihuela et al., 1995; Fernández-Teruel et al., 1997; Martínez-Membrives et al., 2015; Río-Álamos et al., 2019).

Another characteristic of RHA rats is represented by deficits in latent inhibition (LI) and prepulse inhibition (PPI) of the startle response (Esnal et al., 2016), two attention-related endophenotypes of schizophrenia, (Gray et al., 1991; Jones et al., 2011; Lubow and Weiner, 2010; Powell and Miyakawa, 2006; Swerdlow and Light, 2016).

Finally, RHA rats exhibit several neurochemical and molecular traits involving dopaminergic, glutamatergic and serotonergic neural systems. Such traits are considered to play a role in the control of behavioral phenotypes that are reminiscent of core symptoms of schizophrenia.

Concerning dopaminergic transmission in the mesolimbic system, it has been shown that (1) the acute administration of morphine or psychostimulants determines a larger increment in DA output in the nucleus accumbens shell (AcbSh) of RHAs vs. RLAs (Lecca et al., 2004), (2) the repeated administration of morphine and cocaine elicits behavioral (i.e., enhanced hyperlocomotion) and neurochemical sensitization in RHA, but not RLA rats (Giorgi et al., 2005, 2007), (3) compared with their RLA counterparts, RHA rats display a faster acquisition of cocaine self-administration (SA) behavior, higher resistance to extinction and more robust drug-induced reinstatement of cocaine-seeking behavior (Fattore et al., 2009). Collectively, these findings support the hypothesis that the functional tone of the mesolimbic dopaminergic system is more robust in RHAs vs. RLAs, a condition that resembles the hypothesized hyperactivity of the mesolimbic dopaminergic system of schizophrenics. This hypothesis is consistent with the density distribution pattern of DA receptors in the mesolimbic system of RHA rats. Thus, the density of DA D_1 receptors in the Acb is higher in RHA vs. RLA rats (Giorgi et al., 1994; Guitart-Masip et al., 2006a, b). Furthermore, it has been shown that the density of D_2 DA auto-receptors (D_2-autoR) is lower in the substantia nigra/VTA of RHA vs RLA rats (Tournier et al., 2013), in keeping with reports relating novelty seeking with low midbrain DA D_2-autoR density in humans (Gjedde et al., 2010; Zald et al., 2008). On the other hand, the density of postsynaptic DA D_2/D_3 receptors is lower in the striatum and Acb of RHA vs. RLA rats, and it has been shown that striatal DA D_2 receptor availability is inversely correlated with novelty-seeking behavior of Roman rats in the hole-board test (Tournier et al., 2013).

As previously mentioned, RHA rats exhibit also important molecular alterations involving serotonergic and glutamatergic transmission in the central nervous system. Thus, due to a stop codon mutation, the gene encoding the metabotropic glutamate-2 receptor ($mGlu_2$-R), is not expressed in the brain of RHA rats (Klein et al., 2014; Elfving et al., 2019). Of note, as discussed in section 1.4.2, since $mGlu_2$-Rs are associated with 5-HT_{2A} receptors forming a heterodimer (Klein et al., 2014; Elfving et al., 2019), the absence of $mGlu_2$-Rs leads to adaptative changes in 5-HT_{2A} receptors and serotonergic transmission (Elfving et al., 2019; Giorgi et al., 2019; Fernández Teruel et al., 2021) resembling alterations observed in postmortem brain samples from untreated schizophrenic subjects (González-Maeso et al., 2008).

The neurochemical profile of RHA rats goes along with several distinctive structural and functional neural characteristics that are reminiscent of alterations observed in schizophrenics (see section 1.4.5) including increased volume of the lateral ventricles and decreased volume and function of the mPFC, the HC, and the AMYG (Río-Álamos et al., 2017, 2019; Tapias-Espinosa et al., 2019; Wood et al., 2017).

Altogether, these neurochemical, functional and structural alterations may be involved in the wide range of endophenotypes that distinguish RHA rats, including impulsive novelty seeking (Tournier et al., 2013), impaired impulse control (Dalley et al., 2007; Klein et al., 2014; Moreno et al., 2010), vulnerability to drug sensitization and addiction (Dimiziani et al., 2019; Fattore et al., 2009; Giorgi et al., 2007; Tournier et al., 2013), and schizophrenia-relevant attentional/cognitive and social interaction impairments (Oliveras et al., 2015; Río-Álamos et al., 2019; Tapias-Espinosa et al., 2019; Wakabayashi et al., 2015; reviewed by Swerdlow and Light, 2016). Hence, compared with RLAs and other rat strains, RHAs appear to be unique in that they exhibit all the full range of schizophrenia-related phenotypes mentioned above, and this makes this rat strain a promising heuristic tool to investigate relationships among those innate traits and their neurobiological/genetic underpinnings.

1.7 Interaction between genetic and environmental factors in the etiology of psychiatric disorders

As previously discussed, exposure to trauma and stress is known to be one of the main predisposing factors to major depression, which is often viewed as a manifestation of an inability to cope with stress (Cryan and Holmes, 2005). Accordingly, many animal models of depression are based on the exposure to acute or chronic stressors of different nature and intensity to induce a depression-like phenotype. The tail suspension test (TST) and the forced swimming test (FST) are probably the most widely and most frequently used behavioral despair tests based on the exposure of mice or rats to acute stressors (Cryan and Holmes, 2005; Piras et al., 2010, 2014). Likewise, the chronic unpredictable stress (CUS) is one of the most valid and most frequently used models of depression-like behavior based on the chronic exposure of the animals to a range of aversive conditions (Willner et al., 1992).

On the other hand, exposure of individuals to early-life adverse events is also known to have an important role in the pathogenesis of schizophrenia (Jones et al., 2011). Thus, a favoured neurodevelopmental working hypothesis posits that exposure of rodents to stressful

environmental conditions in early postnatal life may cause alterations of neurogenesis resulting in permanent changes in the developing central nervous system that lead to the manifestation, in the post-pubertal period, of phenotypic traits resembling symptoms of schizophrenia (Jones et al., 2011).

Notably, not all early life environmental manipulations have detrimental effects on behavior. For instance, neonatal handling (NH) is an environmental stimulation treatment typically administered during the first three weeks of life that has beneficial effects on behavior, inasmuch as it elicits long-lasting anxiolytic-like effects and, more specifically, improves the ability to cope with stressful situations in rats, including the Roman rats (Fernández-Teruel et al., 2002b; Raineki et al., 2014; Río-Álamos et al., 2015, 2017, 2019). Moreover, recent studies have shown that NH normalizes in adult RHA rats some inborn phenotypic traits reminiscent of schizophrenia symptoms, such as reduced PPI and impaired spatial working memory (Río-Álamos et al., 2019).

Although stress has a critical role in the pathophysiology of psychiatric disorders, it is well known that not all individuals subjected to severe environmental adverse effects develop depression or schizophrenia. Epidemiological studies, in fact, underline the existence of a complex relationship between genotype and environment in the etiology of these disorders. Thus, genetic factors influence overall risk of psychiatric disorders but also influence the sensitivity of individuals to depression or schizophrenia-inducing effects of environmental adversities (Kendler et al., 1995). The combination of genetics, early life stress (for schizophrenia) or ongoing stress (for depression) may ultimately determine individual responsiveness to stress and the vulnerability to these psychiatric disorders. (Charney and Manji, 2004, van Os et al., 2010).

Consequently, genetically-derived animal models based on selective breeding for specific phenotypic traits associated with core symptoms of depression and schizophrenia, like the Flinders sensitive (FSL) and resistant (FRL) rats (Overstreet et al., 1979, 2005) and the RHA and RLA lines/strains of rats (Giorgi et al., 2019; Fernández Teruel et al., 2021) are valuable experimental tools to investigate the role of genetic factors and their interaction with the environment in the neural underpinnings of both depression and schizophrenia. These genetic models have heuristic potential and better translatability than lesion models, chemically manipulated or genetically engineered animals, since they use intact animals and are based on a forward genetics approach. Thus, these models apply the strategy used by genome wide association studies (GWASs) of depressed and schizophrenic patients (Maul et al., 2020; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Flint and Munafo, 2014). GWASs are consistently providing genetic and neurobiological evidence pointing to depression and schizophrenia as

polygenic disorders whose etiology involves multiple gene variants, each one conferring a small percentage of total susceptibility, and the interactions of these gene variants with the environment (Maul et al., 2020; Schizophrenia Working Group of the Psychiatric Genomics Consortium 2014; Flint and Munafo, 2014).

1.8 Objectives

The etiology and pathogenesis of depression (major depressive disorder, MDD) and schizophrenia are poorly understood, but a large body of experimental evidence indicates that the individual vulnerability to these disorders depends on two major determinants, namely, genetics and environmental conditions. Thus, genetic factors influence overall risk of psychiatric disorders but also influence the sensitivity of individuals to MDD- or schizophrenia-inducing effects of environmental adversities (Kendler et al., 1995).

Hence, genetic animal models are valuable experimental tools to investigate the role of genetic factors and their interaction with the environment in the neural underpinnings of both depression and schizophrenia. The psychogenetic selection of the Roman lines has generated two phenotypes characterized by differential specific behavioral and neurochemical traits. Thus, when exposed to stressors, RLA rats behave as reactive copers displaying depression-like and anxiety-related behaviors while RHA rats behave as proactive copers and are resistant to stress-induced depression.

Different forms of stress can induce depression-like symptoms in humans and in experimental animals, and accumulating experimental evidence indicates that stressors may hinder the synthesis, release and binding to their specific receptors of different brain neurotrophins like the Brain Derived Neurotrophic Factor (BDNF), which modulates the growth and differentiation of neural networks. The disruption of BDNF function by stressors may induce the alterations of synaptic plasticity processes underlying MDD.

It has been recently reported that, under basal conditions, the BDNF-like immunoreactivity (LI) is lower in the Ammon's horn whereas trkB-LI is lower in the dentate gyrus (DG) of the dorsal hippocampus (dHC) of depression-prone RLA rats as compared with their RHA counterparts (Serra et al., 2017).

The aforementioned experimental evidence led us to undertake **STUDY I** to evaluate in RLA and RHA rats the effects of two modalities of acute stress, forced swimming (FS), a robust stressor and tail pinch (TP), a mild stressor, on the expression of BDNF and its receptor trkB, using western blot (WB) assays. BDNF-LI and trkB-LI were measured in two brain areas, the Hippocampus (HC),

which plays a major role in the control of emotions and in the consolidation of new memories and the prefrontal cortex (PFC), which is involved in the process of decision-making.

It has been recently shown that RHA rats exhibit behavioral and neurochemical traits reminiscent of positive and cognitive symptoms of schizophrenia, like impaired PPI and deficits in spatial working memory and cognitive flexibility (Oliveras et al., 2015); however, it remains to be established whether RHA rats also exhibit traits resembling negative symptoms of schizophrenia such as asociality.

Neonatal handling (NH) is an environmental stimulation treatment typically administered to rodents during the first three weeks of life that has beneficial effects on behavior. For instance, NH has long-lasting anxiolytic-like effects on adult RLA rats and normalizes behavioral traits related to schizophrenia symptoms such as impaired pre-pulse inhibition (PPI) and deficits in latent inhibition (LI), spatial working memory, and cognitive flexibility in RHA rats (Río-Álamos et al., 2015, 2019).

Therefore, in **STUDY II**, we evaluated whether RHA rats display an inborn deficit in social interaction (SI) (which resembles asociality in schizophrenics). Furthermore, in view of the long-term beneficial effects of early-life environmental stimulation, we studied whether NH may impact, later in adulthood, on social interaction and anxiety/fear related behaviors of RHA and RLA rats.

2. Study I: Effect of acute stress on the expression of BDNF and trkB in the hippocampus and prefrontal cortex of RHA and RLA rats

2.1 Introduction

Despite significant advances over the last decades, the etiopathology of depression is still poorly understood. As described in Section 1.2, various hypotheses have been proposed to account for the overall pathophysiological state or particular core symptoms of depression, based on the dysfunction of monoamine neurotransmission (Schildkraut, 1965), the HPA axis (Holsboer, 2001) or the neuroimmune processes (Miller, 2010). Another hypothesis posits that depression may be caused by a dysfunction of the mechanisms underlying the plasticity of the neuronal networks (Duman et al., 1999), and that the susceptibility to depression results from the abnormal expression of genes that encode the brain-derived neurotrophic factor (BDNF) in neurons which are modulated by monoaminergic inputs (Nestler et al, 2002). A key tenet of this hypothesis is that the hippocampal expression of BDNF is negatively modulated by stressors and positively modulated by chronic antidepressant treatments (Duman et al., 1999). It is noteworthy, however, that the way by which BDNF is involved in the reactivity to stress and in the pathogenesis of depression has not yet been precisely established. Thus, the type or severity of stressors may impact differently on the expression of BDNF in discrete brain areas. For instance, repeated social defeat stress increases both short-term BDNF expression in prefrontal cortical regions and delayed, prolonged BDNF expression in medial AMYG and ventral tegmental area (VTA) (Fanous et al., 2010). Similarly, the finding that the local infusion of BDNF in the hippocampus mimics the behavioral effects of antidepressants (Shirayama et al., 2002) but elicits depression-like behaviors if infused in the VTA (Eisch et al., 2003), is consistent with the hypothesis that different molecular mechanisms and neuronal pathways are involved in the effects of BDNF in depression.

Several animal models have been designed to investigate the impact of the interactions between genetic and aversive environmental factors on the neural substrates of depression. One of these models, the Roman High- (RHA) and Low-Avoidance (RLA) rats, were psychogenetically selected for rapid (RHA) vs. extremely poor (RLA) acquisition of active avoidance in a shuttle-box (Broadhurst and Bignami,1964; Driscoll and Bättig, 1982). Subsequent studies have shown that rather than cognitive processes, emotional reactivity is the most prominent behavioral difference between the two lines, with RLA rats being more fearful/anxious and prone to develop stress-induced depression than their RHA counterparts (Fernández-Teruel et al., 2002a; Steimer et al., 2007; Piras et al., 2010). Interestingly, recent western blot and immunohistochemistry studies have shown that, under baseline conditions, the expression of BDNF and its receptor trkB is significantly lower in the hippocampus of depression-prone RLA rats compared with their RHA counterparts (Serra et al., 2017). This result is in line with previous studies showing that the availability and/or

responsiveness to BDNF are reduced in the hippocampus of depressed patients and of experimental animals exposed to acute or chronic stressful conditions.

On the basis of the above findings, the present study was undertaken to investigate in RLA and RHA rats the impact of two modalities of acute stress, Forced Swimming (FS), a robust stressor and Tail Pinch (TP), a mild stressor, on the expression of BDNF and its receptor trkB, using western blot assays. BDNF-LI and trkB-LI were measured in the dorsal and ventral hippocampus (HC), which plays a major role in the control of emotions and in the consolidation of new memories, and in the prelimbic/infralimbic (PL/IL) and anterior cingulate (AC) sub territories of the prefrontal cortex (PFC), which is involved in the process of decision-making and shows functional alterations in depressed patients (Drevets et al., 1997; Drevets, 2000).

2.2 Materials and methods

2.2.1 Animals

Outbred male Roman rats (N = 44 for each line) were used throughout and were four months old, weighing 400-450 g at the beginning of the experiments.

Rats were housed in groups of four per cage and maintained under temperature- and humidity-controlled environmental conditions (23°C ± 1°C and 60% ± 10%, respectively) and with a 12-h light–dark cycle (lights on at 8:00 a.m.). Standard laboratory food and water were available ad libitum. To avoid stressful stimuli resulting from manipulation, the maintenance activities in the animal house were carried out by a single attendant and bedding in the home cages was not changed on the two days preceding the test. All procedures were performed according with the guidelines and protocols of the European Union (Directive 2010/63/EU) and the Italian legislation (D.L. 04/04/2014, n. 26) and were approved by the Ethical Committee for Animal Care and Use of the University of Cagliari (authorization No. 684/2015 PR). Every possible effort was made to minimize animal pain and discomfort and to reduce the number of experimental subjects.

2.2.2 Selective breeding

The animals used in the present study belonged to the 145th to 146th generations of RHA and RLA rats bred in the colony of the Department of Health and Environmental Sciences (DiSVA), University of Cagliari, Italy. These animals are direct descendents of RHA/Verh and RLA/Verh rats that have

been selected and bred in Switzerland since 1972, on the basis of their divergent performance in active, two-way avoidance behavior (Driscoll, 1986; Giorgi et al., 2005). Since 1998, three mating cycles have been carried out each year in February, June, and October. In order to assess the stability of the selected phenotypes, two rats from each litter and line (one male and one female) born after the first mating cycle of each year are tested, at postnatal age 3 months, for their ability to acquire active avoidance behavior in an automated shuttle-box (Letica Institute, Barcelona, Spain). The shuttle box consists of two equally sized compartments (25 x 25 x 28 cm), connected by an opening (8 x 10 cm). A 2400-Hz, 63-dB tone, plus a light (from a small 7-W lamp), function as the conditioned stimulus (CS). The unconditioned stimulus (US), which starts at the end of the CS, is a scrambled electric footshock of 0.7 mA delivered through the grid floor. Once rats are placed into the shuttle box, a 4 min habituation period is allowed before starting training. After this period, 40 acquisition trials are administered. Each trial consists of a 10- s CS, followed by a 20-s US. The CS or US are terminated when the animal crosses to the other compartment. Crossings during the CS are considered avoidance responses and crossings during the intertrial interval (ITI) are recorded as intertrial crossings. Once a crossing has been made and/or the shock (US) discontinued, a 1 min fixed ITI is presented. The total number of trials, crossings, avoidances and escapes are automatically registered, allowing for the additional calculation of escape failures and intertrial responses (Giorgi et al., 2005). In a representative 40 trial session in animals from the 145th generation, the total number of avoidances were as follows (mean ± SEM) : RHA males, 24.7 ± 2.0 (N = 13); RHA females, 24.8 ± 1.5 (N = 13); RLA males, 0.4 ± 0.2 (N = 14); RLA females, 0.5 ± 0.2 (N = 14). Similar values are obtained across generations (not shown).

2.2.3 Forced swimming stress

Experimental procedure. RHA and RLA rats were randomly assigned to the control or FS groups and were processed in parallel according with a schedule that was counterbalanced for animal line and treatment. All animals (N = 16 for each line) were naïve at the beginning of the experiments. Rats in the FS groups (N = 8 for each line) were singly moved from the animal house to an adjacent sound attenuated, dimly illuminated test room whereas controls (N = 8 for each line) were kept in their home cages in the animal house until sacrifice. All testing was performed between 10:00 a.m. and 6:00 p.m., and the experimental conditions were as previously described (Morello et al., 2017). Briefly, rats were placed individually in plastic cylinders (58 cm tall × 32 cm in diameter) which were filled with water at 24–25 °C to a 40-cm depth to ensure that they could

not touch the bottom of the cylinder with their tails or hind paws. At the end of the 15 min swimming session, rats were removed from the cylinders, gently dried with paper towels, placed in a heated cage for 15 min, and singly transferred to an adjacent room where they were sacrificed. The water in the cylinders was replaced before starting the next test session.

Behavioral measures. All the behaviors were quantified by a single well-trained observer who was blind to rat line. A time-sampling technique was used to record the predominant behavior in each 15-s period of the FS session. The following behaviors were recorded: (1) ***immobility***, defined as floating passively in the water without struggling and doing only those movements necessary to keep the head above water, (2) ***swimming***, i.e., showing moderate active motions around in the cylinder, more than necessary to simply keep the head above water; (3) ***climbing***, i.e., making active, vigorous movements with the forepaws in and out of the water, usually directed against the walls; (4) ***diving***, i.e., swimming under water looking for a way out of the cylinder.

Forty five min after the end of the FS sessions rats were killed by decapitation and their brains were used for the WB assays.

2.2.4 Tail pinch stress

Experimental procedure. RHA and RLA rats (N = 28 for each line, naïve at the beginning of the experiments) were randomly assigned to the control or tail pinch (TP) groups and were tested in parallel according with a schedule that was counterbalanced for animal line. Rats in the TP groups (N = 14 for each line) were singly moved from the animal house to an adjacent sound attenuated, dimly illuminated test room whereas controls (N = 14 for each line) were kept in their home cages in the animal house until sacrifice. All testing was performed between 10:00 a.m. and 6:00 p.m. and the experimental conditions were as previously described (Giorgi et al., 2003). Briefly, rats in the TP groups were placed individually in a macrolon cage (40 cm x 28 cm x 20 cm) with saw dust bedding, and a rubber-padded clamp was delicately tightened at a distance of 6 cm from the tip of their tails for 40 min to induce discomfort, but not pain.

Behavioral measures. Along the 40 min in which the clamp tightened the rat's tail, a trained observer that was blind to rat line recorded the behaviors occurring during 5 s time windows at 1 min intervals. The following behaviors were recorded: (1) ***biting*** the clamp in an attempt to loosen

and remove it from the tail, (2) ***licking*** the tail near the clamp, (3) ***freezing***, characterized by complete immobility, including the vibrissae, and with the exception of respiratory movements, and (4) ***grooming*** of the head with the forelimbs, of the body with the head, or of the body with the hindlimbs. The behaviors were recorded on pre-prepared check lists, according to the time-sampling method described above, and the total time each rat was engaged in any of the behaviors listed above throughout the TP session was recorded and used for statistical analysis.

At the end of the TP session rats were killed by decapitation and their brains were rapidly removed from the skull. The brains of 8 rats in each experimental group were used for WB assays and the rest were used for immunohistochemistry assays that are currently under way.

2.2.5 Western blot assays

Tissue sampling. Immediately after sacrifice the brains were rapidly removed from the skull and cooled in dry ice for 15 seconds, placed in a brain matrix and cut in 2 mm thick coronal slices using the stereotaxic coordinates of the rat brain atlas (Paxinos and Watson, 1998) as a reference. The AP coordinates (from bregma) were approximately 2.2 mm for Prelimbic/Infralimbic Cortex, 1.0 mm for the Anterior Cingulate Cortex, -3.30 mm and -6.04 mm for the Dorsal and Ventral Hippocampus. Bilateral punches of the different areas were taken as described by Palkovits (1983). For each rat, tissue punches from both hemispheres were pooled and stored at -80°C until used. The punches were homogenized in distilled water containing 2% sodium dodecyl sulfate (SDS) (300 µl/100 mg of tissue) and a cocktail of protease inhibitors (cOmpleteTM, Mini Protease Inhibitor Cocktail Tablets, Cat# 11697498001, Roche, Basel, Switzerland).

Assays. Total protein concentrations in the homogenates were determined as described by Lowry et al., (1951) using bovine serum albumin as a standard. Proteins from each tissue homogenate (40 µg), diluted 3:1 in 4X loading buffer (NuPAGE LDS Sample Buffer 4X, Cat# NP0008, Novex by Life Technologies, Carlsbad, CA, USA), were heated to 95 °C for 7 min and separated by sodium dodecyl sulfate (SDS)-polyacrilamide gel electrophoresis (SDS-PAGE) using precast polyacrylamide gradient gel (NuPAGE 4-12 % Bis-Tris Gel Midi, Cat# NP0321, Novex by Life Technologies) in the XCell4 Sure LockTM Midi-Cell chamber (Life Technologies). Internal mw standards (Precision Plus Protein Western C Standards, Cat# 161-0376, Bio-Rad, Hercules, CA,

USA) were run in parallel. Blots were blocked by immersion in 20 mM Tris base and 137 mM sodium chloride (TBS) containing 0.1% Tween 20 (TBS-T) and 5% milk powder for 60 min at room temperature. The primary antibodies were rabbit polyclonal antibodies against BDNF (Cat# N-20 sc-546, RRID:AB_630940, Santa Cruz Biotechnology) and trkB (Cat# (794) sc-12, RRID:AB_632557, Santa Cruz Biotechnology), both diluted 1:1,000 in TBS containing 5% milk powder and 0.02% sodium azide. Incubations with primary antiserum were carried out for two nights at 4°C. After rinsing in TBS/T, blots were incubated at room temperature for 60 min with peroxidase-conjugated goat anti-rabbit serum (Cat#9169, RRID:AB_258434, Sigma Aldrich, St Louis, MO, USA), diluted 1:10,000 in TBS/T. Controls for equal loading of the wells were obtained by immunostaining the membranes as above, using a mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (MAB374, RRID:AB_2107445, EMD Millipore, Darmstadt, Germany), diluted 1:1,000, as primary antiserum, and a peroxidase-conjugated goat anti-mouse serum (AP124P, RRID:AB_90456, Millipore, Darmstadt, Germany), diluted 1:5,000, as secondary antiserum. In order to control for non-specific staining, blots were stripped and incubated with the relevant secondary antiserum. In order to check for antibody specificity and cross-reactivity, the anti-BDNF antibody was challenged with 200 ng of rhBDNF (Cat# B-257, Alomone Labs, Jerusalem, Israel). After rinsing in TBS/T, protein bands were developed using the Western Lightning Plus ECL (Cat# 103001EA, PerkinElmer, Waltham, Massachusetts, USA), according to the protocol provided by the producer, and visualized by means of ImageQuant LAS-4000 (GE Healthcare, Little Chalfont, UK). Approximate molecular weight (mw) and relative optical density (O.D.) of labeled protein bands were evaluated by a blinded examiner. The ratio of the intensity of BDNF- and trkB-positive bands to the intensity of GAPDH-positive ones was used to compare relative expression levels of these proteins in the brain areas of RHA and RLA lines. The O.D. was quantified by Image Studio Lite Software (RRID:SCR_014211, Li-Cor, http://www. licor.com/bio/products/software/ image studio_lite/).

2.2.6 Statistical analyses

Behavioral measures were evaluated using the Student's t test for independent samples and WB data were assessed with the two-way ANOVA (main factors: line and treatment). Before performing Student’s t tests and ANOVAs, data sets of each experimental condition were inspected for normal distribution and homogeneity of variances with the Shapiro-Wilk’s test and the Bartlett’s test, respectively. Among the FS behavioral measures, the diving data showed statistically significant

unequal variances and, therefore, were analysed with the Welch's t test. As for the immunochemical O.D. values, most data sets (with the exception of WB data of trkB-LI in the dHC and vHC) showed significant differences in variances. Hence, these data sets were log-transformed and then analyzed by two-way ANOVA. When two-way ANOVAs revealed statistically significant interactions, the sources of significance were ascertained by pair wise post hoc contrasts with the HSD Tukey's test. In all the other cases pair wise comparisons were performed using two-tailed t tests with Sidak's corrected alpha values. Statistical analyses were all carried out with PRISM, GraphPad 6 Software (San Diego, USA) with the significance level set at $p < 0.05$.

2.3 Results

2.3.1 Forced swimming stress

Behavioral measures. In line with previous studies (Piras et al., 2010, 2014), RHA and RLA rats exhibited markedly different coping styles when exposed to a 15 min session of FS (**Fig. 1**). Thus, the behavioral performance of RLA rats was characterized by a significantly longer immobility time vs. RHA rats ($p < 0.001$) (**Fig. 1A**), while the RHA rats spent more time climbing ($p < 0.001$) (**Fig. 1C**) and diving ($p < 0.01$) (**Fig. 1D**), than their RLA counterparts. No significant differences between the lines were found in swimming (**Fig. 1B**).

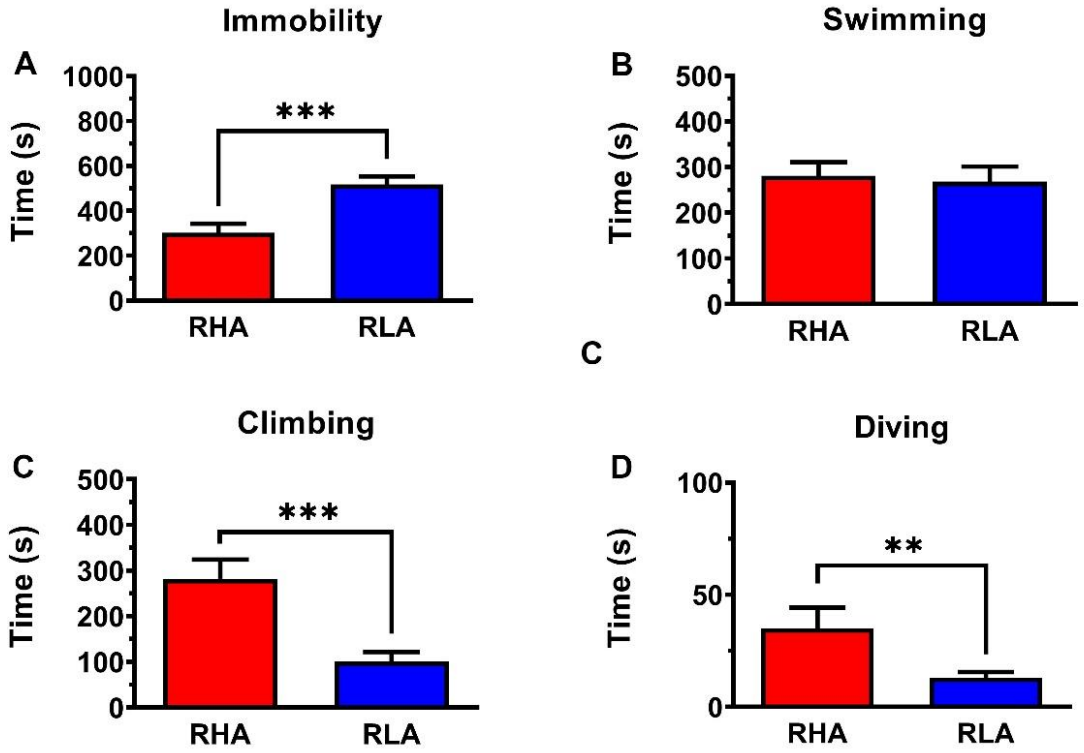

Figure 1. Behavioral performance of Roman High- (RHA) and Low-Avoidance (RLA) rats, during the 15 min forced swimming session. The columns and bars represent the mean ± SEM of 8 rats per line. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$ (Student's *t* test for independent samples).

Western blots of BDNF in dorsal and ventral hippocampus after FS. The anti-BDNF antibody recognized a protein band with a relative molecular weight (mw) of about 13 kDa (**Figs. 2A and B**), in agreement with the reported mw of the monomeric form of the protein (Rosenthal et al., 1991). Assessment of the densitometric values of BDNF-like immunoreactivity (BDNF-LI) in homogenates from the dHC by two-way ANOVA (between groups factors: rat line and treatment, i.e., FS) revealed a significant interaction line x FS ($F_{1,28}$: 8.52, $p < 0.01$) but no significant effects of line and FS. Consistent with a previous study (Serra et al., 2017), the basal BDNF-LI levels in the control groups were tendentially lower in RLA rats (**Fig. 2C**). Additional pair-wise contrasts showed that the basal level of BDNF-LI of RLA rats increased by 175% after FS, while no significant changes were observed in RHA rats. In the vHC, a two-way ANOVA revealed a significant effect of FS ($F_{1,28}$: 14.41, $p < 0.001$) but not of line or the interaction line × FS.

Post hoc contrasts showed that FS induced a decrease (-88%) in the basal BDNF-LI of RLA rats but had no significant effect on the basal level of BDNF-LI of RHA rats (**Fig. 2D**).

Western blots of trkB in dorsal and ventral hippocampus after FS. The antibody against the full-length form of the BDNF receptor, trkB, labeled a protein band with a relative mw ≅ 140 kDa (**Figs. 2A and B**), consistent with the reported mw of the receptor protein (Klein et al., 1989). Statistical evaluation of the densitometric values of trkB-LI in the dHC by two-way ANOVA revealed a significant line x FS interaction ($F_{1,28}$: 6.086, $p < 0.01$), but no significant effects of line or FS. Moreover, no significant changes were observed in the basal levels of trkB-LI of either line (**Fig. 2E**). On the other hand, in the vHC, the two-way ANOVA showed no significant effects of line, FS, or their interactions (**Fig. 2F**).

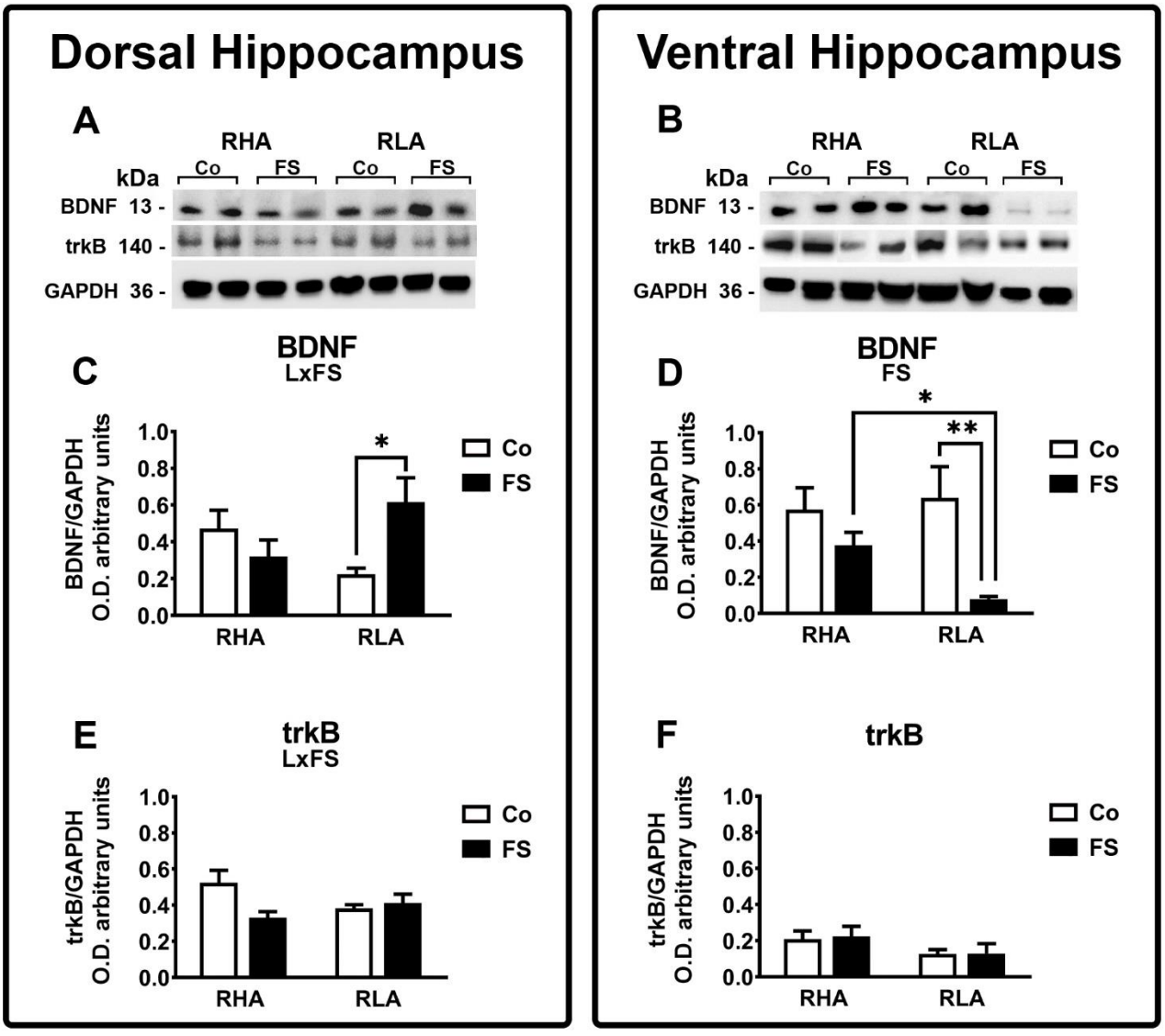

Figure 2. Western blot assays of BDNF-LI and trkB-LI in the dorsal (left panel) and ventral hippocampus (right panel) of RHA and RLA rats, under baseline conditions (Co), and after forced swimming (FS). Shown are immunostained BDNF and trkB blots of representative samples from the dorsal (**A**) and ventral (**B**) hippocampus of two rats and the densitometric analyses of the BDNF/GAPDH (**C, D**) and trkB/GAPDH (**E, F**), band gray optical density (O.D.) ratios. Columns and bars denote the mean ± S.E.M. of eight rats in each experimental group. *: $p < 0.05$; **: $p < 0.01$.

Western Blots of BDNF in prelimbic/infralimbic cortex and anterior cingulate cortex after FS. In the PL/IL CX, the statistical evaluation of the densitometric values of BDNF-LI by two-way ANOVA revealed a significant interaction line x FS ($F_{1,28}$: 32.45, $p < 0.0001$) but no significant effects of line and FS. Post hoc pairwise comparisons showed that the basal BDNF-LI levels in the control groups were markedly lower in RLA rats ($p < 0.0001$; **Fig. 3C**). Interestingly, additional contrasts indicated that FS induced a decrease in the basal level of BDNF-LI of RHA rats (- 47%) but increased by 72% the basal level of BDNF-LI of their RLA counterparts (**Fig. 3C**). On the other hand, in the AC CX, a two-way ANOVA revealed a significant effect of FS ($F_{1,28}$: 4.267, $p < 0.05$) and of the interaction line x FS ($F_{1,28}$: 7.956, $p < 0.01$); moreover, post hoc contrasts showed that FS elicited a significant increase (+ 98%) of the basal BDNF-LI of RLA rats but failed to modify the basal level of BDNF-LI of RHA rats (**Fig. 3D**).

Western Blots of trkB in prelimbic/infralimbic cortex and anterior cingulate cortex after FS. The Statistical evaluation of trkB-LI in the PL/IL CX by two-way ANOVA revealed significant effects of FS ($F_{1,28}$: 6.481, $p < 0.05$) and the interaction line x FS ($F_{1,28}$: 8.165, $p < 0.01$), but no significant effect of line. Additionally, post hoc comparisons showed that FS elicited a significant increment (+ 90%) of the basal trkB-LI of RLA but not RHA rats (**Fig. 3E**). On the other hand, in the AC CX, the alterations induced by FS on the basal trkB-LI paralleled those observed in BDNF-LI (**Fig. 3F**). Thus, a two-way ANOVA indicated a significant L x FS interaction ($F_{1,28}$: 18.98, $p < 0.001$) but no effect of L or FS, and post hoc contrasts revealed that the baseline trkB-LI was lower in RLA rats (- 51%, $p < 0.05$). Notably, FS induced a decrease in the basal level of trkB-LI of RHA rats (- 48%, $p < 0.05$) but increased by 102% the basal level of trkB-LI of their RLA counterparts ($p < 0.05$, **Fig. 3F**).

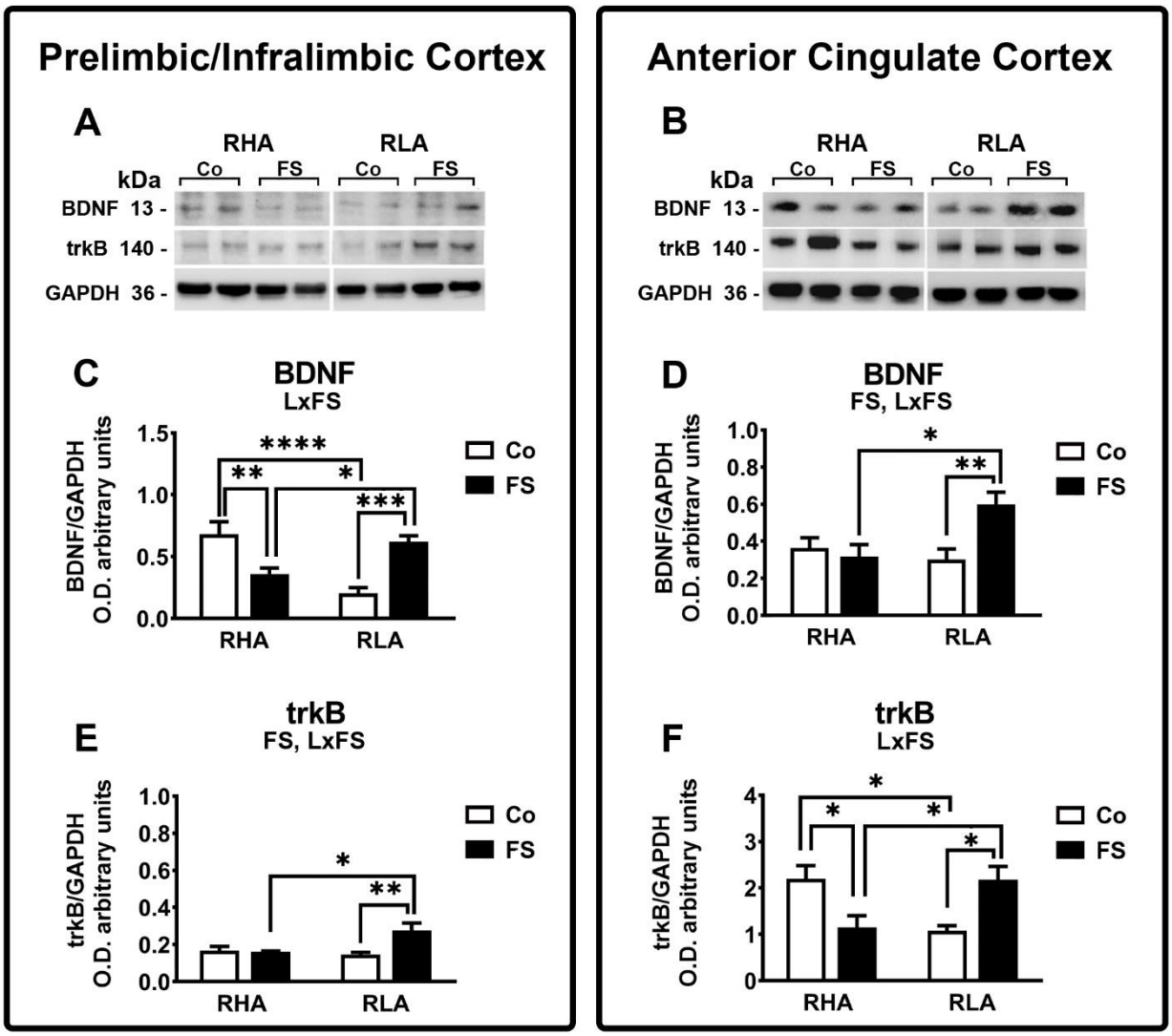

Figure 3. Western blot assays of BDNF-LI and trkB-LI in the PL/IL CX (left panel) and AC CX (right panel) of RHA and RLA rats, under baseline conditions (Co), and after FS. Shown are immunostained BDNF and trkB blots of representative samples from the PL/IL CX (**A**) and AC CX (**B**) of two rats and the densitometric analyses of the BDNF/GAPDH (**C, D**) and trkB/GAPDH (**E, F**), band gray optical density (O.D.) ratios. Columns and bars denote the mean ± S.E.M. of eight rats in each experimental group. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

2.3.2 Tail pinch stress

Behavioral measures. Corroborating previous studies (Giorgi et al., 2003), when submitted to tail pinch (TP) for 40 min, RHA displayed intense proactive coping behavior while RLA rats exhibited a reactive coping activity (**Fig. 4**). Thus, RHA rats spent a longer time biting the clamp in an attempt to remove it from their tails ($p < 0.001$, **Fig. 4A**), whereas the performance of RLA rats was characterized by spending significantly longer times licking their tails ($p < 0.001$, **Fig. 4B**), freezing ($p < 0.0001$, **Fig. 4C**) and grooming ($p < 0.05$, **Fig. 4D**) than RHA rats.

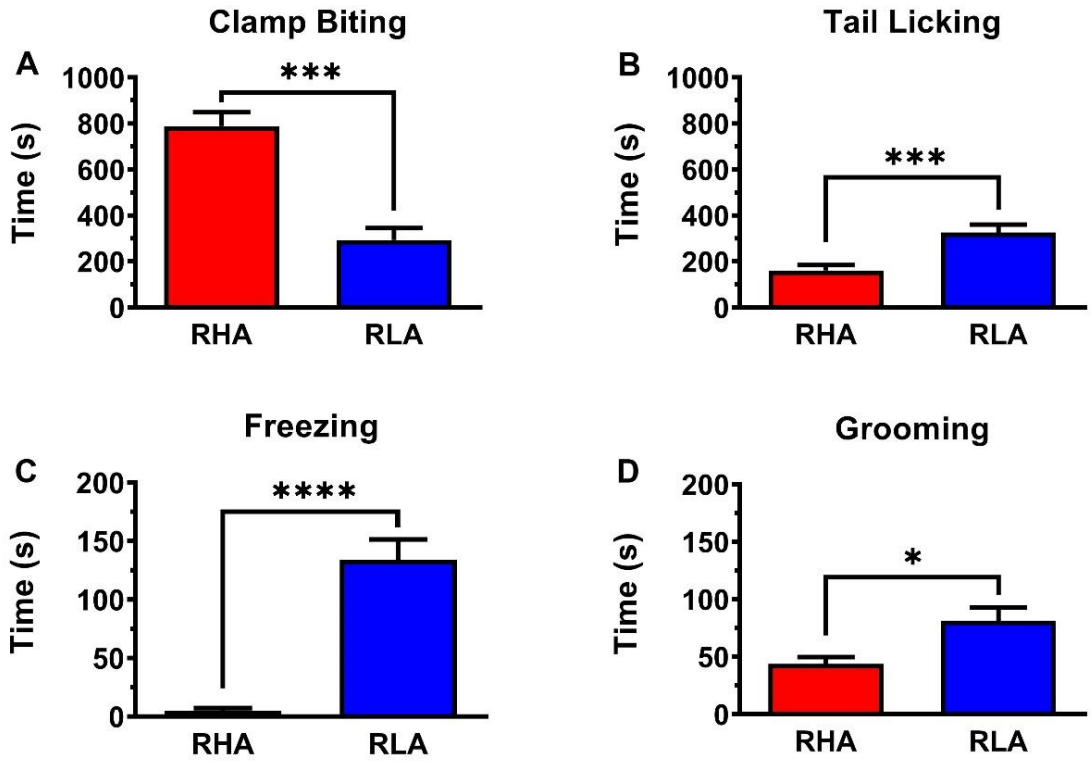

Figure 4. Behavioral performance of RHA and RLA rats, during the 40 min tail pinch session. The columns and bars denote the mean ± SEM of 14 rats per line. *: $p < 0.05$, ***: $p < 0.001$, ****: $p < 0.0001$ (Student's *t* test for independent samples).

Western blots of BDNF in dorsal and ventral hippocampus after TP. Assessment of the densitometric values of BDNF-like immunoreactivity (BDNF-LI) in homogenates from the dHC by two-way ANOVA (between groups factors: rat line and treatment, i.e., TP) revealed a significant effect of TP ($F_{1,28}$: 24.98, $p < 0.0001$) but no significant effects of line or the interaction line x TP (**Fig. 5C**).

Post hoc pairwise contrasts showed that TP increased the basal level of BDNF-LI by 77% in RHAs and 98% in RLA rats. Likewise, in the vHC, a two-way ANOVA revealed a significant effect of TP ($F_{1,28}$: 0.7489, $p < 0.01$) but not of line or the interaction line × FS (**Fig. 5D**). Post hoc contrasts showed that TP elicited a relatively small albeit significant decrease of the basal BDNF-LI of RHA rats (-33%, $p < 0.05$) but had no significant effect on the basal level of BDNF-LI of RLA rats (**Fig. 5D**). It is noteworthy that, at variance with a previous study (Serra et al., 2017) the WB assays of the basal levels of BDNF-LI in the dHC showed no significant line dependent differences.

Western blots of trkB in dorsal and ventral hippocampus after TP. As shown in **Fig. 5E**, in the dHC, the alterations induced by TP on the basal trkB-LI paralleled those observed in BDNF-LI (**Fig. 5C**). Thus, a two-way ANOVA assessment of trkB-LI in the dHC revealed a significant effect of TP ($F_{1,28}$: 26.69, $p < 0.0001$), but no significant effects of line or the line x TP interaction, while pairwise contrasts showed that TP increased the basal trkB-LI by 31% ($p < 0.05$) in RHA rats and 53% ($p < 0.001$) in their RLA counterparts. On the other hand, a two-way ANOVA in the vHC indicated a line effect ($F_{1,28}$: 7.049, $p < 0.05$) and a line x TP interaction ($F_{1,28}$: 6.271, $p < 0.05$); in addition, post hoc contrasts indicated that the levels of trkB-LI in the vHC of stressed RLA rats were significantly lower than those of their own RLA controls (-40%, $p < 0.05$, **Fig. 5F**).

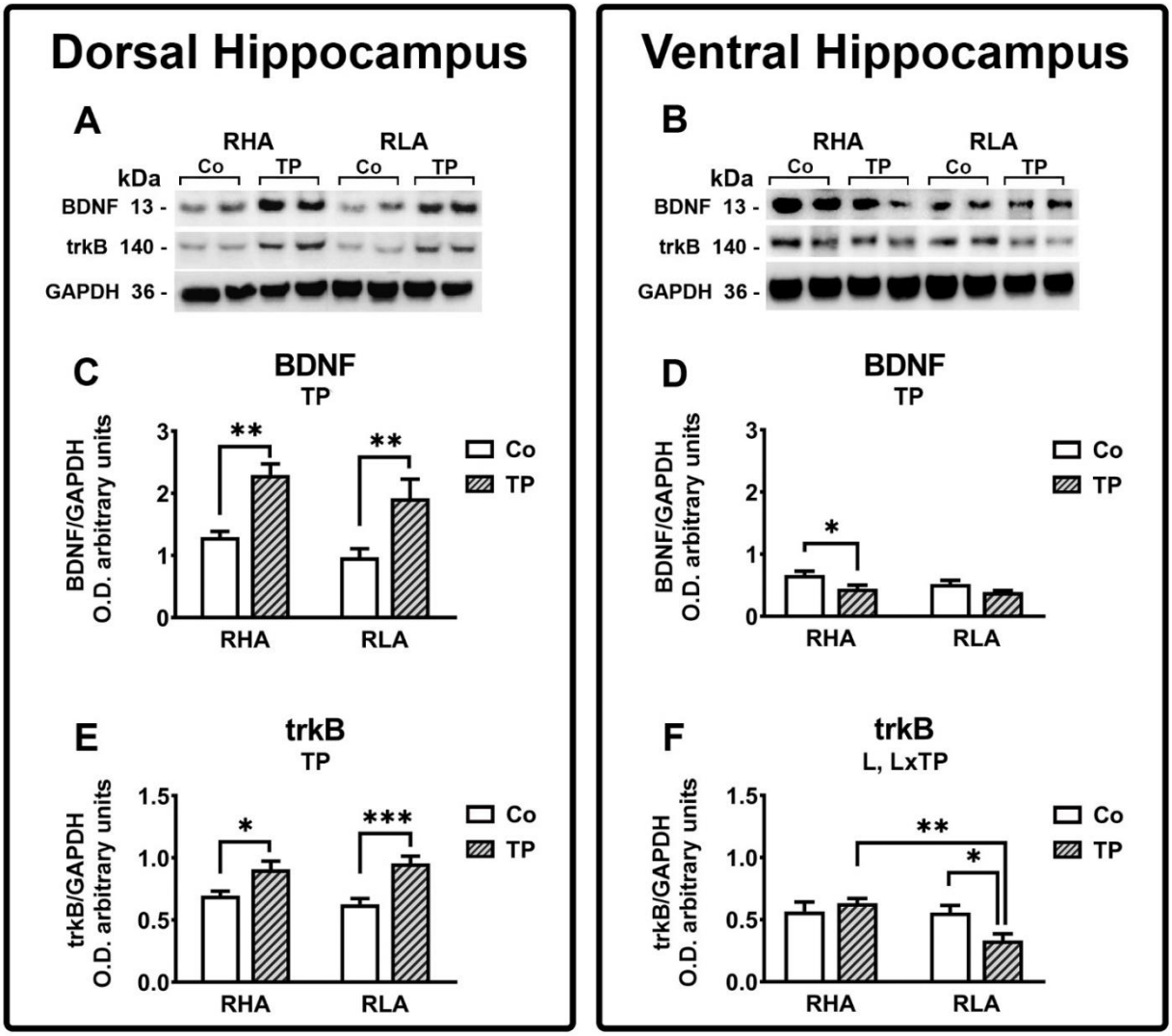

Figure 5. Western blot assays of BDNF-LI and trkB-LI in the dorsal (left panel) and ventral hippocampus (right panel) of RHA and RLA rats, under baseline conditions (Co), and after a 40 min tail pinch (TP) session. Shown are immunostained BDNF and trkB blots of representative samples from the dorsal (**A**) and ventral (**B**) hippocampus of two rats and the densitometric analyses of the BDNF/GAPDH (**C, D**) and trkB/GAPDH (**E, F**), band gray optical density (O.D.) ratios. Columns and bars denote the mean ± S.E.M. of eight rats in each experimental group. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Western Blots of BDNF in prelimbic/infralimbic cortex and anterior cingulate cortex after TP. In the PL/IL CX, the statistical evaluation of the densitometric values of BDNF-LI by two-way ANOVA revealed a significant TP ($F_{1,28}$: 35.07, $p < 0.0001$) but no significant effects of line or interaction line x TP. Notably, at variance with the results of the WB assays of the FS experiment showing that the basal BDNF-LI in the PL/IL CX is lower in RLA vs. RHA rats (**Fig. 3C**), post hoc pairwise comparisons showed no line related differences of the basal BDNF-LI in the PL/IL CX (**Fig. 6C**). On the other hand, additional contrasts indicated that TP induced a significant increment of the basal BDNF-LI of both, RHA (+88%, $p < 0.001$) and RLA rats (+59%, $p < 0.01$) (**Fig. 6C**).

In the AC CX, a two-way ANOVA revealed significant effect of line ($F_{1,28}$: 8.417, $p < 0.01$), TP ($F_{1,28}$: 14.45, $p < 0.001$) and line x TP interaction ($F_{1,28}$: 4.345, $p < 0.05$); moreover, post hoc contrasts showed that TP elicited a marked increase (+ 168%, $p < 0.01$) of the basal BDNF-LI of RHA rats and a tendential increment of the basal level of BDNF-LI of RLA rats (**Fig. 6D**). Furthermore, after TP, the BDNF-LI was 113% higher in RHA vs. RLA rats ($p < 0.01$; **Fig. 6D**).

Western Blots of trkB in prelimbic/infralimbic cortex and anterior cingulate cortex after TP. The Statistical evaluation of trkB-LI in the PL/IL CX by two-way ANOVA revealed a significant effect of TP ($F_{1,28}$: 10.61, $p < 0.01$), but no significant effects of line or the line x TP interaction; moreover, no significant differences were revealed by post hoc contrasts (**Fig. 6E**). Finally, assessment of the influence of TP on trkB-LI in the AC CX by two-way ANOVA showed no significant effects of line, TP or their interaction (**Fig. 6F**).

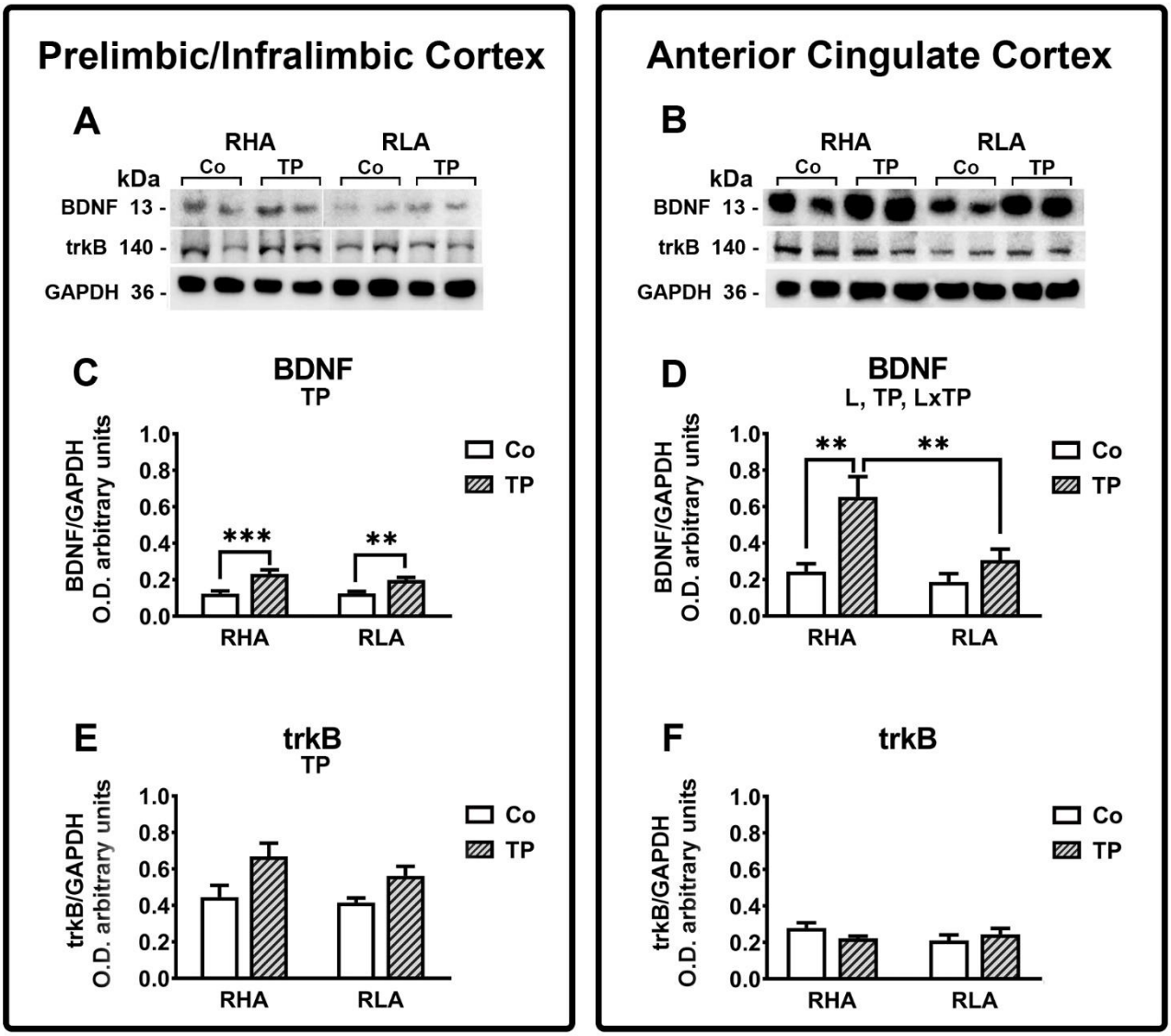

Figure 6. Western blot assays of BDNF-LI and trkB-LI in the PL/IL CX (left panel) and AC CX (right panel) of RHA and RLA rats, under baseline conditions (Co), and after TP. Shown are immunostained BDNF and trkB blots of representative samples from the PL/IL CX (**A**) and AC CX (**B**) of two rats and the densitometric analyses of the BDNF/GAPDH (**C, D**) and trkB/GAPDH (**E, F**), band gray optical density (O.D.) ratios. Columns and bars denote the mean ± S.E.M. of eight rats in each experimental group. **: $p < 0.01$; ***: $p < 0.001$.

2.4 Discussion

RLA and RHA rats represent two divergent phenotypes displaying respectively reactive and proactive coping styles in the face of aversive environmental conditions (Fernández-Teruel et al., 2002a; Steimer et al., 2007; Giorgi et al, 2003; Piras et al., 2010). Notably, the more fearful/anxious RLA rats are also more prone than RHA rats to develop stress-induced depression that is normalized by chronic treatment with antidepressant drugs (Piras et al., 2014), thus adding experimental evidence on the key role of stressful environmental conditions in the etiology of this psychiatric disorder.

The results of the present study confirm and extend previous behavioral data (Piras et al., 2010) demonstrating that, during an acute 15 min session of forced swimming (FS), RLA rats exhibit longer lasting immobility and fewer climbing and diving counts when compared to their RHA counterparts. Similarly, when exposed for 40 min to tail pinch (TP), a milder form of stress than FS (Giorgi at al., 2003), RLA rats exhibited a reactive coping activity mainly characterized by freezing, grooming, and tail licking, whereas RHA rats displayed intense proactive coping behavior, spending a longer time biting the clamp in an attempt to remove it from their tails.

To characterize the effects of these two modalities of stress on BDNF/trkB transmission in RHA and RLA rats, Western blot assays were carried out in the hippocampus (HC) and the prefrontal cortex (PFC), two brain areas that play key roles in the consolidation of new memories, the control of emotions, and in the process of decision-making.

The intrinsic organization of the HC is highly conserved, but its afferent and efferent projections are markedly different along the septo-temporal axis. Functionally, the dorsal hippocampus (dHC) is predominantly involved in the processing of sensory signals into memories; in contrast, the ventral hippocampus (vHC) has distinct afferent/efferent connections, including pathways to the amygdala (AMYG) which are considered to be involved in the attribution of emotional salience to memories (Tanti and Belzung, 2013).

In agreement with a previous study (Serra et al., 2017), the densitometric analysis of the WBs of tissue homogenates showed that the basal BDNF levels are lower in the dHC and vHC of RLA than RHA rats. Moreover, in RLA rats, FS elicited opposite changes on the BDNF levels in the hippocampal subregions examined: an increment in the dHC vs. a decrease in the vHC, with no alterations in trkB-LI in either subregion. This finding provides compelling evidence that an acute stressor can modulate hippocampal plasticity in opposite directions along the longitudinal septotemporal axis. Accordingly, it has been shown that in rats that had experienced juvenile stress

long term potentiation (LTP) was impaired in the dHC but enhanced in the vHC (Maggio and Segal, 2007). In addition, the same stressor induced a reduction in the sensitivity to the β-adrenergic receptor agonist noradrenaline in the dHC whereas in the vHC the sensitivity to noradrenaline was increased (Grigoryan et al., 2015). Moreover, our data are consistent with ample evidence suggesting that a dynamic and rapid regulation of BDNF expression and signaling is implicated in the effect of acute stressors on hippocampal structure and connectivity (Murakami et al., 2005; Nair et al., 2007; Pittenger and Duman, 2008). In particular, the increase in the BDNF protein levels in the dHC of RLA rats upon FS is in agreement with the increment in the BDNF mRNA or protein levels caused by different types of acute stress (Shi et al., 2010; Uysal et al., 2012; Marmigère et al., 2003) and may be considered as a rapid and adaptive neuronal plasticity response to stress. On the other hand, in the vHC of RLA rats the BDNF protein levels were decreased upon FS, and this effect was associated with a reduction in the levels of Polysialilated-Neural Cell Adhesion Molecule (PSA-NCAM), an adhesion molecule that may promote clustering and aggregation of the trkB receptor molecules, thereby facilitating the BDNF signaling (Serra et al., 2018). The concurrent marked decrease in the expression of BDNF, its receptor trkB, and the PSA-NCAM in the vHC suggests that this kind of acute stress of moderate intensity exerts a strong disruptive effect on the capability of vHC neurons to engage in neuroplastic processes, such as neuronal migration, neurite extension/retraction, and synaptogenesis. Conversely, no significant alterations in the levels of BDNF and trkB were observed upon FS in the RHA rats, suggesting that this type of stress may hinder plastic events, in the vHC of the RLA rats, but not of their stress-resistant RHA counterparts.
The effects of TP on BDNF and trkB in the dHC and vHC were only partially consistent with those produced by FS. Thus, in RLA rats, the increase induced by TP on the basal BDNF-LI in the dHC paralleled that observed following FS and was associated with an increase in trkB-LI, whereas in the vHC TP caused a decrease in trkB-LI but failed to modify BDNF-LI. Notably, in RHA rats, TP increased the basal level of BDNF-LI and trkB in the dHC, and produced a relatively small albeit significant decrease of the basal BDNF-LI in the vHC.
Collectively, these results demonstrate that TP stress interferes with the baseline BDNF signaling, eliciting different changes in the dHC vs. the vHC, and between the two rat lines. Interestingly, in RHA rats this stress modality, which induces a proactive behavior characterized by frequent and robust biting bouts aimed at removing the clamp from the tail but not anxiety-like behaviors, is associated with alterations in BDNF signaling that are not present during FS.

Hodological and functional studies have identified a reciprocal interaction between the HC and the target areas of the mesolimbic/mesocortical DA projections (i.e. Acb and PFC) originating in the

VTA. A dense glutamatergic projection originating in the vHC (Groenewegen et al., 1987, 1997) passes through the fimbria–fornix and diffusely innervates the entire anterior–posterior extent of the Acb (Kelley and Domesick, 1982), where it forms synapses on the dendrites of medium spiny neurons in close proximity with VTA DA axon terminals, suggesting that physiologic interactions occur at the level of individual dendrites (Sesack and Pickel, 1990). Besides this direct hippocampal input to the Acb, other indirect polysynaptic pathways involve vHC projections to the PFC, which in turn project to Acb and VTA DA neurons (Floresco et al., 2001). Interestingly, in rats submitted to bilateral neurotoxic lesions of the ventral HC in the early neonatal period, a significant reduction in BDNF mRNA is observed in the PFC in the postpubertal period (Lipska et al., 2003).

The present results also indicate that the expression of BDNF and trkB is differentially regulated after acute stress in frontocortical subregions of RHA vs. RLA rats. Thus, FS for 15 min is sufficient to elicit in the mPFC of RLA but not RHA rats an increased expression of both BDNF and trkB in the PL/IL cortex and the AC cortex. The selective increment in BDNF signaling in the PFC of RLA rats may be interpreted as a functional underpinning of their high emotionality/fear. In keeping with this hypothesis, the pyramidal neurons of the PFC project to the hypothalamus and the periaqueductal gray, two brain areas that play a key role in the visceral and motor activity associated with emotion, including freezing (Yu and Chen, 2011), and may therefore be involved in the behavioral manifestations of the reactive coping strategy displayed by RLA rats during FS. To test this hypothesis, we assessed the expression of BDNF and trkB in the PFC of RHA and RLA rats submitted to TP, a low intensity stressful condition in which RHA rats exhibit a robust proactive coping activity while their RLA counterparts display a reactive coping strategy. Interestingly, TP elicited an increase in BDNF signaling in the PL/IL cortex and the AC cortex of both lines but the effect was more pronounced in RHA rats. This result argues against the possibility that the activation of the frontocortical BDNF signaling represents only a neurochemical correlate of fear or anxiety.

It has been demonstrated that dopaminergic neurons in the VTA express high levels of BDNF mRNA and protein, which is transported anterogradely to the projection areas in the Acb and PFC (Conner et al., 1997). Previous *in vivo* brain microdialysis studies have shown that a 40 min TP session increases DA output in the PFC of RHA but not RLA rats, suggesting that the activation of this neural system by aversive stimuli may be causally related to the cognitive processes aimed at gaining control over the stressor (Giorgi et al., 2003). Since BDNF modulates DA signaling in encoding responses to acute stress (Koo et al., 2019) it appears likely that the increment in frontocortical BDNF expression elicited by TP is involved in the activation of DA transmission and in the execution of cognitive processes directed at actively coping with the stressor.

A same event may be more or less stressful or have pathophysiological consequences for one individual compared with another depending on multiple factors, including the modality, intensity, duration (i.e., acute vs. chronic) of the stressor, and period of life when the individual is exposed to aversive conditions. The genetic makeup is another important determinant of the individual's vulnerability or resilience to the effects of stressors (Feder et al., 2009). The neurobiological mechanisms whereby the factors described above may elicit depression and other psychiatric disorders in susceptible individuals exposed to stressors are not completely understood. Likewise, there is a relative paucity of information regarding the neural underpinnings of stress resilience.

Nevertheless, in the last decades intensive research has begun to identify the environmental, genetic, epigenetic and neural mechanisms that underlie vulnerability and resilience, and has shown that both conditions are mediated by adaptive changes in several neural circuits involving numerous neurotransmitter and molecular pathways. These changes shape the functioning of the neural circuits that regulate reward, fear, emotion reactivity and social behavior, which together are thought to mediate successful coping with stress or lack thereof. In this scenario, the Roman lines represent a valid approach to investigate the genetic factors underlying the reactive and proactive coping strategies respectively displayed by RLA and RHA rats in the face of stressors. In addition, the investigation of the neurobehavioral effects of stressors in the Roman rats may provide important leads for the development of novel and more effective therapies of depression.

3. Study II: Effect of neonatal handling on social interaction and anxiety related behaviors of adult RHA and RLA rats

3.1 Introduction

Schizophrenia is a chronic and disabling psychiatric disorder, characterized by the high complexity of its numerous symptoms which can be classified into 3 categories: positive, negative and cognitive (NIMH, 2011; Owen et al., 2016). Positive symptoms include disorganized speech, hallucinations and delusions, negative symptoms include apathy, social withdrawal, anhedonia and lack of initiative, and cognitive symptoms include impairments in visual-verbal learning, poor ability to understand the information learned and to make decisions. Epidemiologic studies indicate that schizophrenia is predominantly a genetic disorder with heritability estimated to be around 80% (Jones et al., 2011). Therefore, several animal models used to assess the influence of the genetic make up on the vulnerability to schizophrenia are based on the selective breeding of rodents for differences in the responses to a specific test or drug, allowing the differentiation into distinct lines or strains (Del Río et al., 2014; Ellenbroek and Karl, 2016).

The Roman-Low (RLA) and High-Avoidance (RHA) rat strains are bidirectionally selected and bred, for extremely poor vs. rapid acquisition of the two-way active avoidance task, respectively. Over 50 years of selective breeding have led to the characterization of many specific phenotypes that distinguish RHA from RLA rats. Thus, RLA rats display increased levels of anxiety in both unconditioned and conditioned tests, and exhibit intense hormonal responses to stress (i.e., pronounced increments of the plasmatic concentrations of ACTH, corticosterone, and prolactin) vs. their RHA counterparts (Río-Álamos et al., 2015, 2017, 2019). On the other hand, compared with RLAs, RHA rats are characterized by (i) impulsive behavior in the 5-choice serial reaction time test (5-CSRTT) and delay discounting task (Moreno et al., 2010); (ii) novelty/sensation seeking and NMDA-antagonist-induced hyperactivity (Escorihuela et al., 1999; Oliveras et al., 2017); (iii) psychostimulant-induced locomotor and mesolimbic dopaminergic sensitization (Giorgi et al., 2007); and (iv) enhanced vulnerability to drug abuse (reviewed by Giorgi et al., 2019). Moreover, compared with their RLA counterparts, RHA rats display deficits in PPI and impaired LI of the fear-potentiated startle response and the two-way active avoidance task (Esnal et al., 2016). On the basis of the above mentioned and other findings, RHA rats have been proposed as a potential genetic model of schizophrenia-relevant features.

Clinical and preclinical studies demonstrate that the exposure to severe adverse experiences in early-life deeply affects brain development and may contribute to the occurrence of psychiatric disorders, such as depression and schizophrenia in genetically predisposed individuals**.** Although the molecular mechanisms underlying the long-term consequences on mental health elicited by

adverse events in early-life are not yet completely understood, there is experimental evidence supporting the involvement of a disruption of the normal development of neural systems that control stress responses and emotionality (Lukkes et al., 2009).

Notably, whereas early adverse experiences may negatively affect brain development thereby leading to psychiatric disorders, positive environmental experiences are able to modulate complex behavior and eventually compensate for gene-dependent abnormalities. Neonatal handling (NH) is an environmental treatment administered to pups usually during the first weeks of life (Levine, 1956). Studies on NH indicate that it has long-lasting anxiolytic-like and anti-stress effects and, more specifically, it improves the ability to cope with stressful situations in rats, including the Roman rats (Fernández-Teruel et al., 1997, 2002b; Raineki et al., 2014; Río-Álamos et al., 2015, 2017, 2019). Besides its anxiolytic-like effects, NH affects attentional/cognitive functions that are altered in schizophrenia. Accordingly, adult RHA rats submitted to NH displayed improved PPI, showed better working memory and a more efficient cognitive flexibility in a reversal spatial learning task (Aguilar et al., 2002; Raineki et al., 2014; Río-Álamos et al., 2019). However, there is a relative paucity of preclinical studies aimed at assessing the enduring effects of NH on processes or behavioral responses that are reminiscent of the negative symptoms of schizophrenia, such as social withdrawal (Del Río et al., 2014), which up to now have not been characterized in detail. Therefore, the aim of the present study was twofold: 1) to examine whether RHA rats display behavioral traits resembling asociality in schizophrenics, using a social interaction test, and 2) to determine whether NH can improve social behavior in adult RHA vs RLA rats.

3.2 Materials and methods

3.2.1 Animals

A total of 79 naïve male rats from the RHA and RLA strains were used (40 RLAs and 39 RHAs). All rats belonged to the colony maintained at the Laboratory of the Medical Psychology Unit, Department of Psychiatry and Forensic Medicine, Autonomous University of Barcelona, Spain, since 1996. Animals were approximately 3-4 months old at the beginning of the experiments (body weight: 390 – 420 g).

Each experimental group consisted of rats belonging to 10 to 15 different litters. Animals were housed in same-sexed pairs in standard macrolon cages (50 x 25 x 14 cm) and maintained under a 12:12 h light-dark cycle (lights on at 08:00 a.m.), with controlled temperature (22 ± 2º C) and

humidity (50-70%). They had food and water available ad libitum. All testing was carried out between 09:00 h and 14:00 h. All procedures were carried out in accordance with the Spanish Legislation (Royal Decree 53/2013, February 1st, 2013) and the current regulation related to "Protection of Animals used for Scientific Purposes" established by the European Union (2010/63/UE, 22 September 2010).

3.2.2 Neonatal handling (NH) treatment

Neonatal handling (NH) treatment was administered twice a day (at 9:30 h and 17:30 h) between postnatal days (PND) 1-21. At the beginning of each handling session, the mother was first removed from the litter. Then, the pups were placed individually in plastic cages (35 x 15 x 25 cm) lined with a paper towel, placed in a room with a temperature of 22 $\pm$ 2 ºC, and were gently stroked for 3-4 seconds at 0, 4 and 8 minutes. After these 8 minutes of individual separation from the mother, the pups were returned to their home cages with their mother and the rest of the litter. Male rats from at least 10-15 different randomly selected litters per strain were included in each experimental group (RLA-C, n = 21; RLA-NH, n = 19; RHA-C, n = 21; RHA-NH, n = 18). Non-handled rats ("C" groups) were left undisturbed, except for the regular home cage cleaning once a week. After weaning on PND 21, rats were housed in pairs of the same experimental group in standard macrolon cages (50 x 25 x 14 cm).

3.2.3 Novel object exploration (NOE) test

A Novel Object Exploration (NOE) test was carried out to test anxiety-related behavior (i.e. behavioral inhibition) in response to novelty and to compare with previous results (Río-Álamos et al., 2015). At PND 60, food was removed from the home cage (except 4 food pellets that were left in every cage), and each cage was pulled 20 cm out of the rack. One hour later, the novel object (graphite pencil Staedtler Noris, HB nº 2) was vertically introduced in the cage through the grid until it touched the cage bedding. The latency to the first exploration (NOE Latency Time) and the total time of exploration (NOE Exploration Time) were scored in a 3-min test by a trained observer standing approximately 50 cm away from the cage front (Rio-Álamos et al., 2015).

3.2.4 Social interaction (SI) experiments

Apparatus. Social interaction (SI) was assessed using a set-up adapted from Gururajan et al. (2012), in a room dimly illuminated with red light. Two acrylic boxes (65 x 23 x 20 cm) were placed in front of each other 12 cm apart to prevent physical contact between the animals. Each box

had two holes at the ends of 3 cm diameter. The hole facing the other box was named “social hole”, while the hole on the opposite side of the box was named “non-social hole” (**Fig. 1**). All the procedure was recorded by a camera placed on the ceiling and connected to a TV set placed outside the experimental room.

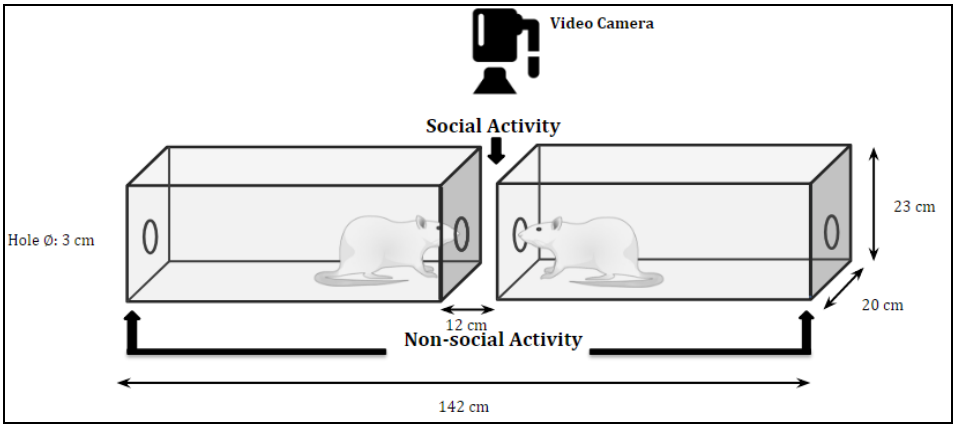

Figure 1. Schematic drawing of the experimental Social Interaction set up (modified from Deak et al., 2009 and Gururajan et al., 2012).

Experimental procedure. The SI procedure was carried out approximately 30 days after the NOE test, using randomly selected rats from the four groups used in that test (RHA-C, n=8; RHA-NH, n=7; RLA-C, n = 8; RLA-NH, n = 7).

Animals were habituated to the testing room and the apparatus for 30 min 24 h before assessing SI. To prevent the exploration of the adjacent box during the habituation period the four holes were covered with tape and a screen was placed between the two boxes. A pair of non-familiar weight-matched animals were placed into the boxes (one rat in each box) for the 30-min habituation period. For SI assessment, in the test day the holes were opened and two weight-matched non-familiar rats of the same strain were placed (one in each box) into the set-up for a 15-min test. The time spent exploring (i.e. nose poking) the social hole (Social Time) and the non-social hole (Non Social Time) were recorded by two trained observers blind to strain and NH conditions (reliability between their measurements, $r > 0.97$).

Social preference, i.e., the percentage (%) of total time spent in the social hole was calculated according to the following formula:

$$\%\text{Social Time} = \frac{Social\ Time}{(Social\ Time + Non\ Social\ Time)} \times 100$$

The time spent self-grooming of the head with the forelimbs, of the body with the head, or of the body with the hindlimbs (Grooming Time) was also recorded. The boxes were cleaned with a 70% ethanol solution and dried with a paper towel after each test.

3.2.5 Statistical analyses

Statistical analyses were performed using GraphPad 6 Software (San Diego, USA). The data were evaluated by two-way ANOVAs (2 "strain" x 2 "treatment" levels). If the two-way ANOVA revealed "Strain", "Treatment" or "Strain x Treatment" effects, post hoc pair wise contrasts were performed with the Tukey's multiple range test, since we hypothesized *a priori* that NH groups would show higher levels of social behavior and/or social preference, as well as higher levels of exploration of the novel object in the NOE test and lower levels of self-grooming behavior. Significance level was set at $p \leq 0.05$.

3.3 Results

3.3.1 Novel object exploration test

The different behaviors displayed by RHA and RLA rats in the NOE test were clear-cut and consistent with the differences in emotionality that distinguish one strain from the other. Thus, the elapse of time until the first exploration of the novel object was markedly longer in control RLA rats vs. their control RHA counterparts and, notably, NH elicited a dramatic decrease in the latency time of RLA rats so that it was statistically indistinguishable from that of the control RHA rats. In contrast, NH did not affect the latency time of RHA rats (**Fig. 2A**). Accordingly, assessment of the latency times with the two-way ANOVA (main factors: strain and treatment, i.e., NH) revealed significant effects of strain ($F_{1,75}$: 34.66, $p < 0.0001$), NH ($F_{1,75}$: 34.62, $p < 0.0001$), and the strain x NH interaction ($F_{1,75}$: 30.19, $p < 0.0001$). As regard exploration time (**Fig. 2B**), control RLA rats spent less time exploring the novel object than control RHA rats. Importantly, NH induced a marked increase of the exploration time of RLA but not RHA rats, so that in animals submitted to NH RLA rats spent more time exploring the novel object than RHA rats (**Fig. 2B**). Thus, the assessment of the novel object exploration time by a two way ANOVA revealed a tendential effect of strain ($p = 0.08$) and significant effects of NH ($F_{1,75}$: 115.20, $p < 0.0001$) and the strain x NH interaction ($F_{1,75}$: 59.49, $p < 0.0001$).

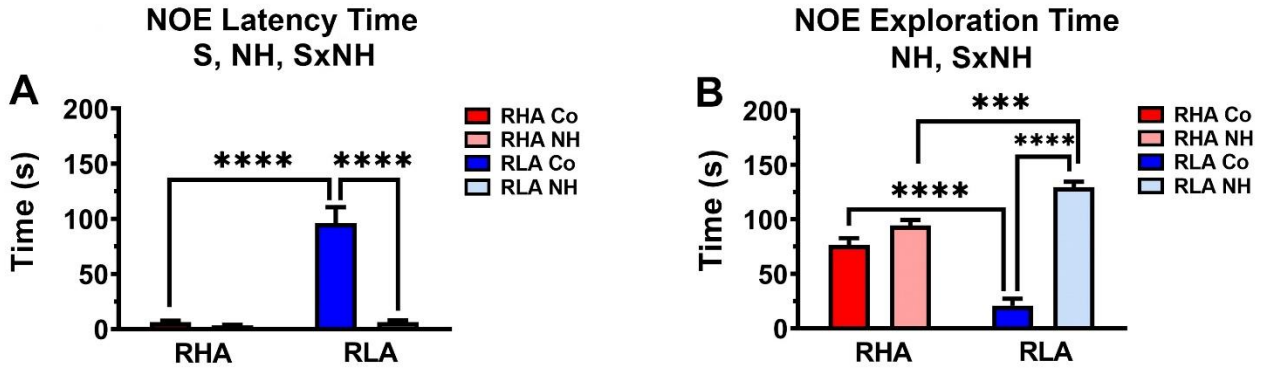

Fig. 2. Different behavioral patterns of the Roman strains in the novel object exploration (NOE) task. **A**, **NOE latency time**. **B**, **NOE exploration time**. Values are the mean ± SEM of the following number of animals in each experimental group: RLA Co, 21; RLA NH, 19; RHA Co, 21; RHA NH, 18. S: strain; NH: neonatal handling; SxNH: strain x neonatal handling). ***p < 0.001, ****p < 0.0001 (two way ANOVA followed by Tukey's multiple range test).

3.3.2 Social interaction test

The graphs plotted in the left side panels of **Fig. 3** illustrate the non social time (**A**), social time (**C**) and social preference (**E**) of RHA and RLA rats recorded during the 15 min of the SI test. Assessment of non social time data by two way ANOVA (main factors, strain and treatment, i.e., NH) showed no significant effects of strain, NH or the interaction strain x NH (**Fig. 3A**) while evaluation with ANOVA of social time revealed a significant effect of NH ($F_{1,26}$: 15.62, $p < 0.001$) but not of strain or the interaction strain x NH. Moreover, post hoc contrasts revealed that NH increased by 49% social time of adult RHA but not RLA rats (**Fig. 3C**). Finally, the statistical analysis of the social preference data showed no significant effects of strain, NH or the interaction strain x NH.

The lack of strain related differences in social interaction time between the control groups prompted us to evaluate whether the behavioral measures captured during the initial 5 min of the test revealed significant differences between RHA and RLA rats in terms of the basal social time and of the potential impact of NH on this behavioral measure.

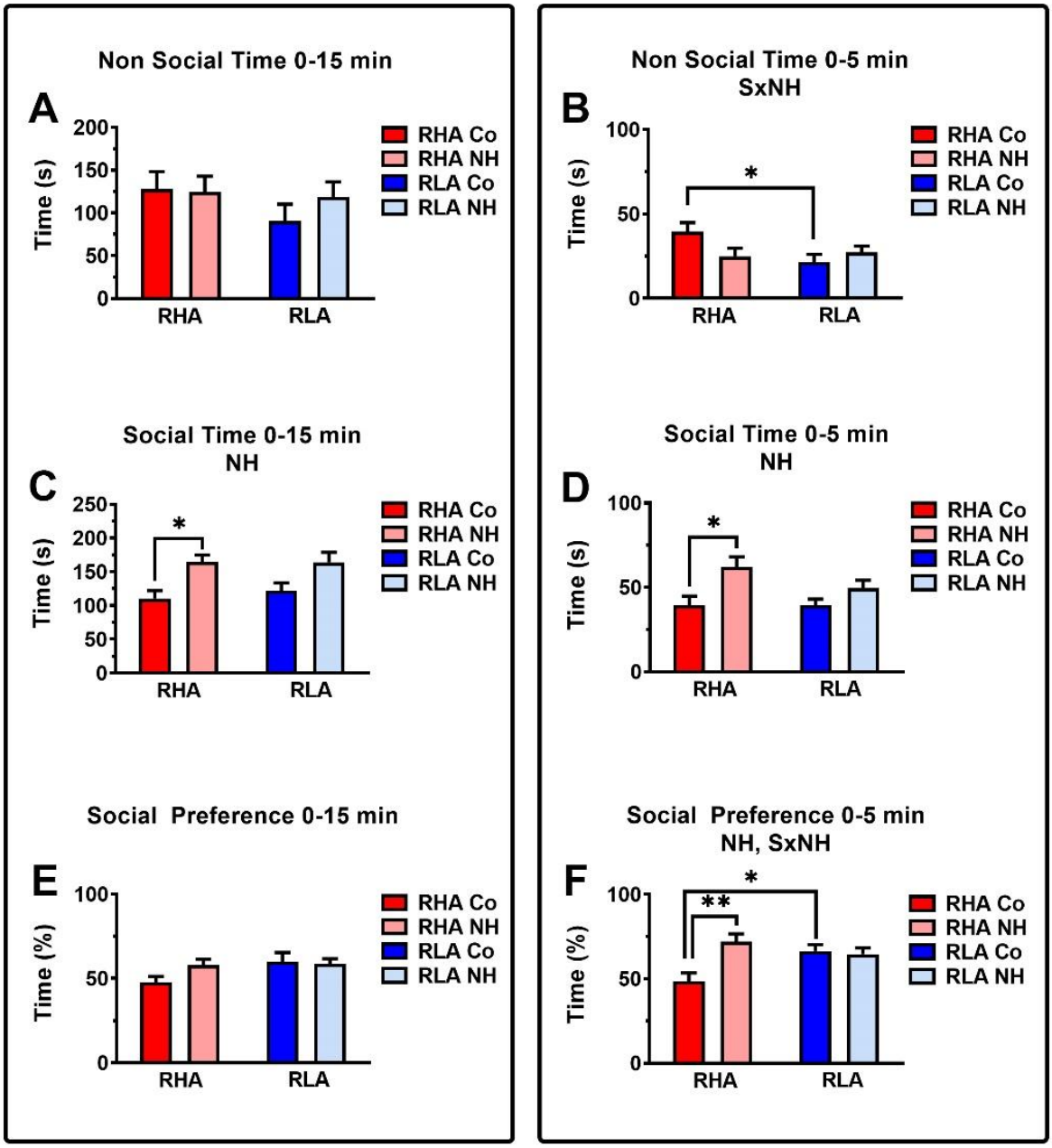

Figure 3. **Effect of NH on the behavioral performance of RHA and RLA rats in the social interaction (SI) test**. ***Left panels***: 15 min test. **A**, **Non social time**, **C**, **Social time**, and **E**, **Social preference.** ***Right panels***: 5 min test. **B**, **Non social time**, **D**, **Social time**, and **F**, **Social preference.** Shown are the mean ± SEM of the following number of animals in each experimental group: RLA Co, 8; RLA NH, 7; RHA Co, 8; RHA NH, 7. S: strain; NH: neonatal handling; SxNH: strain x neonatal handling interaction). ***p < 0.0001, ****p < 0.0001 (two way ANOVA followed by Tukey's multiple range test).

As shown in **Fig. 3B**, assessment by two way ANOVA of non social time during the initial 5 min of the SI test revealed a significant strain x NH interaction ($F_{1,26}$: 4.52, $p < 0.05$) but no effect of strain or NH. Moreover, post hoc comparisons showed that RHA rats spent more non social time than RLA rats during the first five minutes of the test (+82%). Furthermore, ANOVA of social time data revealed a significant effect of NH ($F_{1,26}$: 10.90, $p < 0.01$), but not of strain or the strain x NH

interaction and post hoc contrasts showed that NH induced a significant increment of social time in adult RHA rats (+ 58%, $p < 0.05$) but not in their RLA counterparts (**Fig. 3D**).

Most important, **Fig. 3F** shows that assessment of social preference data with two way ANOVA showed significant effects of NH ($F_{1,26}$: 6.42, $p < 0.05$) and the interaction strain x NH ($F_{1,26}$: 8.58, $p < 0.01$), and post hoc contrasts indicated that social preference was 48% (i.e. random exploration of both holes) in control RHA rats whereas it was 66% in control RLA rats (i.e. preferential exploration of the social hole). Furthermore, NH increased by 49% ($p < 0.01$) the social preference of RHAs but did not modify this parameter in RLA rats .

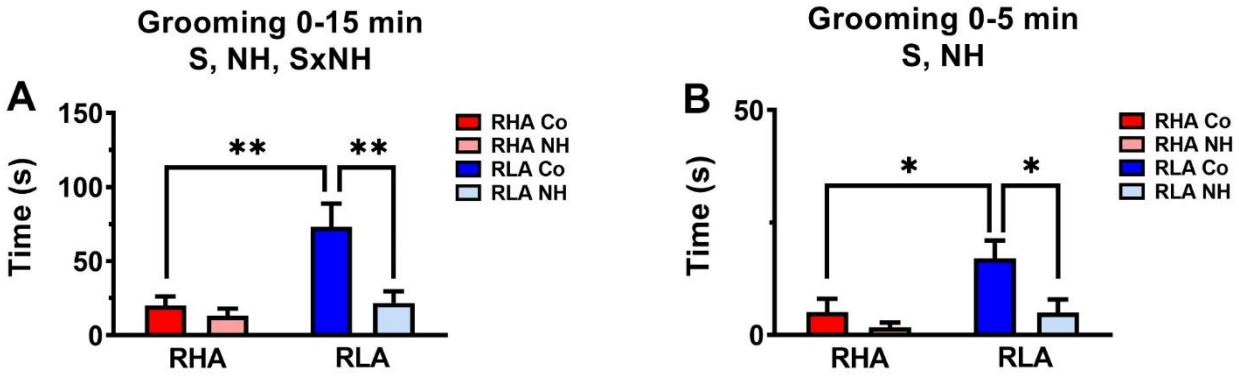

Figure 4. **Effect of NH on self grooming of RHA and RLA rats in the social interaction (SI) test**. **A**: 15 min test, **B**: 5 min test. Shown are the mean ± SEM of the following number of animals in each experimental group: RLA Co, 8; RLA NH, 7; RHA Co, 8; RHA NH, 7. S: strain; NH: neonatal handling; SxNH: strain x neonatal handling interaction). *p < 0.05, **p < 0.01 (two way ANOVA followed by Tukey's multiple range test).

The statistical analysis of grooming activity throughout the 15 min of the SI test revealed significant effects of strain ($F_{1,26}$: 9.43, $p < 0.01$), NH ($F_{1,26}$: 8.35, $p < 0.01$) and the strain x NH interaction ($F_{1,26}$: 4.84, $p < 0.05$). Subsequent post hoc pairwise contrasts indicated significant differences between the time spent grooming by adult control RHA rats and their RLA counterparts and between the grooming times of control RLA rats vs. line-matched rats submitted to NH. Thus, as shown in **Fig. 4A**, adult control RLA rats spent a > 3-fold longer time grooming than their RHA counterparts and, importantly, NH reduced markedly grooming time of adult RLA but not RHA rats so that following NH the time spent grooming by adult RLA rats was indistinguishable from RHA rats' grooming time.

The results of the statistical evaluation of grooming behavior during the initial 5 min of the SI test were completely superimposable to the results of the analysis of grooming activity throughout the 15 min of the SI test (compare **Fig. 4A** with **Fig. 4B**). Accordingly, a two way ANOVA of the data indicated significant effects of both, strain ($F_{1,26}$: 6.35, $p < 0.05$) and NH ($F_{1,26}$: 6.49, $p < 0.05$).

3.4 Discussion

The present work was aimed at exploring whether RHAs display phenotypes resembling negative schizophrenia symptoms, such as reduced social interaction, and whether such traits can be ameliorated by NH. It was also evaluated whether NH affects anxiety-related behaviors in the two strains, as observed in previous studies. Remarkably, RHA rats showed for the first time a decrease in social preference during the first 5 min of the SI test compared with RLAs, evidencing that RHAs display a behavioral repertoire which is thought to model social withdrawal or asociality, one of the negative symptoms of schizophrenia. NH increased absolute levels of social behavior in both strains, but with a more marked effect in RHA rats. On the other hand, compared with RHAs, RLA rats displayed increased anxiety-related behaviors in the NOE test, as reflected by more intense behavioral inhibition and lesser exploration of the novel object, and also in the SI test, as indicated by higher levels of self-grooming during the initial 5 min and throughout the 15 min of the SI test. These behaviors were dramatically reduced by NH treatment, supporting the long-lasting anxiolytic-like effect of this neonatal behavioral manipulation. Thus, it is hypothesized that NH might have long-lasting positive effects on behavioral and neurobiological processes that are impaired in schizophrenia, in addition to its well-known beneficial effect on anxiety-related behaviours.

In line with the hypothesis that the RHA strain presents a relative degree of asociality, the % social preference displayed by control RHA rats during the first 5 min of the SI test was very close to 50%, indicating a random exploration of the holes, that is, no side preference. In contrast, control RLA displayed a clear cut social preference inasmuch as they spent more than 66% of the time exploring the social hole.

This finding adds a negative symptom-like phenotype (i.e. decreased social preference) to the list of traits reminiscent of positive symptoms of schizophrenia that characterize the RHA strain, including impulsivity (Moreno et al., 2010; Coppens et al., 2012, 2013), novelty/sensation seeking (Siegel and Driscoll, 1996; Escorihuela et al., 1999; Manzo et al., 2014), NMDA antagonist-induced

hyperactivity (Oliveras et al., 2017), psychostimulant- induced behavioral and mesolimbic DA sensitization and vulnerability to substance use disorder (Giorgi et al., 2007, 2019), as well as deficits in working memory and cognitive flexibility (Escorihuela et al.,1995; Fernández-Teruel et al., 1997). Latent inhibition (LI) and prepulse inhibition (PPI) of the startle response are attention-related processes that are impaired in schizophrenia (Gray et al., 1991; Kohl et al., 2013; Lubow and Weiner 2010; Swerdlow et al., 1996). The experimental procedures used to evaluate LI and PPI in rodents are similar to those used in humans, and both processes are currently considered as endophenotypes of schizophrenia (Jones et al., 2011; Swerdlow and Light, 2016). It has been reported that, compared with RLAs, RHA rats show impaired LI of the fear-potentiated startle (Esnal et al., 2016) and also clear-cut and consistent deficits of PPI of the acoustic startle response (Oliveras et al., 2017; Del Río et al., 2014; Río-Álamos et al., 2015; Tapias-Espinosa et al., 2018). Notably, structural magnetic resonance imaging reveals that, compared with RLAs, RHA rats present enlarged lateral ventricles, reduced volumes of the of medial PFC and HP (Río-Álamos et al., 2019; Tapias-Espinosa et al., 2019) and deficits of neural activity in PFC and HP linked to alterations in PPI and in behavioral performance during different novelty tests (Meyza et al., 2009; Tapias-Espinosa et al., 2019). Altogether, these studies reveal behavioral and neurofunctional traits of RHA rats that have also been found in schizophrenic patients (reviewed by Giorgi et al., 2019), thus conferring face and construct validity to the RHA model of this psychiatric disorder.

Exposure of individuals to early-life adverse events is known to have an important role in the pathogenesis of schizophrenia (Jones et al., 2011). Thus, it has been hypothesized that exposure of prepubertal rats to repeated stress may cause long lasting alterations of neurogenesis in the developing central nervous system that lead to the manifestation later in adulthood of phenotypic traits resembling symptoms of schizophrenia. Accordingly, isolation rearing (IR) of rats, usually starting after weaning and lasting a few weeks, induces detrimental effects on brain development and adult behavior which mimics some symptoms of schizophrenia (Jones et al., 2011). Interestingly, IR produces PPI deficits, cognitive/learning impairment and hyperactivity in RHA but not RLA rats (Oliveras et al., 2016; Sánchez-González et al., 2019). Whereas early adverse experiences may have detrimental effects on brain development, positive environmental experiences are able to modulate complex behavior and eventually compensate for gene-dependent abnormalities. Neonatal handling (NH) is an environmental treatment administered to pups usually during the first weeks of life (Levine, 1956) that has long-lasting anxiolytic effects and improves the ability to cope with stressful situations in rats (Raineki et al., 2014). Confirming previous studies (Fernández-Teruel et al., 1997; Río-Álamos et al., 2015, 2017, 2019), this treatment reduced

the behavioral inhibition preferentially in the RLA strain, as indicated by an increase above control values of the time spent exploring the novel object in the NOE test and by a reduction in the self-grooming time during the SI test.

NH also increased absolute levels of social behaviour in both strains, but this effect was more pronounced in RHA rats. Thus, the positive effect of NH was observed on both, the time spent in proximity of the social hole of the SI apparatus and the preference for the social hole. The effects of NH on SI are very specific, since this treatment did not modify non-social behavior or activity (i.e., crossings) in any rat strain. Thus, NH does not increase general activity or general exploration of the holes, but it acts selectively by increasing "sociability" ("social time" and "% social preference"), particularly in RHA rats.

In conclusion, this study shows the RHA rat strain displays relatively reduced social interaction preference (a model of schizophrenia's negative symptomatology, i.e. asociality) as compared to RLA rats. Moreover, NH treatment long-lastingly increases social interaction preference in the Roman rats, and this effect is more pronounced in the RHA strain.

The present findings add experimental evidence to the beneficial effects of NH on other aspects of schizophrenia-related phenotypes. Accordingly, NH improves PPI deficits and increases working memory and cognitive flexibility in RHA rats (Río-Álamos et al., 2019). Similarly, Pryce et al., (2001) also reported that NH attenuates apomorphine-induced impairment in PPI and improves latent inhibition (Peters et al., 1991; Pryce et al., 2001; Shalev et al., 1998). Based on the above findings, it can be proposed that some types of early-life stimulation, such as NH, may produce long-lasting beneficial effects on psychological and neurobiological processes related to schizophrenia spectrum disorders (Fernández-Teruel et al., 2002b; Río-Álamos et al., 2019).

4. Conclusions

Depression and schizophrenia (SCZ) are major contributors to the enormous burden in terms of health care costs and major social and economic issues of psychiatric illnesses.

Animal models of complex and heterogeneous psychiatric disorders such as depression (i.e., Major Depressive Disorder, MDD) and SCZ are all-important preclinical tools for the investigation of the neurobiological basis of these disorders. Notably, animal models have several strengths: (1) they provide a heuristic platform to monitor the progression of the disease more rapidly than in humans, (2) they allow performing invasive assessments of the structural and molecular underpinnings of the disease and (3) they permit studies aimed at testing novel therapies that cannot be administered to patients.

According to epidemiologic studies depression is approximately 35% heritable, suggesting that genetic factors play an important role in its etiology (Sullivan et al., 2000), while tween studies and a genome wide association study (GWAS) unequivocally demonstrate that SCZ is predominantly a genetic disorder with heritability estimated to be around 80% (Sullivan et al., 2003; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). However, the identification of specific genes is a difficult task for different reasons. Firstly, both depression and SCZ are complex disorders that involve a large number of genes each of which contributes a small fraction of the total risk (Sullivan et al., 2000; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Second, epidemiological data underline the existence of a complex relationship between genotype and environment. Thus, genetic factors influence the overall risk of these illnesses but also influence the sensitivity of individuals to the depression- or schizophrenia-inducing effects of environmental adversities (Kendler et al., 1995). The combination of genetics, early life stress and ongoing stress may ultimately determine the individual responsiveness to stress and the vulnerability to depression or SCZ. It is likely that genetic factors and life stress contribute not only to the neurochemical alterations, but also to the impairments of cellular plasticity observed in these pathologies (Charney and Manji, 2004).

Several genetic animal models have been developed taking into account the pivotal role of genetic factors in the pathogenesis of depression and SCZ. The Roman High- (RHA) and Low-Avoidance (RLA) rats represent a model that provides a valid approach to the investigation of the contribution of the genotype, and its interactions with environmental factors on the neural substrates of depression and schizophrenia (Giorgi et al., 2019). Thus, there is considerable converging

neurobehavioral and pharmacological evidence suggesting that RLA rats may be considered as a genetic model of vulnerability to stress-induced depression with face, construct and predictive validity, whereas RHA rats have been proposed as a potential genetic model of schizophrenia-relevant features (Giorgi et al., 2019; Fernández Teruel et al., 2021).

Among the different hypotheses proposed to account for the pathophysiological state or particular symptoms of depression, and to identify the neurobiological substrates for its treatment, the neurotrophic hypothesis has received particular attention in the last two decades. It proposes that depression may be caused by dysfunction of the mechanisms underlying the plasticity of neuronal networks (Duman et al., 1999; Stahl, 2000), and that the vulnerability to stress-induced depression results from the abnormal expression of genes that encode trophic factors, such as BDNF, in neurons that are modulated by monoaminergic inputs (Barde et al., 1982; Nestler et al., 2002). A growing body of experimental evidence indicates that the appearance of depression-like behaviors is associated with a decreased hippocampal concentration of BDNF that normalizes when such behaviors are ameliorated by antidepressant treatments (Duman and Monteggia, 2006; Jacobsen and Mork, 2006; Nibuya et al., 1995; Koponen et al., 2005). In keeping with the experimental findings described above, post-mortem studies have demonstrated that in not-treated depressed patients the concentrations of BDNF and trkB in the HC and PFC are reduced (Castren and Rantamaki, 2010; Thompson et al., 2011). Conversely, post-mortem brain samples of patients successfully treated with antidepressants show an increase in the hippocampal and cortical concentrations of BDNF and trkB (Duman and Monteggia, 2006), suggesting that the concentrations of BDNF in the HC and PFC may be correlated with both, the appearance of depressive symptoms and their resolution with antidepressant treatment.

The results obtained in **Study I** are in line with the above findings: (*i*) BDNF-LI is lower in the HC of depression-prone RLA vs. RHA rats, (*ii*) when submitted to aversive conditions RLA rats exhibit a typical reactive coping strategy resembling depression-like behavior during forced swimming (FS) and anxiety/fear-like behavior characterized by intense freezing, (*iii*) in contrast, RHA rats do not display depression-like behavior during FS and behave as proactive copers during TP. Remarkably, the differential modifications observed in BDNF-LI- and trkB-LI depended on brain area, stress modality, and rat line, suggesting that the stress-induced increase in BDNF-LI in the PFC may reflect emotionality/fear in RLA rats on the one hand and cognitive processes underlying motor activity aimed at gaining control over the stressor in RHA rats on the other. In particular, the TP-induced activation of BDNF signaling in the PFC of RHA rats is as important as that observed

in RLA rats, since it may represent a neurochemical phenotype of RHA rats involved in their resistance to display stress-induced depression-like behavior.

In addition to their proactive coping style, RHA rats show a variety of behavioral and neurochemical traits reminiscent of positive symptoms of schizophrenia, including impulsivity (Moreno et al., 2010; Coppens et al., 2012, 2013), novelty/sensation seeking (Siegel and Driscoll, 1996; Escorihuela et al., 1999; Manzo et al., 2014), hyperactive response to the NMDA receptor antagonist dizocilpine (Oliveras et al., 2017), psychostimulant-induced behavioral and mesolimbic DA sensitization, vulnerability to substance use disorder (Giorgi et al., 2007, 2019), as well as impaired LI of the fear-potentiated startle (Esnal et al., 2016) and deficits of PPI of the acoustic startle response (Oliveras et al., 2017; Del Río et al., 2014; Río-Álamos et al., 2015; Tapias-Espinosa et al., 2018).

The results of **Study II** provide further experimental support to the view that RHA rats may be regarded as a valid model of SCZ-related symptoms; thus, we have shown that % social preference during the first 5 min of the SI test is lower in RHAs vs. RLAs. This finding adds to the behavioral repertoire of RHA rats a phenotype that resembles social withdrawal or asociality, a core negative symptom of SCZ.

Exposure of individuals to early-life adverse events is known to have an important role in the pathogenesis of SCZ (Jones et al., 2011). Thus, it has been hypothesized that the repeated administration of a stressor to prepubertal rats may cause long lasting alterations of neurogenesis in the developing central nervous system that lead to the postpubertal manifestation of phenotypic traits resembling symptoms of schizophrenia. Whereas early adverse experiences may have detrimental effects on brain development, rewarding environmental conditions are able to modulate complex behavior and eventually compensate for gene-dependent abnormalities. Neonatal handling (NH) is an environmental treatment administered to pups during the first weeks of life (Levine, 1956) that has long-lasting anxiolytic effects and improves the ability to cope with stressful situations in rats (Raineki et al., 2014). More recently it has been found that NH improves PPI deficits and increases working memory and cognitive flexibility in RHA rats (Río-Álamos et al., 2019). Likewise, Pryce et al. (2001) reported that NH attenuates the apomorphine-induced impairment in PPI and improves latent inhibition (Peters et al., 1991; Pryce et al., 2001; Shalev et al., 1998). Accordingly, we showed in **Study II** that NH increased social time and % social preference in RHA rats; moreover NH reduced anxiety-related behaviors in the two strains, supporting the long-lasting anxiolytic-like effect of this neonatal behavioral manipulation. Thus, it appears likely that NH may have long-lasting beneficial effects on behavioral and neurobiological

processes that are impaired in SCZ, in addition to its well known therapeutic effect on anxiety-related behaviors.

A same event may be more or less stressful or have pathophysiological consequences for one individual compared with another depending on multiple factors, including the modality, intensity, duration (i.e., acute vs. chronic) of the stressor, and period of life when the individual is exposed to aversive conditions. The genetic makeup is another important determinant of the individual's vulnerability or resilience to the effects of stressors (Feder et al., 2009). The neurobiological mechanisms whereby the factors described above may elicit depression and other psychiatric disorders, including schizophrenia in susceptible individuals exposed to stressors are not completely understood. Likewise, there is a relative paucity of information regarding the neural underpinnings of stress resilience.

Nevertheless, in the last decades intensive research has begun to identify the environmental, genetic, epigenetic and neural mechanisms that underlie vulnerability and resilience, and has shown that both conditions are mediated by adaptive changes in several neural circuits involving numerous neurotransmitter and molecular pathways. These changes shape the functioning of the neural circuits that regulate reward, fear, emotion reactivity and social behavior, which together are thought to mediate successful coping with stress or lack thereof. In this scenario, the Roman lines represent a valid approach to investigate the genetic factors underlying the reactive and proactive coping strategies respectively displayed by RLA and RHA rats in the face of stressors. In addition, the investigation of the neurobehavioral alterations elicited in the Roman rats by the exposure to stressors during the perinatal period in the case of SCZ or during adulthood in the case of depression may provide important leads for the development of novel and more effective therapies for these disorders.

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7. Publications

Fernández-Teruel, A., Oliveras, I., Cañete, A., Rio-Álamos, C., Tapias-Espinosa, C., Sampedro-Viana, D., Sánchez-González, A., **Sanna, F.**, Torrubia, R., González-Maeso, J., Driscoll, P., Torres, C., Aznar, S., Tobeña, A., Corda, M.G., Giorgi, O., 2021. Neurobehavioral and molecular profiles of a heuristic genetic model of schizophrenia-relevant features and dual diagnosis: fifty-five years of research with the Roman rat strains. Neurosci Biobehav Rev, submitted.

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*** Shared First Authorship**