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Characterization of the Roman lines/strains of rats as a genetic model of psychiatric disorders: a behavioral and brain dialysis study

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Presentata da:	Dott.ssa Mª. Antonietta Piludu
Coordinatore Dottorato:	Prof.Elias Maccioni
Tutor:	Prof. Osvaldo Giorgi

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Contents

Abstract

State of the art Depressive disorders are fairly *prevalent* in the general population, with a higher rate in women compared to men, are *disabling*, since they can significantly impair psychosocial functioning, and are typically associated with *high mortality* due mainly to the high rate of suicides but also to the negative impact that depression has on the course of co-occurring illnesses. In addition, pharmacological and psychological antidepressant therapies in use have a limited efficacy and/or are associated with side effects that reduce the compliance in many patients. Although the etiology and pathogenesis of depression are poorly understood, it is most likely that the combination of genetics, early life adverse events, and ongoing stress may ultimately determine the individual vulnerability to stress-related disorders, such as depression. Therefore, the development and characterization of animal models of vulnerability and resistance to the effects of stress, including early life stress, is a major challenge for depression research.

One such model is represented by the Roman high- (RHA) and low-avoidance (RLA) lines/strains of rats, which are psychogenetically selected for, respectively, rapid versus extremely poor acquisition of active avoidance in a shuttle box. A large body of evidence indicates that a major reason for their divergent performance in this test is their different reactivity to stressful stimuli, that is, their coping style. Thus, when exposed to aversive stimuli, RLA rats display a reactive coping strategy, associated with a strong activation of the HPA axis; moreover, they display robust depressive-like behaviors in the forced swim test (FST) that are normalized by the subacute administration of antidepressants. In contrast, compared with their RLA counterparts, RHA rats display a proactive coping strategy in the face of aversive conditions, associated with higher baseline levels of impulsivity, a more robust sensation/novelty seeking profile, a marked preference for, and intake of, natural and drug rewards along with a greater responsiveness of the mesolimbic dopaminergic system.

Aims The behavioral and neurochemical traits that distinguish the two lines/strains suggest that RLA rats may represent a model of vulnerability to stress-induced depression, whereas RHA rats may model resistance to stress-induced depression-like behaviour.

To test this hypothesis, in the first study we evaluated the performance of RLA and RHA rats in the FST in response to chronic antidepressant treatments, since clinical evidence indicates that several weeks of treatment with antidepressant drugs are required to achieve an adequate therapeutic response.

Furthermore, one of the cardinal symptoms of depression observed in many patients is anhedonia, which is defined as the loss of interest in once enjoyable activities, including sexual activity. Accordingly, depressive episodes are frequently associated with sexual dysfunctions. In consideration of this clinical evidence, and given the well-established role of dopamine in sexual behavior, the second study was aimed at characterizing the sexual behavior of RHA and RLA rats and its correlation with the functional state of their mesolimbic dopaminergic system.

In keeping with the long-term consequences on mental health elicited by early-life adverse events, it has been observed that post weaning social isolation in rodents may lead to a later increment in the prevalence of anxiety/fear related behaviors. Thus, in the third study we evaluated the impact of post weaning isolation on the anxiety-related behaviors of inbred RHA and RLA rats in the Elevated Zero Maze, and in motility cages used to asssess locomotor activity in a new environment.

Results In study I we demonstrated that chronic treatments with low doses of antidepressants, that were ineffective when given subacutely, were able to decrease immobility and also to increase climbing (desipramine) or swimming (fluoxetine) in RLA rats. Conversely, neither subacute nor chronic antidepressant treatments affected the behavior of RHA rats in the FST.

In addition, the results of study II showed that, compared with their RLA counterparts, RHA rats displayed higher levels of sexual motivation and a better copulatory performance, associated with a greater release of DA in the AcbSh. These line-related differences were attenuated but not abolished by sexual experience. Moreover, RLA rats were more responsive than their RHA counterparts to both, the facilitatory effect of apomorphine and the inhibitory effect of haloperidol on sexual behavior.

Finally, in study III we found that the isolation-rearing procedure significantly increased the level of anxiety of RHA-I rats in the EZM, as reflected by a smaller number of entries and a shorter time spent in the open space, associated with decreased head dipping, increased latency to enter in the open space, and reduced novelty-induced locomotor activation, whereas it failed to produce significant changes in the behavior of RLA-I rats.

Conclusions The results of these studies show that the Roman lines/strains of rats may represent a valid experimental approach to investigate the neural substrates and molecular mechanisms involved in the individual vulnerability and resistance to stress-induced depression, with the aim of identifying both, potential biomarkers for an early diagnosis of depression and potential molecular targets for novel antidepressant treatments. Moreover, the Roman lines/strains may be used to study the neurophysiology of the appetitive and consummatory aspects of sexual behaviour, in order to better understand the mechanisms underlying the psychological and pathological causes of sexual dysfunctions. Finally, the Roman rats may provide a useful model to identify the mechanisms whereby early-life adverse events interact with the genetic make up to induce psychiatric disorders in adulthood.

1. Introduction

1.1 Depression: diagnosis and social impact

Depression is a psychiatric condition belonging to the category of mood disorders. Notably, mood is not the only aspect that is dysregulated in this complex illness, since also neurovegetative, psychomotor and cognitive functions are impaired in depressed patients. Mood disorders are classified by the Diagnostic Statistical Manual-V (DSM-V) of mental disorders **[1]** into two major categories:

- Major Depressive Disorder (MDD) or '*unipolar*' depression, characterized by only depressive episodes;
- *Bipolar* disorder, previously termed Manic-Depressive illness, when the extremely low mood typical of depressive episodes alternates with states of excessive euphoria typical of mania.

Each of these forms of mood disorders includes several subtypes that differ mainly for the severity, frequency and duration of the symptoms.
As regards depressive disorders, the cardinal symptoms identified by the DSM-V include:

- Persistent depressed mood more days than not for at least two weeks;
- Feelings of hopelessness, worthlessness and excessive or immotivate guilt;
- Anhedonia (i.e., loss of interest in once pleasurable activities, including sex);
- Fatigue and decreased energy;
- Psychomotor agitation or retardation;
- Appetite increment or loss;
- Insomnia or excessive sleeping;
- Difficulty concentrating, remembering, making decisions;
- Recurring thoughts of suicide.

The simultaneous presence of 5 or more of these symptoms, experienced most of the day nearly every day for at least two weeks, with a severity that compromises the daily life of the affected individual, justifies the diagnosis of Major Depressive Disorder (MDD). Milder cases are classified as "dysthymia," although there is no clear distinction between the two **[2]**.

Another distinction that should be done is based on the etiology of the disorder. In this context, the **DSM-V** identifies two types of mood disorders: Mood Disorder Due to a General Medical Condition and Substance-Induced Mood Disorder. The former is diagnosed when the mood disturbance is judged to be the direct physiological consequence of a specific general medical condition (e.g., multiple sclerosis, stroke, hypothyroidism); the latter when a substance (e.g., a drug of abuse, a medication, or exposure to a toxin) appears to be etiologically related to the mood disturbance.

This differential diagnosis may be not easy because depression can be either the consequence or the cause of another illness, or even the two illnesses may simply co-exist without etiological relationship. Among the most common medical conditions or illnesses that co-occur with depression, we can consider anxiety disorders and substance (especially alcohol) abuse, but also other serious diseases such as diabetes, AIDS, heart disease, cancer and Parkinson's disease. There is evidence that patients affected by chronic illnesses have a higher risk for depression than the general population. Similarly, depressed patients present substantial rates of comorbidity with chronic medical diseases **[3]**. For this reason, in case of co-existence of depression and another illness, an early diagnosis of both conditions assumes a great importance for a better treatment.

Comorbidity is only one of the aspects that contributes in rendering depression a significant public health issue. Depressive disorders and especially MDD are fairly *prevalent* in the general population, with a higher rate in women compared to men, are *disabling*, since they can

significantly impair psychosocial functioning, and are typically associated with *high mortality* due mainly to the high rate of suicides but also to the negative impact that depression has on the course of co-occurring illnesses.
In addition, depression does not discriminate on account of age: it can arise in childhood, adolescence, adulthood and even in elderly, albeit with differences in the variety of symptoms. Furthermore, antidepressant (pharmacological and psychological) therapies in use have a limited efficacy and/or are associated with side effects that reduce the compliance in many patients.

Hence, the study of the neurobiological mechanisms underlying depressive disorders is extremely important in order to develop more effective strategies for their prevention and treatment.

1.2 The neurochemistry of depression

1.2.1 The monoamine hypothesis

Among the several neurochemical hypotheses formulated to explain the etiology and pathogenesis of depression, the monoaminergic hypothesis is the one that has received most attention. This hypothesis was proposed in 1965 by Schildkraut, who asserted that depression is associated with a deficiency of monoamine activity at important sites in the brain, whereas mania is associated with a relative excess **[4]**.

This hypothesis derives from the observation that reserpine, a drug used 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 accumulation in the cytosol where they are converted into inactive metabolites by the monoamineoxidases (MAOs).

Other events that contributed to support the monoaminergic hypothesis were the serendipitous discoveries that the potential antitubercular iproniazid **[5, 6]** and the potential antipsychotic imipramine **[7]** could relieve many symptoms of depression. Thus, iproniazid and imipramine became the founders of two classes of antidepressants: the MAO-Inhibitors (MAO-I) and the Tricyclic antidepressants (TCA), respectively. They both increase the availability of monoamines (mainly NE), MAO-Is by inhibiting their catabolic enzyme, and TCAs by blocking their reuptake into presynaptic neuron terminals **[8]**.

These first antidepressants were not selective for NE or 5-HT and they produced an increment in the levels of many other neurotransmitters, even not catecholaminergic, provoking a series of side effects. Much effort was devoted over the following years to the development of selective antidepressants, such as the NRIs (Norepinephrine Reuptake Inhibitors) and the SSRIs (Selective Serotonin Reuptake Inhibitors).
NE is well known to play a major role in the control of neuroendocrine activity, in the mechanisms of reward, attention and stress-response, all of which are altered in mood disorders. Similarly, the influence of serotonin on emotivity, thermoregulation, food intake, sleep and nociception, is accountable for its involvement in the pathophysiology of depression and explains the therapeutic success attained by SSRIs.

1.2.2 The role of dopamine

Another important monoaminergic neurotransmitter to take into account, is dopamine (DA). Although DA has not received as much attention as serotonin and norepinephrine in the pathophysiology of depression, several lines of evidence support the involvement of the dopaminergic system in depression:

- the concentration of homovanillic acid (HVA), a major metabolite of dopamine, is decreased in the cerebrospinal fluid (CSF) of depressed patients **[9-13]**;

- prominent symptoms of depression may precede, or co-exist with, Parkinson's disease (which affects the nigrostriatal dopaminergic system) **[14, 15]**;
- psychostimulants (which are known to elevate DA concentrations in the mesolimbic circuitry) induce euphoria and increased energy in depressed and euthymic individuals **[16, 17]**;
- inhibitors of DA reuptake, like bupropion, are effective antidepressants **[18]**;
- dopamine antagonists are able to elicit depression-like symptoms **[19]**.

In this context, it is well established that the mesocortical and mesolimbic dopaminergic systems are critically involved in the mechanisms of aversion and motivation/gratification. In particular, the dopaminergic neurons originating in the ventral tegmental area (VTA) and projecting, among others, to the nucleus accumbens (Acb), amygdala (AMYG) and prefrontal cortex (PFCx), are able to react to stimuli with a certain motivational value.
The limbic system allows the individual to recognize those natural rewarding stimuli that are essential for the survival of the species, such as food and sexual activity. The mesolimbic dopaminergic system is also activated by addictive drugs; however, at variance with natural rewards, the activation induced by drug reward is much stronger because it does not undergo habituation. The activation of the mesolimbic dopaminergic system in response to consummatory behaviors associated with feelings of pleasure and satisfaction, suggests that a hypofunction of this system may reduce the ability to appreciate pleasurable activities thereby leading to anhedonia and lack of motivation which are two cardinal symptoms of depression **[18]**.

As already mentioned, also aversive stimuli are able to activate the mesocortical and mesolimbic dopaminergic systems. In particular, the mesocortical projections respond to mild aversive stimuli **[20, 21]**, while stronger and longer-lasting stressors are required to activate the mesolimbic system **[22, 23]**.

Moreover, the mesocortical system is involved in the control of cognitive processes. Therefore, an alteration of this system may contribute to the cognitive deficits observed in depressed patients, such as the reduction of attention and ability to concentrate and memorize **[1]**.

Finally, since DA is involved in the control of motor activity, it has been proposed that a dopaminergic hypofunction may be involved in the psychomotor retardation observed in some depressed patients **[19]**.

Even though the monoamines are the neurotransmitters that have been most extensively studied in relation to the pathophysiology of depression and represent the principal target of the currently used antidepressant drugs, other neurotransmitters cannot be ruled out, given the complexity of the depressive syndrome that implies an interaction among different neurotransmitters and also between neurotransmitters and endocrine systems.

1.2.3 The glucocorticoid hypothesis

Beyond the hypotheses concerning the function of different neurotransmitters in the pathophysiology of depression, there are other hypotheses that take into account the role of the neuroendocrine systems.
One of such hypotheses assigns a fundamental role to the hypothalamic-pituitary-adrenal (HPA) axis, which is intensely activated by acute and chronic stress **[2]**.

Thus, many neurotransmitters, such as NE, 5-HT, acetylcholine (Ach) and γ-aminobutiric acid (GABA), modulate the secretion of corticotropin-releasing factor (CRF) by the neurons of the hypothalamic paraventricular nucleus (PVN) in response to stress. CRF stimulates the anterior pituitary to produce and release adrenocorticotropin (ACTH) which, in turn, stimulates the synthesis and release of glucocorticoids (cortisol in humans, corticosterone in rodents) from the adrenal cortex.

Glucocorticoids have profound effects on both, general metabolism and behavior that are necessary to tackle the adverse situation, like the consumption of energy and the promotion of certain cognitive abilities **[2]**. Normally, glucocorticoids exert an inhibitory feedback, via the hippocampus, on the HPA axis thereby ensuring a transitory activation of this cascade. 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 **[24].**

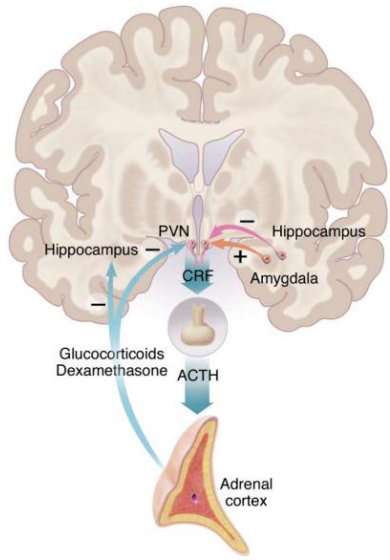

Fig.1 Regulation of the Hypothalamic-Pituitary-Adrenal (HPA) Axis. CRF-containing parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN) integrate information relevant to stress. Prominent neural inputs include excitatory afferents from the amygdala and inhibitory (polysynaptic) afferents from the hippocampus. Other important inputs are from ascending monoamine pathways (not shown). CRF is released by these 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 bloodstream, where it stimulates the release of glucocorticoids. 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. At higher levels, glucocorticoids also impair, and may even damage, the hippocampus, which could initiate and maintain a hypercortisolemic state related to some cases of depression. **[2]**

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 (a synthetic analog of cortisol) 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. Finally, the intracerebral administration of CRF in experimental animals produces effects that are similar to those observed in humans, such as a reduction in locomotor activity, loss of appetite, insomnia and anxiety. These preclinical and clinical findings suggest a correlation between the hyperactivity of the HPA axis and depression **[25]**.

The mechanisms by which sustained high levels of glucorticoids are able to alter the inhibitory feedback on the HPA axis are not yet completely understood but *in vitro* studies have revealed a damage of hippocampal neurons which involves a reduction in dendritic branching and a loss of highly specialized dendritic spines important for the reception of glutamatergic synaptic inputs **[26, 27]**. Moreover, the damage triggers a positive feedback because the reduction in the inhibitory control exerted by the hippocampus on the HPA axis leads to a further increase in circulating glucocorticoid levels and subsequent hippocampal damage **[2]**.

The results of studies aimed at characterizing the pathologic effects of stress on the hippocampus have led to another recent hypothesis that proposes a role for neurotrophic factors in the etiology of depression and in its treatment **[28, 29]**.

1.2.4 The neurotrophic factor hypothesis and the role of BDNF

Neurotrophic factors are so named because they were first characterized as peptides involved in the modulation of neural growth and

differentiation during development, but now they are known to be required also for plasticity and survival of adult neurons and glia **[2]**. 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 **[30]**. Recent data suggest that pro-BDNF and mature BDNF activate different intracellular signaling pathways **[31-33]**. Pro-BDNF signals through the low-affinity neurotrophin receptor p75 that is believed to be involved in apoptosis **[30, 34]**, whereas mature BDNF signals through its high-affinity tropomyosin related kinase B (TrkB) receptor **[35]**. When BDNF is bound to TrkB, it induces its dimerization and the receptor tyrosine kinase is autophosphorylated, leading to the activation of intracellular signaling cascades, as well as augmentation of N-methyl-D-aspartate (NMDA) receptor-mediated currents **[36]**.

The BDNF-TrkB activation can regulate at least three signaling transduction pathways (**Fig. 2**): (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; and (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 (for review, see **[37, 38]**). Briefly, rapid synaptic and ion channel effects are thought to depend on PLCγ-mediated release of intracellular calcium stores, and longer-lasting genomic effects are considered to be downstream of PI3K and MAPK pathways. In addition, there is also evidence that BDNF may directly activate voltage-gated sodium channels to mediate rapid depolarization of target neurons **[39]**.

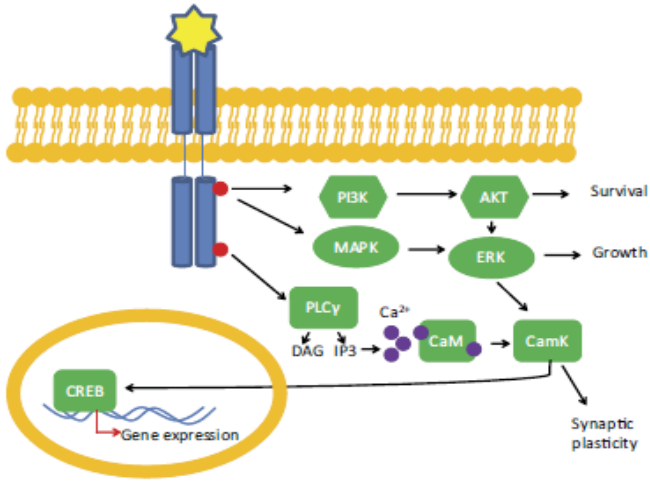

Fig.2 BDNF signaling through TrkB receptors All three pathways converge on transcription factor CREB (cAMP response element-binding protein), which can up-regulate gene expression. CaM: calmodulin; CaMK: calmoduline kinase; DAG: diacylglicerol. [**35]**

There are several lines of preclinical and clinical evidence supporting a role for BDNF in depression and in the mechanisms of action of antidepressants.

Clinical studies in depressed patients have revealed decreased plasma levels of BDNF, a condition that is normalized by antidepressant treatments **[40, 41]**. However, it is not clear if the plasma levels of BDNF are indicative of its cerebral concentration. Additional clinical evidence derived from post-mortem investigations comparing depressed patients without treatment (i.e., suicide victims or with other causes of death) with depressed patients that were good responders to antidepressants. The results of those studies show that in not treated patients there are low concentrations of BDNF and TrkB in the hippocampus (HIPP) and prefrontal cortex (PFCx) **[42, 43]**, and even a reduced hippocampal volume **[35]**. Conversely, patients successfully treated with antidepressants show an increase in the hippocampal and cortical concentrations of BDNF and TrkB **[44]**.

In keeping with the results obtained in humans, several preclinical studies have been perfomed in a variety of animal models of depression, in which the depressive-like behavior can be the result of a genetic selection or it can be induced by environmental or pharmacological manipulations during the development or in adulthood **[45]**.

In particular, the results of preclinical studies show that:

- chronic mild stress or unpredictable stress can lead to decreases in hippocampal mRNA and protein levels of BDNF in mice and rats **[44]**;

- similarly, long-term administration of corticosterone is sufficient to produce decreases in BDNF expression in the rat hippocampus **[46]**;

- in contrast, chronic and subacute treatment with antidepressants increases the expression of BDNF in the hippocampus **[47, 48]**. Moreover, electroconvulsive stimuli increase the concentrations of BDNF in the hippocampus, striatum, and occipital cortex **[49]**;

- infusion of BDNF into the midbrain, ventricles, or hippocampal regions results in increased antidepressant-like behavior **[50-53]**. In particular, Siuciak and collaborators have demonstrated that the infusion of BDNF into the midbrain produces antidepressant-like effects in learned helplessness paradigms and in the forced swim test **[54]**.

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.

1.2.5 Beyond the hippocampus

Neuroimaging studies in patients with the diagnosis of major depressive disorder have identified structural and functional abnormalities in the orbital and dorsal PFCx, the amygdala, and related parts of the striatum and thalamus. 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, post mortem studies demonstrate reductions in orbital and medial PFCx volume even in the brain of patients who died during symptom remission. Conversely, changes in physiological activity (CBF and glucose metabolism) are mood state-dependent and generally normalize with antidepressant drug treatment **[55]**.

In particular, a decreased CBF and metabolism have been observed during depressive episodes in dorsal PFCx areas implicated in language, selective attention, visuospatial or mnemonic processing **[55]**. Conversely, in the ventrolateral, orbital and pregenual anterior cingulate portions of the PFCx abnormal elevations of CBF and metabolism have been recorded **[56-58]**. 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 amygdala **[57]**. Regarding the amygdala, regional CBF and glucose metabolism consistently correlate positively with depression severity **[59-61]**, but this abnormally elevated activity decreases toward normal levels during antidepressant drug treatment **[62]**. The amygdala participates in the assignment of emotional significance to experimental stimuli and in the organization of behavioral, autonomic, and neuroendocrine manifestations of emotional expression **[63]**. In particular, during depressive episodes, the metabolic rate of the amygdala is positively correlated with plasma cortisol concentrations, suggesting that pathological amygdala activity may increase HPA axis activity in depression **[64]**.

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 mood disorders.

1.3 Animal models of depression

Animal models remain indispensable tools for the advancement of medicine as a whole, and for psychiatry in particular. It is most important to keep in mind that the use of animals in medical research must be based on ethical principles, which can be summarized by the so called "rule of 3 Rs":

- **Reduction**: researchers should use the lowest number of animals sufficient to guarantee statistic reliability). This objective may be met by improving experimental techniques and sharing information among researchers;
- **Refinement**: it is necessary to refine the experiment or the way the animals are taken care for so as to minimize their suffering;
- **Replacement**: when possible, experiments with animals should be replaced with alternative techniques.

Ideally, the animal model created should mimic the human condition of interest with respect to its etiology/biological basis (***construct* validity**), symptomatology (***face* validity**) and response to treatment (***predictive* validity**) **[65]**.
Clearly, meeting such requirements is difficult, especially for depression, since many of the core symptoms of this disorder (e.g., feelings of worthlessness, guilt and suicidal ideation) cannot be modeled in an animal. Furthermore, the multifactorial pathogenesis of depression does not allow an easy creation of animal models with a good construct validity. Nevertheless, since depression is often described as an abnormal reaction to stress, most models have exposed healthy animals to adverse experiences for prolonged periods of time in order to induce a depressive-like phenotype.

A clear example of model based on stress-response is the **chronic mild stress** (CMS) paradigm. In the CMS the animal is exposed to a series of different unpredictable stressful conditions, such as mild unescapable foot shock, changes in housing conditions, food and water deprivation, reversal of light/dark periods, and exposure to noise and bright light. After exposure to these stressors for 2 or 3 weeks, rats show a number of behavioral changes that are maintained for months and are reminiscent of depressive symptoms. These include not only changes in psychomotor behavior (e.g., reduced open field activity), but also a reduced sensitivity to rewards, as evidenced by a decrease in the consumption of sucrose solution in comparison to tap water **[66]**. The strength of the CMS model is its predictive validity, because long-term treatment with various antidepressants causes a return to initial levels of sucrose intake **[67, 68]**.

Another kind of stressor is represented by **early maternal separation**, in which the pups are separated from their mothers for brief periods of time, every day, during the first weeks of their life. Given the important role played by the close contact with the dam in the emotional and cognitive development of the offspring, early maternal separation results in a traumatic event that causes acute disturbances and produces long-lasting effects. These effects, including the repeated activation of stress mediators such as glucocorticoids and catecholamines, can influence the behavior and neurochemistry over the lifetime of the mammal, leading to psychiatric disorders in adulthood **[69]**. Similarly, it has been observed that in humans early stressors such as neglect or abuse lead to persistent elevations of CRH, rendering an individual vulnerable to depression or anxiety, or both **[67]**.

These examples make use of normal rodents (rats or mice) in which the behavioral changes bearing a resemblance to depression are induced by the stressful experience. Another approach is represented by the **olfactory bulbectomy**. It is based on the observation that the olfactory system in the rat forms a part of the limbic region in which the amygdala and hippocampus contribute to the emotional and mnemonic components of

behavior. These neuroanatomical areas seem to be dysfunctional in the patient with major depression. Thus, bilateral olfactory bulbectomy causes a major dysfunction of the cortical-hippocampal-amygdala circuit leading to changes in behavior, and in the endocrine, immune and neurotransmitter systems. These changes resemble many of those seen in patients with major depression and are normalized by chronic antidepresssant treatments **[70]**.

However, none of these models accounts for the genetic vulnerability in the development of depression. Genetic factors strongly influence the pathogenesis of this illness (as well as of the majority of illnesses), 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 overall risk of illness but also influence the sensitivity of individuals to the depression-inducing effects of environmental adversities **[71]**. 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 **[72]**.

Considering the pivotal role of genetic factors in the pathogenesis of depression, several genetic animal models of depression have been developed. Some of these make use of **genetically engineered mice**, such as DAT, NET, and SERT (respectively: Dopamine-, Norepinephrine- and Serotonin- Transporter) knockout (KO) mice, to study the contribution of specific genes, in particular those encoding for monoamine transporters that are the targets of the most widely used antidepressant drugs **[73]**. Other models are based on the use of lines/strains of rats or mice selected for their differences in phenotypic characteristics associated (in a more or less fortuitous way) to depression.

One such model is represented by the **Flinders Sensitive Line (FSL) of rats**. This model was developed at the Flinders University in Australia with the purpose of creating a line of rats that was genetically resistant to the effects of di-isopropyl-fluoro-phosphate (DFP), an organophosphate cholinesterase inhibitor **[74]**. However, the selective breeding program failed in this aim because the Flinders Resistant Line (FRL) rats are not more resistant to DFP relative to outbred control rats but only with reference to the FSL rats **[75, 76]**. Thus, FSL rats are more sensitive to DFP and cholinergic agonists than their FRL counterparts. Since depressed individuals are more sensitive to cholinergic agonists than normal controls, the FSL rats were proposed as an animal model of depression **[77-79]**. Accordingly, this line of rats exhibits some behavioral features resembling those seen in depressed patients, such as reduced locomotor activity, reduced body weight, increased REM sleep and cognitive deficits **[80]**. This model has been very useful as a screen for antidepressants since many depressive-like behaviors (such as the immobility in the forced swim test) exhibited by FSL rats are reverted by chronic antidepressants, including those without anticholinergic effects **[81]**. However, this model (as well as the majority of animal models) has some limitations, such as the inconsistency with other hypotheses about the pathophysiology of depression, (e.g., the involvement of the HPA axis) **[74]**.

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 is represented by the Roman High- and Low-Avoidance rats.

1.4 The Roman high- and low-avoidance rats

1.4.1 Background

The Roman high- (RHA) and low-avoidance (RLA) rats were psychogenetically selected from a Wistar stock of Rattus Norvegicus for rapid (RHA) versus extremely poor (RLA) acquisition of the active avoidant behavior in a shuttle box.

The process of selection started in 1965 in Rome **[82]** where the first five generations were bred, and it was subsequently carried on in Birmingham (U.K.) **[83]**. In 1972, some couples of RHA and RLA rats belonging to the 22nd generation, were sent to the Department of Psychology and Psychiatry of the University of London, where the study of the genetic mechanisms involved in the avoidance response continued for several years **[84]**. In 1972 some of the animals bred in Birmingham were also sent to Zurich where the Swiss subcolony was established (i.e., RHA/Verh and RLA/Verh) **[85]**. More recently, breeding nuclei from the Swiss colony were used to establish two other colonies, one at the Autonomous University of Barcelona, started in 1993, and the other at the University of Cagliari (1998). The rats in the Spanish colony are inbred **[86]**, whereas the initial outbreeding process was continued in the Italian colony **[87]**.

At present three colonies of Roman rats exist: the Swiss colony, which was moved from Zurich to Geneva in 1998 **[88]**; the Spanish colony at the Autonomous University of Barcelona and the Italian colony established at the Department of Life and Environmental Sciences of the University of Cagliari.

1.4.2 The selection criterion: Two-way Active Avoidance test

The criterion used to select the Roman high- and low-avoidance rats is represented by their behavioral response in the two-way active avoidance test.

Briefly, this test consists of placing the animal in a shuttle box divided in two compartments communicating by an opening. The floor of the shuttle box is a metallic grid connected to an instrument that provides a tone and a light as a conditioned stimulus (CS) followed by an electric shock to the paws as unconditioned stimulus (US). These stimuli are always presented in the compartment occupied by the rat, so that it has to flee to the safe chamber before the onset of the unconditioned stimulus to avoid the shock. Such response of the animal is considered as an ***avoidance***, while if the rat flees to the safe chamber after the onset of the US, it is considered as an ***escape***. Finally, if the animal stays in the same compartment during the entire shock, the behavior is called ***failure***.

As shown in these graphs, the two lines differ markedly in the number of avoidances, which is significantly higher in RHA rats as compared with RLA rats. Conversely, the number of failures is much higher in RLA than RHA rats.

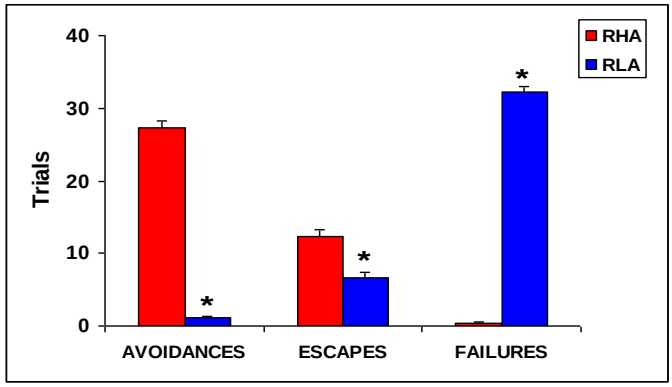

Fig.3 Performance in the active avoidance test of RHA and RLA rats. Columns and bars indicate the mean ± S.E.M. of 20 rats per line. *: P<0,0001 vs the corresponding value of RHA rats.

Although the performance in the two-way active avoidance test is widely considered to depend on learning and memory, there is growing evidence that other mental processes may modify the ability to acquire the avoidant response.

Actually, the initial phase of the acquisition in the shuttle box seems to generate a conflict situation in the animal that affects its ability to learn the avoidant behavior. Due to this conflict, the animal shows an emotional response called ***conditioned fear*** which is responsible for the tendency to remain immobile (***freezing***). Such response prevents the acquisition of the active avoidance **[89]**. Accordingly, it has been demonstrated that behavioral **[90, 91]** and pharmacological **[89]** manipulations reducing fear/anxiety promote avoidance acquisition, whereas other factors, such as anxiogenic drugs **[89]** or environmental conditions that induce fear/anxiety have the opposite effect **[92]**.

These results validate the use of the active avoidance test to study anxiety in rodents **[89]**, as well as the ***elevated plus-maze test*** **[93]**, the ***conflict test*** **[94, 95]** and the ***social interaction test*** **[96]**.

1.4.3 Phenotypic characteristics of RHA and RLA rats

On the basis of the above mentioned findings, it has been proposed that the factor that prevents the acquisition of the avoidance response in RLA rats is their high emotionality **[97]** rather than a learning deficit, as described also in other low-avoidance strains **[98]**.

Not only the RLA rats are not "dumber" than RHA rats, but in several learning tests that imply only a marginal or moderate component of stress **[91, 98-100]**, as in the case of the ***Hebb-Williams Maze test*** **[101]**, RLAs perform significantly better than their RHA counterparts.

Thus, the opposite reactivity to stressful stimuli, defined as coping style or coping strategy, displayed by RLA and RHA rats accounts for their divergent performance in the active avoidance test and also for many other behavioral traits that distinguish the two lines. In particular, in a

variety of tests used to assess emotionality and coping styles in rodents, the hyperemotional RLA rats show a reactive coping, with a behavioral repertoire characterized by hypomotility and robust self-grooming and freezing, while the hypoemotional RHA rats display a proactive coping strategy aimed at taking control over the stressor **[21, 88, 91, 97, 102-104]**. A divergence in the response to stress is also observed in the neuroendocrine function, with RLA rats showing higher activation of the hypothalamus-pituitary-adrenal (HPA) axis than RHA rats, as reflected by a larger increase in corticotropin and corticosterone secretion following exposure to mild stress **[88, 105-107]**. Moreover, the combination of the dexamethasone suppression test (DST) with a corticotropin-releasing hormone (CRH) challenge, has shown that RLA rats are more responsive to CRH administration than RHA rats **[108]**, as observed in many symptomatic depressed patients **[25]**.

The behavioral and neuroendocrine characteristics of RLA rats described above suggest that this rat line may be more prone to develop depression-like behavior under adverse experimental conditions **[108]**. On the other hand, RHA rats display a number of traits such as low HPA axis reactivity and emotionality, high impulsivity and novelty/sensation seeking, and proactive coping **[21, 88, 100, 102, 109, 110]** that may be involved in their resistance to undergo stress-induced depression-like behavior **[111]**.

In this context, the performance of RLA and RHA rats in the Forced Swim Test, one of the most widely used methods to examine depression-like behavior and to evaluate the efficacy of antidepressant drugs in rodents, was evaluated by Piras et al. **[111]**. Two main findings emerged from that study: (i) under baseline conditions RLA rats displayed greater immobility and fewer active behaviors than RHA rats, (ii) the subacute treatment with antidepressant drugs reduced the immobility and increased the active behaviors in RLA rats, leaving the performance of RHA rats unaffected.

The coping strategy is also correlated with the prefrontal dopaminergic function. In this context, it is interesting to note that studies in gerbils indicate that a transient increment in dopamine (DA) output in the medial prefrontal cortex (mPFCx) is required for the acquisition of the avoidant behavior in the shuttle box **[112, 113]**. Accordingly, in vivo voltammetry and microdialysis studies conducted in Roman rats, have shown that a variety of stressors activate the mesocortical dopaminergic pathway of RHA, but not of RLA, rats **[21, 114]**. This and other lines of evidence support the view that the behavioral patterns that distinguish the Roman lines may be mediated, at least in part, by differences in the functional properties of their mesotelencephalic dopaminergic projections (see **[115]** and references therein).

The differences between RHA and RLA rats in terms of the functional tone of mesocorticolimbic dopaminergic transmission may also account for their different responsiveness to a variety of drugs of abuse. Thus, intracerebral microdialysis studies have demonstrated that (i) acute administration of morphine or psychostimulants determines a larger increment in DA output in the nucleus accumbens shell (AcbSh) of RHA rats vs. the AcbSh of RLA rats; (ii) the repeated administration of morphine and cocaine elicits behavioral (i.e., enhanced locomotor activity) and neurochemical sensitization in RHA, but not RLA rats **[87, 115]**. In addition, in self-administration (SA) studies with cocaine it has been observed that, compared with their RLA counterparts, RHA rats display (I) faster acquisition of the SA behavior; (II) larger amount of drug consumed; (III) higher resistance to extinction; (IV) more robust drug-induced reinstatement of cocaine-seeking behavior **[116]**. The greater propensity to consume drugs of abuse displayed by RHA vs RLA rats, may also be influenced by the high levels of impulsivity **[100]** shown by the former, since it is well known that impulsivity is one of the susceptibility factors for the development of drug addiction **[117, 118]**.

Hence, RHA rats may represent a genetic animal model to study the individual vulnerability to undergo a transition from recreational and controlled drug use to compulsive and uncontrolled drug abuse.

Moreover, RHA rats may also represent a model of resilience to stress-induced depression, whereas RLA rats, with their low responsiveness to natural and drug rewards **[87, 109, 116]** resembling the anhedonic state of depressed patients, may be considered as resistant to drug addiction. Nevertheless, it has been shown that RLA rats are able to increase ethanol intake to correct for an aversive emotional state induced by incentive loss **[119]**.

The increased behavioural responsiveness to psychostimulants upon repeated administration **[115, 120, 121]**, the augmented mesocortical dopaminergic response to stress **[21]**, the poor performance in several learning/memory tasks not involving electric shocks **[101, 122-124]** and the enhanced impulsive behavior in the 5-choice serial reaction time task (5-CSRTT) and DRL-20 operant tasks **[99, 100, 125]** shown by RHA rats suggest that this strain/line of rats model some core symptoms of schizophrenia. Considerable experimental evidence supports this view. For instance, in inbred Roman rats (RHA-I and RLA-I) it has been demonstrated that, compared with the RLA strain and/or with standard rat strains, RHAs display deficits in latent inhibition threshold **[126]**, as well as in Pre-Pulse Inhibition (PPI), spatial working memory, and reference memory **[127, 128]**, resembling the cognitive deficits and sensorimotor gating impairments seen in schizophrenic patients. Furthermore, in the study of Klein and colleagues **[125]**, RHA-I rats showed no detectable expression of mGluR2 in the frontal cortex, hippocampus and striatum (for the implications of the mGluR2 in schizophrenia see the review **[129]**). These behavioral and neurochemical characteristics of RHA rats are reminiscent of some core features of the schizophrenic phenotype.

1.5 Depression and anhedonia: implications for sexual behavior

1.5.1 The biological value of pleasure

One of the core features of depression is represented by ***anhedonia***, which was originally defined as the inability to experience pleasure **[130]**, but is now considered to be a wider concept that includes decreased motivation to seek, diminished interest in and inability (or reduced ability) to concentrate on pleasurable activities **[131]**. Undoubtedly, pleasure is a fundamental factor in the control of motivated behaviors in humans and, although the term "pleasure" may be questioned referring to rodents, a similar variable may control motivated behaviors also in animals. Through motivated behavior, animals (and humans) adapt to external or internal changes and reduce their bodily needs that act as ***driving forces***. Thus, some ***drives***, such as thirst or hunger, are part of the regulatory process of homeostasis and act to correct an internal change (the deficit of water or food, respectively). In other conditions the control of behavior does not appear to be based on any well-defined physiological deprivation but on external triggering stimuli, as in the case of mating or exploration, which are respectively driven by sex arousal and curiosity. The ability to feel pleasure associated to motivated behaviors, for instance in response to food or during sexual activity, is innate since such behaviors are fundamental for the survival of the individual and the species. This kind of pleasure is connected to the ***consummatory*** aspect of the motivated behavior that manifests itself with a specific behavioral repertoire depending on the nature of the stimulus. However, in a motivated behavior the ***consummatory*** phase is usually preceded by adaptive, flexible forms of ***appetitive*** behavior, which enables an animal to come into physical contact with its goal. In the ***appetitive*** phase, pleasure consists in a state of incentive arousal which reinforces the seeking of and the approaching to the goal. This "anticipatory" pleasure is not innate, but learned, since it implies a classical conditioning in order to

associate the predictive stimulus (e.g., the pheromones coming from a potential partner) with the possibility to gain the goal (e.g., copulation).

1.5.2 The brain reward circuitry

The motivational properties of pleasure depend on the activity of anatomically and functionally interconnected brain areas that define the so-called ***brain reward circuitry***. This circuitry includes limbic structures (such as the infralimbic portion of PFCx, the shell of the Acb (AcbSh), the medial portion of the ventral pallidum (VP), the AMYG, and the VTA) which play a pivotal role in the control of motivated behavior, and motor structures (the prelimbic portion of PFCx, the Acb core (AcbCo), the dorsal VP and the substantia nigra) which are more relevant for habitual behaviors. As previously described, both natural rewarding stimuli and drugs of abuse are able to activate this circuitry; in particular, they share the ability to induce the release of dopamine (DA) in the AcbSh. More precisely, dopamine release from the nucleus accumbens is modulated by various characteristics of the stimulus and by the motivational state.

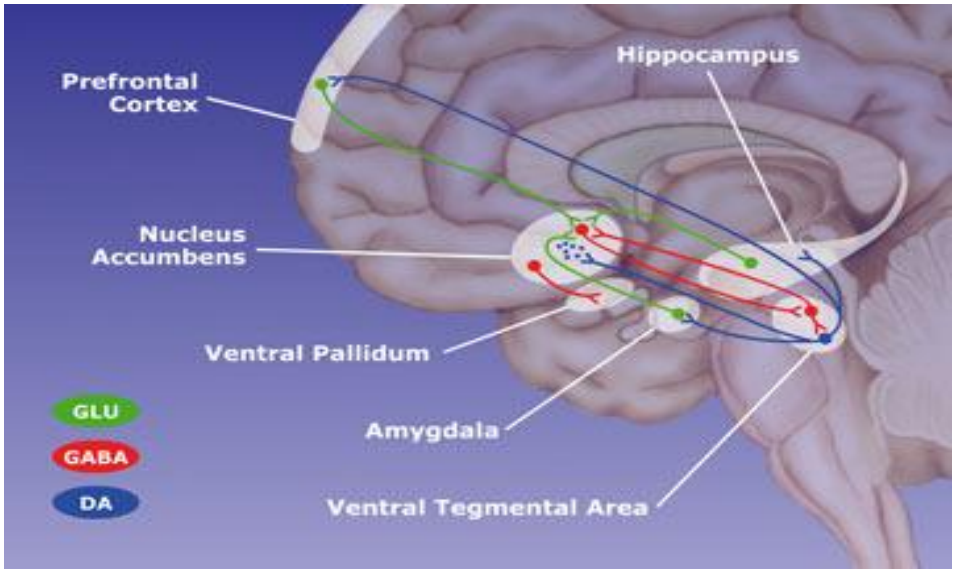

Fig. 4 Schematic representation of the human brain reward circuitry

1.5.3 Reward-related impairments

A dysfunction in processing rewarding stimuli is characteristic of some psychiatric conditions. For instance, in substance abuse disorders there is an excessive activation of the mesolimbic dopaminergic system that promotes incentive learning, a mechanism by which otherwise emotionally neutral stimuli acquire a motivational value. On the contrary, in depressive disorders, previously attractive stimuli lose their rewarding properties thereby leading to ***anhedonia***. Anhedonia in humans can be measured in various ways (for an extensive review see **[132]**). Behavioral **[133]**, electrophysiological **[134]**, hemodynamic **[135]**, interview-based measures **[136]**, and self-reports are frequently cited in research related to anhedonia in major depressive disorder (MDD) **[137]**. Such variety of measurements is justified by the complex nature of anhedonia, that may be defined as a composite symptom, consisting of at least two dimensions (i.e. motivational/appetitive and consummatory) **[138]**, each one based on distinct biological mechanisms **[139]**. It is noteworthy in this context that depression seems to be characteristically linked to motivational anhedonia which has been consistently correlated with mesolimbic dopaminergic signalling **[138, 139]**.

1.5.4 Depression and sexual activity

The loss of interest in once enjoyable activities (i.e., anhedonia) observed in many depressed patients often includes sexual activity. Several studies have demonstrated that the prevalence of sexual dysfunction is higher in patients with MDD than in the general population **[140-147]**. Moreover, the frequent coexistence with anxiety symptoms, which are also associated with sexual problems, can exacerbate this condition **[148-153]**. Depressed persons may experience a series of sexual problems: frequently they express a reduction of sexual desire (***libido***) and excitement (***arousal***), a diminished ability to maintain sexual arousal or achieve orgasm, and in males also erectile dysfunction can occur **[154]**. Unfortunately, in addition to ameliorating the majority of depressive symptoms, almost all antidepressants fail to improve sexual dysfunction

and can actually cause a decrease in libido and other sexual side-effects that reduce the compliance of the patient. Notably, an exception is represented by bupropion, which increases central dopaminergic transmission **[155]**. In this context, it is noteworthy that dopamine transmission in the hypothalamus **[156-163]** and in the mesolimbic projection **[164-169]** has a facilitatory effect on both the ***anticipatory*** (sexual motivation/arousal) and the ***consummatory*** (penile erection and copulation) phases of sexual behaviour in laboratory animals and humans.

Therefore, the study of the neurophysiology of sexual function (in its ***appetitive*** and ***consummatory*** aspects) is warranted in order to better understand the mechanisms underlying the psychopathological and pharmacologic causes of sexual dysfunctions.

1.6 Early life adverse experiences and psychiatric disorders

As mentioned in the section "animal models of depression", the ***early maternal separation paradigm*** may be used to induce a depression-like phenotype in rodents. Thus, such a traumatic event experienced in early life may produce long-lasting effects, including the sensitization of the HPA axis, which increase the risk to develop anxiety and/or depressive disorders in adulthood.

A large body of preclinical and clinical evidence supports the view that exposure to either rewarding or traumatic experiences in early life plays a pivotal role in the development of the central nervous system thereby influencing the behavior and neurochemistry over the lifetime of the individual. Therefore, the exposure to adverse experiences in early-life may contribute to the occurrence of psychiatric disorders, such as

depression, schizophrenia, and substance abuse in genetically predisposed individuals [170-172].

Notably, the eventual development of a psychiatric disorder, as well as the type of disorder, may be determined not only by the genotype of the individual who experiments the adverse event but also by the characteristics of the adverse experience. For instance, severe, horrendous stress, such as that experienced during combat, does not typically induce depression, but instead causes post-traumatic stress disorder (PTSD).

Similarly, different experimental paradigms used to model early life stress in laboratory animals may induce differential neurochemical and behavioral changes depending on the species, the line/strain and the sex of the animal used for the test.

Thus, while early maternal separation has been proposed as a paradigm to induce a depression-like phenotype, the role of post weaning social isolation appears to be more related to the later development of schizophrenia-like symptoms, although some controversial issues remain unclear.

It is noteworthy in this context that the Roman lines/strains of rats may model the genetic predisposition for depression (RLA), as well as the vulnerability to develop schizophrenic or substance abuse disorders (RHA). Exposing RLA and RHA rats to adverse early-life events (such as maternal separation or social isolation) may therefore contribute to the identification of the molecular mechanisms underlying the long-term consequences of adverse early-life events, in order to improve our knowledge on the pathogenesis of several psychiatric disorders and to identify new targets for their treatment.

1.7 Objectives

Although exposure to stressful and/or traumatic events, especially in early life, is considered to be a major predisposing factor to depression and other psychiatric disorders, not all humans exposed to severe stress develop a psychopathology. This suggests that the individual sensitivity to stress plays a pivotal role in determining vulnerability or resistance to depression and other psychiatric disorders.

Notably, the selective breeding of Roman lines/strains of rats has generated two phenotypes that differ markedly in the reactivity to stressful stimuli.

The vulnerability of RLA rats and, viceversa, the resistance of RHA rats to develop stress-induced depression, as suggested by their divergent behavioral and neurochemical traits, was assessed in a previous study in which the performance of the two lines was compared in the Forced Swim Test (FST) under baseline conditions and after subacute treatments with antidepressant drugs. However, that study showed the positive response of RLAs to subacute antidepressants, whereas it is well known that several weeks of treatment with antidepressant drugs is required to obtain an adequate clinical response.

On the bases of the results obtained in the study by Piras et al., and considering the clinical reports, the aim of study I was to evaluate the performance of RLA and RHA rats in the FST in response to chronic antidepressant treatments at doses that were ineffective when given subacutely.

One of the cardinal symptoms of depression observed in many patients is anhedonia, which may be defined as a loss of interest in once enjoyable activities, including sexual activity.

Depressive episodes are frequently associated with sexual dysfunctions, especially concerning the motivational aspects of sexual activity. In consideration of this clinical evidence, and given that:

- RHA and RLA rats display different coping styles and behavioral profiles, including a marked difference in the responsiveness to natural and drug rewards;

- these differences may be determined, at least in part, by different functional properties of brain dopaminergic transmission;

- dopamine has a well documented facilitatory effect on both, the motivational and the consummatory aspects of sexual behavior;

<u>study II</u> was aimed at characterizing the sexual behavior of Roman rats and its correlation with the functional state of the mesolimbic dopaminergic transmission.

Finally, in keeping with the long-term consequences on mental health elicited by early-life adverse events, it has been observed that post weaning social isolation in rodents may lead to a later increment in the anxiety/fear related behaviors and to other behavioral and neurochemical alterations that have translational relevance to developmental neuropsychological disorders, in particular to several core symptoms of schizophrenia.

Thus, in <u>study III</u> the impact of an early adverse experience, such as the post weaning isolation, on the anxiety-related behaviors of RHA and RLA (inbred) rats was evaluated in the Elevated Zero Maze and in motility cages used to asssess locomotor activity in a new environment.

2. Study I: Effects of subacute and chronic antidepressant treatments in RLA and RHA rats

2.1 Introduction

As described in the general introduction, the individual vulnerability to depression is determined by a combination of genetic factors, early life stress, and ongoing stress, and the relative contribution of each component may be studied using animal models. In this context, the selective breeding of Roman high- (RHA) and low-(RLA) avoidance rats led to the fortuitous segregation of behavioral traits (such as the reactive coping associated with hyper-reactivity of the HPA axis shown by RLA rats exposed to stressful conditions) that are reminiscent of some key features of clinical depression.

Thus, the behavioral traits and neurochemical characteristics that distinguish the Roman lines suggest that, under stressful conditions, RLA rats may be prone, whereas RHA rats may be resistant to develop depression-like behavior. To test this hypothesis, the performance of these rat lines was evaluated by Piras et al. **[111]** in the Forced Swim Test (FST), a paradigm used to evaluate depression-like behavior and to assess antidepressant activity in rodents **[173-175]**. It was found that RLA rats remained immobile for a longer time and spent less time in active behaviors than RHA rats, either during the 15-min pre-swim session or during the 5-min swim test performed 24 h later. Moreover, subacute treatments with clinically effective antidepressants, such as desipramine, fluoxetine, and chlorimipramine, produced a dose-dependent decrease in immobility and an increase in active behaviors in RLA rats, but did not modify the performance of RHA rats **[111]**.

Because clinical studies consistently show that several weeks of drug administration are required to observe the first improvements in the depressive symptoms, the present study was designed to further evaluate the responses to antidepressants of RLA and RHA rats by comparing the effects of subacute (1 day) and chronic (15 days) administration of desipramine, fluoxetine, and chlorimipramine on their performance in the

forced swim test. To this aim, antidepressant drugs were administered chronically at doses that were ineffective when given subacutely and passive and active behaviors were assessed during a single exposure to the swim test, that is without pretest **[176]**.

2.2 Materials and methods

2.2.1 Animals

A total number of 229 male rats (115 RHA and 114 RLA) bred in the colony established in 1998 at the University of Cagliari were used. The rats were 4-month old at the time of behavioral testing (body weight 400–500 g). After weaning at postnatal day 25, they were housed in groups of four per cage and were maintained in a temperature- and humidity controlled environment (23±1 °C and 60 %, respectively) under a 12-h light–dark cycle (lights on at 8:00 a.m.), with standard laboratory food and water freely available. To minimize stress to the rats before the test, the contact with the animal house maintenance personnel was limited to a single attendant, and bedding in the home cages was not changed in the two days preceding the test. All the procedures were performed in the housing facilities of the Department of Life and Environmental Sciences of the University of Cagliari, in accordance with the guidelines and protocols approved by the European Union (EU Council 86/609; D.L. 27.01. 1992, no.116) and by the Ethical Committee for Animal Care and Use of the University of Cagliari.

2.2.2 Forced Swim Test

The Forced Swim test (FST), described originally by Porsolt et al. **[173, 177]**, consists of placing the animal in an inescapable deep tank of water, since it has been observed that in such condition rats and mice develop immobility after an initial period of vigorous activity **[173]**. The reduction

of escape-directed behavior (such as swimming or climbing) is thought to resemble the inability to adopt proactive coping strategies under stressful situations, which is a core feature of human depression **[178]**. Antidepressant treatments reduce the amount of immobility, or delay its onset, and increase or prolong active escape behaviors displayed during the FST **[178]**.

The procedure used in our study was very similar to that described by Porsolt et al. **[173]** and modified by Detke et al. **[174]**, except that the water was deeper to ensure that rats were unable to touch the bottom of the cylinder with their hind paws or tail. Swim sessions were conducted by placing the rats in individual plastic cylinders 58 cm tall and 32 cm in diameter which were filled with water (24‑25°C) to a height of 40 cm from the bottom (for more details see **[111]**).

The usual paradigm implies a first exposure to inescapable swim for 15 min (pre swim session) which serves as an induction procedure that facilitates passive behaviors on subsequent swim exposure, and is followed, 24 h later, by a 5-min swim test during which the active and passive behaviors displayed by the rats are recorded **[175]**. However, in this study only the 5-min swim test, without the pre swim session, was performed in order to compare more equally the effects of subacute and chronic treatments.

2.2.3 Behavioral measurements

A single well-trained observer, blind to the treatment condition, quantified all the behaviors using a time-sampling technique whereby the predominant behavior in each 5 s period of the test was recorded. The following behaviors were rated by the observer:

- ***immobility***: floating passively in the water without struggling and doing only those movements necessary to keep the head above water;

- ***swimming***: showing moderate active motions around in the cylinder, more than necessary to simply keep the head above water;
- ***climbing***: making active vigorous movements with forepaws in and out of the water, usually directed against the walls.

In all the experiments, the observer recorded also the latency to immobility, defined as the time at which the rat first adopted an immobile posture.

2.2.4 Locomotor activity

In separate cohorts of rats (48 RHA and 48 RLA), the effects of chronic treatments with desipramine, fluoxetine, or chlorimipramine on ambulatory activity were assessed in eight photocell activity monitors (Opto-Varimex Mini, Columbus, Ohio). After a 5 h period of habituation in the test room, animals were placed individually in the center of the Perspex cages of the activity meters (50×30×20 cm), and the number of interruptions of infrared beams (i.e., counts) were recorded at 5 and 30 min after the beginning of the test.

2.2.5 Drug administration

Subacute treatments Two different doses of desipramine (5 and 20mg/kg), fluoxetine (2.5 and 10mg/kg), chlorimipramine (5 and 20mg/kg), or vehicle (2 ml/kg) were administered 24, 5, and 1 h prior to the start of the test session. Desipramine and fluoxetine were administered subcutaneously (s.c.), whereas chlorimipramine was administered intraperitoneally (i.p.) in a constant volume (2 ml/kg) of sterile deionized water. Drug doses are indicated as milligrams per kilogram of the respective salt and were selected on the basis of the previous study by Piras et al. **[111]**.

Chronic treatments Desipramine (5 mg/kg/day), fluoxetine (2.5 mg kg/day), chlorimipramine (5 mg/kg/day), or vehicle (2 mg/kg/day) were administered i.p. once daily for 15 consecutive days. These doses were

selected because they failed to affect the performance of either line in the forced swim test upon subacute administration (see left-side panels in Figs. 1, 2, and 3; **[111]**). Forced swim tests and ambulatory activity measures were performed 24 h after the last injection of drug or vehicle in a counterbalanced order according to drug treatment and animal line.

2.2.6 Data analysis

Forced swim test The results are expressed as the mean±SEM number of behavioral episodes (i.e., immobility, swimming, and climbing) during the 5-min swim test and as the mean±SEM latency time (in seconds) to the first immobility episode. Separate two-way ANOVAs were performed for each behavioral measure, antidepressant drug, and duration of treatment (i.e., subacute or chronic), with line (RLA vs. RHA) and treatment (i.e., drug dose) as the main factors. Whenever appropriate (i.e., P for the main factors and/or their interaction, <0.05), ANOVA was followed by pair wise contrasts with the honest significant difference Tukey test.

Locomotor activity The results are expressed as the mean±SEM counts/5 min and counts/30 min. Separate two-way ANOVAs were carried out on each drug (main factors: line and treatment with repeated measures over time (5 and 30 min)).

2.3 Results

2.3.1 Baseline performance of RLA and RHA rats in the FST

The behavioral patterns of vehicle-treated RLA and RHA rats shown in **Figs. 1, 2,** and **3** allow a comparison of the baseline performance of the two lines in the FST. In line with Piras et al. **[111]**, RLA rats, compared

with their RHA counterparts displayed a shorter latency to immobility and a higher immobility frequency ($P<0.05$), as revealed by two-way ANOVAs and post hoc contrasts with the Tukey test. Moreover, the frequency of climbing episodes was lower in RLA than in RHA rats ($P<0.05$), whereas no statistically significant line-related differences in swimming counts were observed.

2.3.2 Effects of subacute and chronic treatments with antidepressants on the performance of RLA and RHA rats in the FST

Desipramine

Immobility Both subacute and chronic treatments with desipramine showed significant effects on immobility latency, as indicated by two-way ANOVA analyses (subacute: P values<0.01 for line, treatment, and their interaction; chronic: $P<0.05$ for treatment; and $P<0.0001$ for line x treatment) (**Fig. 1a**). Post hoc contrasts revelead that the effects of desipramine were limited to RLA rats. Thus, in this line a significant increase in immobility latency was observed upon either the subacute desipramine administration of 20 mg/kg, or the repeated one at the dose of 5 mg/kg/day. Significant changes were observed also in the frequency of immobility episodes (subacute: effects of line, treatment and interaction with P values<0.0001; chronic: $P<0.01$ for treatment and interaction); in particular, post hoc analyses revealed that immobility frequency was decreased in RLA but not RHA rats after subacute treatment only at the high dose (20mg/kg), while the low dose (5 mg/kg) was sufficient to induce a similar reduction in the frequency of immobility of RLA rats when administered chronically. (**Fig. 1b**).

Swimming No significant effects on swimming counts were detected with either subacute or chronic desipramine treatments (P values for the effects of line, treatment, and their interaction, >0.05) (**Fig. 1c**).

Climbing Climbing scores were significantly affected by both, subacute and chronic desipramine treatments (subacute: P values for the effect of

line, treatment and their interaction, <0.02; chronic: significant effect of line, P<0.05; and treatment, P<0.01). In RLA but not RHA rats, post hoc comparisons revealed a significant increment in climbing frequency upon subacute treatment with 20 mg/kg of desipramine, and upon the repeated administration of desipramine at the dose of 5 mg/kg/day (**Fig. 1d**).

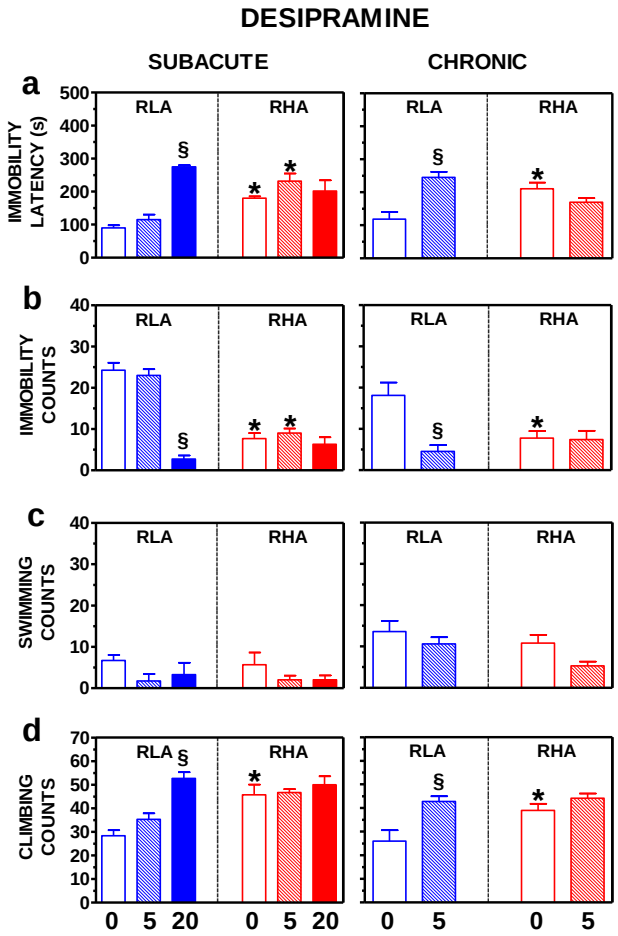

Fig. 1 Effects of desipramine on the behavioral performance of RLA and RHA rats in the FST. Columns and bars indicate the mean ± SEM number of counts of 3 rats per group. Left-hand panels show the effects of the subacute administration of 2ml/kg of vehicle (desipramine = 0), or desipramine (5mg/kg and 20 mg/kg) on immobility latency **(a)**, immobility frequency **(b)**, swimming frequency **(c)**, and climbing frequency **(d)**. Right-hand panels show the effects of the repeated daily administration of vehicle or desipramine (5mg/kg) for 15 consecutive days on the behavioral measures described above. *: P<0.05 vs the treatment-matched RLA line. §: P<0.05 vs the line-matched vehicle-treated controls.

Fluoxetine

Immobility Two-way ANOVA analyses revealed significant effects of line and line x treatment interaction on immobility latency upon subacute and chronic fluoxetine treatments (**Fig. 2a)** (both subacute and chronic: $P<0.01$ for line and $P\leq0.05$ for line x treatment interaction). A tendency towards an increase in immobility latency was observed in RLA but not RHA rats upon the subacute treatment at the dose of 10 mg/k and the chronic administration of 2.5 mg/kg/day ($P<0.16$ and $P=0.12$, respectively). Subacute and chronic fluoxetine treatments significantly changed immobility frequency (subacute: $P<0.01$ for all the main factors; chronic: $P<0.01$ for line, $P=0.05$ for treatment and $P<0.001$ for their interaction). As shown by post hoc contrasts, immobility scores were decreased in RLA but not RHA rats either after subacute treatment at the high dose of 10 mg/kg or after the repeated administration of fluoxetine at the dose of 2.5 mg/kg/day ($P<0.001$ and $P<0.05$ versus the vehicle-treated controls of the same line for subacute and chronic treatments, respectively) (**Fig. 2b**).

Swimming As shown in **Fig. 2c**, the number of swimming episodes was significantly modified after subacute and chronic treatments with fluoxetine (ANOVA analysis revealed all P values <0.001 for the subacute treatment and all P values <0.0001 for the chronic treatment). Post hoc analysis revealed that the subacute treatment at the dose of 10 mg/kg significantly increased swimming frequency ($P<0.001$) in RLA but not RHA rats, whereas the chronic treatment at the dose of 2.5 mg/kg/day induced a similar increment only in RLA rats ($P<0.001$).

Climbing In both lines, climbing counts were not modified by either subacute or chronic treatments with fluoxetine (**Fig. 2d**). However, RHA rats displayed an overal higher frequency of climbing episodes compared with their RLA counterparts: ANOVA analyses indicated a significant effect of line for both subacute ($P<0.0001$) and chronic treatments ($P<0.01$).

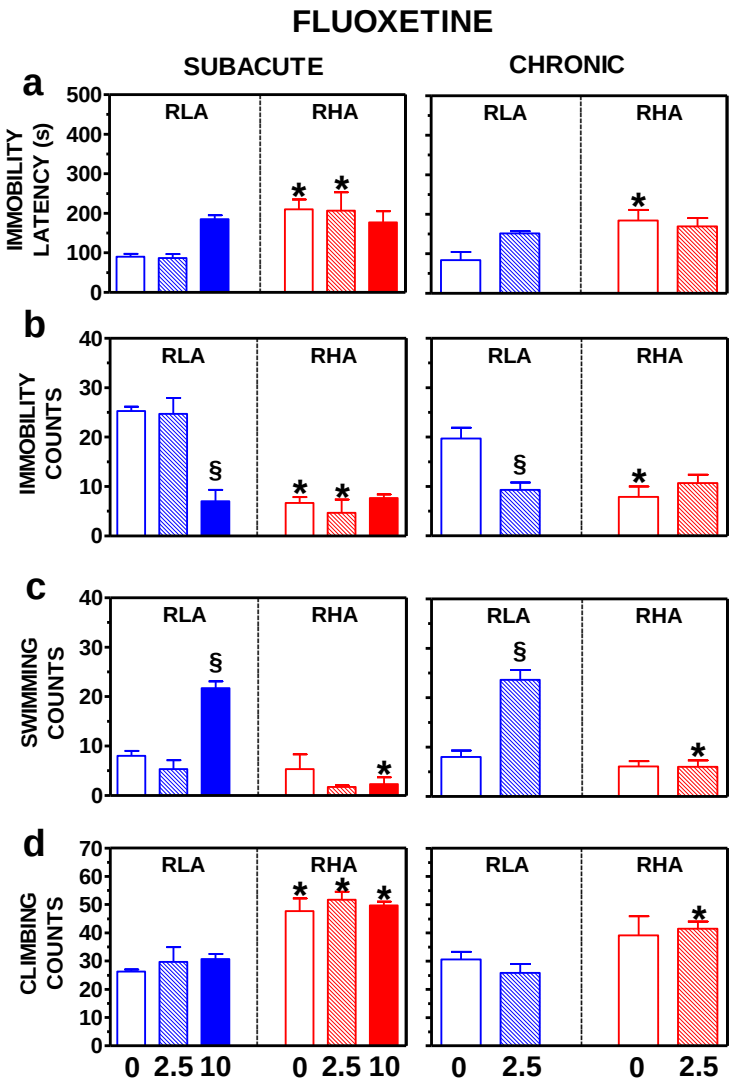

Fig. 2 Effects of fluoxetine on the behavioral performance of RLA and RHA rats in the FST. Columns and bars denote the mean ± SEM number of counts of 3 (subacute) or 7 (chronic) rats per group. Left-hand panels show the effects of the subacute administration of 2ml/kg of vehicle (fluoxetine = 0), or fluoxetine (2.5mg/kg and 10 mg/kg) on immobility latency **(a)**, immobility frequency **(b)**, swimming frequency **(c)**, and climbing frequency **(d)**. Right-hand panels show the effects of the repeated daily administration of vehicle or fluoxetine (2.5mg/kg) for 15 consecutive days on the behavioral measures described above. *: P<0.05 vs the treatment-matched RLA line. §: P<0.05 vs the line-matched vehicle-treated control group.

Chlorimipramine

Immobility As indicated by ANOVA analyses, the latency to the first immobility episode was significantly affected by both, the subacute (all P values <0.025) and the chronic treatments (significant effect of line x treatment interaction: P<0.025) with chlorimipramine (**Fig. 3a**). Subsequent pair wise contrasts showed that after subacute treatment only the high dose (20 mg/kg) induced an increment in immobility latency in RLA (P<0.01) but not RHA rats, whereas the low dose of chlorimipramine (5 mg/kg) given repeatedly for 15 days significantly increased the latency time in RLA rats as compared with their respective vehicle-treated controls (P<0.025). Similarly, chlorimipramine induced significant changes on immobility frequency upon both subacute (all P values <0.001) and chronic treatment (P=0.01 for treatment and P<0.001 for line x treatment interaction). In addition, in RLA but not in their RHA counterparts, post hoc contrasts revealed a significant reduction in immobility frequency following the subacute treatment at the high dose of 20 mg/kg (P<0.001 versus vehicle-treated RLA rats), and the repeated administration of chlorimipramine at the dose of 5 mg/kg/day (P<0.001 versus RLA vehicle-treated) (**Fig. 3b**).

Swimming ANOVA analyses indicated that both subacute and chronic chlorimipramine treatments failed to induce significant changes in the frequency of swimming episodes in either line (P values for the effects of line, treatment, and their interaction, >0.05) (**Fig. 3c**).

Climbing Significant effects on climbing scores were reported after subacute (all P values <0.025) and chronic treatments with chlorimipramine (P<0.025 for the effect of line and P<0.05 for the line x treatment interaction) (**Fig. 3d).** Post hoc contrasts revealed that the subacute treatment increased climbing frequency only in RLA rats (P<0.005) at the dose of 20 mg/kg; on the other hand, only a tendency towards an increase in the climbing scores was observed in RLA rats (P=0.10) but not in their RHA counterparts after the repeated administration of chlorimipramine at the dose of 5 mg/kg/day.

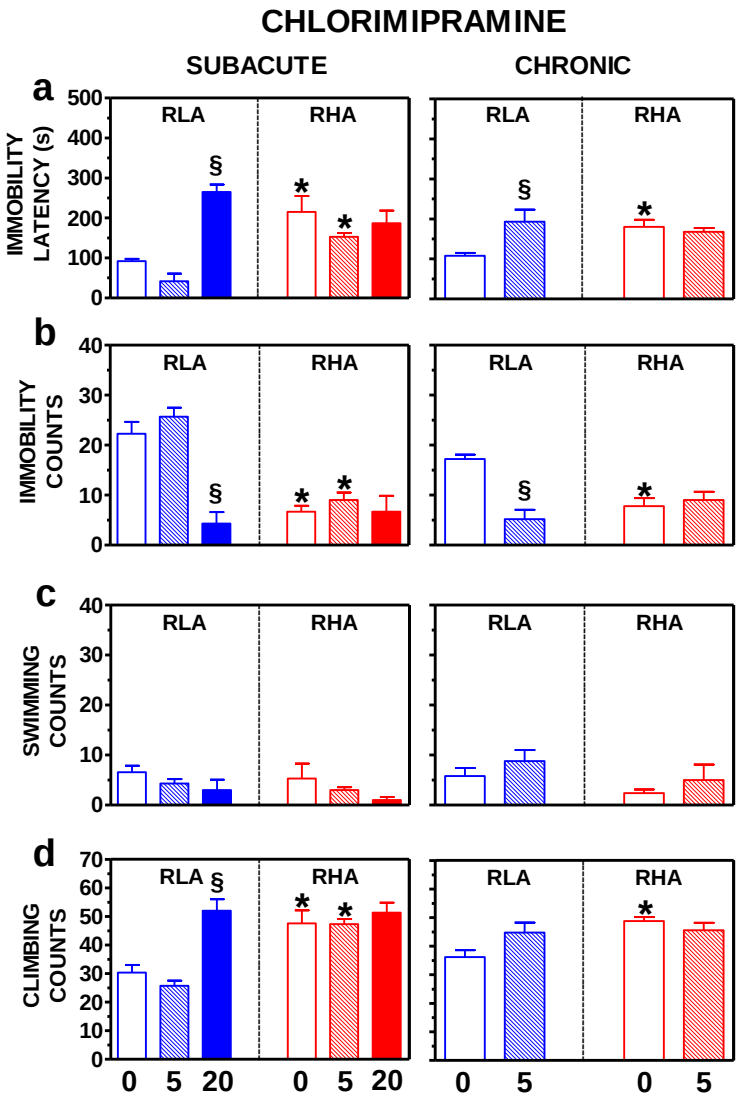

Fig. 3 Effects of chlorimipramine on the behavioral performance of RLA and RHA rats in the FST. Columns and bars indicate the mean ± SEM number of counts of 3 (subacute) or 5 (chronic) rats per group. Left-hand panels show the effects of the subacute administration of 2ml/kg of vehicle (chlorimipramine = 0), or chlorimipramine (5mg/kg and 20 mg/kg) on immobility latency **(a)**, immobility frequency **(b)**, swimming frequency **(c)**, and climbing frequency **(d)**. Right-hand panels show the effects of the repeated daily administration of vehicle or chlorimipramine (5mg/kg) for 15 consecutive days on the behavioral measures described above. *: P<0.05 vs the treatment-matched RLA line. §: P<0.05 vs the line-matched vehicle-treated control group.

Collectively, these results show that chronic treatments with desipramine, fluoxetine, and chlorimipramine reduced immobility and increased active behaviors in RLA but not RHA rats, at doses that were ineffective when administered subacutely.

To exclude potential false positive effects, due to the potentiation of monoaminergic transmission **[173, 174, 179]** induced by these antidepressant drugs, locomotor activity was measured following the same chronic treatment regimens used in the FST.

2.3.3 Locomotor activity

The effects of chronic treatments with desipramine, fluoxetine and chlorimipramine on the locomotor activity of RLA and RHA rats are indicated in **Table 1**. For each drug, two-way ANOVA analyses (main factors, line (RLA vs. RHA) and treatment (saline vs. antidepressant) with repeated measures over time (5 and 30 min)) revealed no significant effects of chronic treatments on the locomotor activity of either line. Notably, the same doses that failed to affect locomotor activity were able to produce reliable behavioral effects in the FST performance of RLA rats. This finding demonstrates that non specific motor effects are not involved in the reduction of immobility and increase in active behaviors observed in RLA rats upon the chronic administration of desipramine, fluoxetine or chlorimipramine.

On the other hand, it is interesting to note that a tendentially higher number of ambulatory counts was observed in control RHA rats (i.e., chronically treated with saline) as compared with their RLA counterparts. This result is in line with several previous reports indicating that RHA rats display a more intense locomotor activity than RLA rats when exposed to a novel environment **[86, 88, 180, 181]**.

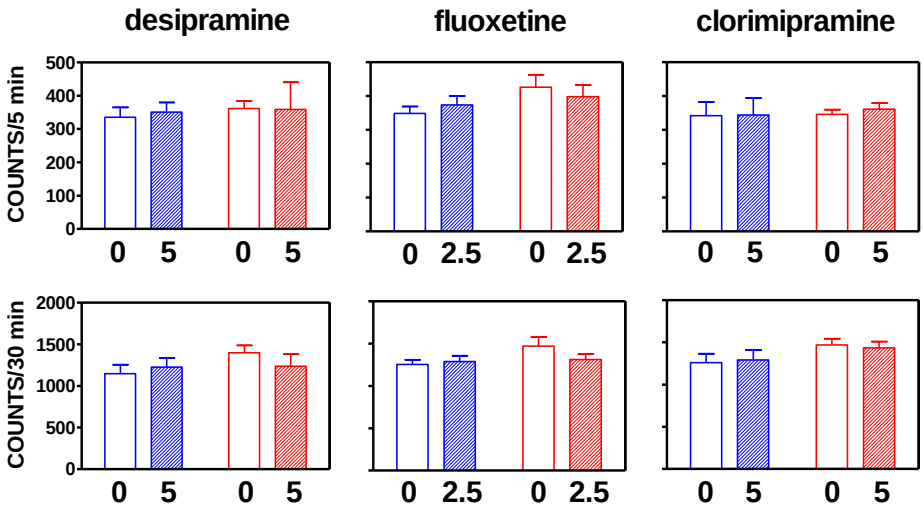

Fig. 4 Effects of chronic treatments with desipramine, fluoxetine and chlorimipramine on the locomotor activity of RLA and RHA rats. Columns and bars indicate the mean ± SEM number of cumulative counts of 8 rats per group recorded at 5 and 30 min after the start of the test. RLA and RHA rats were injected i.p. for 15 consecutive days with either vehicle (2 ml/kg/day: drug = 0) or drug at the following doses: desipramine, 5 mg/kg/day ; fluoxetine, 2.5 mg/kg/day; chlorimipramine, 5 mg/kg/day, and ambulatory activity was measured 24 h after the last injection of the drug or vehicle.

2.4 Discussion

This study confirms the differences between the performance of the Roman lines in the FST under baseline conditions, with RLA rats showing greater immobility and fewer active behaviors than RHA rats and demonstrates that such results are replicable even with a single exposure to the swim test (i.e., the swim test not preceded by the induction pretest). In this context, it is important to keep in mind that the usual paradigm of FST consists of a 5-min swim test preceded, 24 h before, by a pre swim session in which the animal is exposed for the first

time to inescapable swim for 15 min. The pretest serves as an induction procedure that facilitates passive behaviors on subsequent swim exposure thereby allowing a clearer observation of the effects of antidepressants on the performance in the FST. However, the decrease in the latency to immobility elicited on the subsequent test may be interpreted as a "learned immobility" rather than a "behavioral despair". The results obtained in the Roman rats with a single exposure to the FST suggest that the passive strategy displayed by RLA rats in the FST denotes an innate trait, rather than the consequence of a previous exposure to stress.

Moreover, this study shows that low doses of desipramine (5 mg/kg), fluoxetine (2.5 mg/kg) and chlorimipramine (5 mg/kg),which were not effective when given subacutely, became fully effective upon chronic treatment in RLA rats but not in their RHA counterparts.

The pattern of active behaviors displayed by RLA rats chronically treated with low doses of desipramine and fluoxetine was similar to that observed after subacute treatments with higher doses of the same drugs. Thus, in line with several reports on the effects of selective norepinephrine reuptake inhibitors (SNRI) in the FST, desipramine increased climbing behaviors in RLA rats. On the other hand, fluoxetine increased swimming in RLA rats, as reported in studies using selective serotonin reuptake inhibitors (SSRIs) other than fluoxetine. Notably, clinical studies show that selective inhibitors of either NE or 5-HT reuptake induce initial improvements in different symptoms of depression. In particular, SNRIs initially improve motor retardation and depressed mood, while the early effects of SSRIs include a reduction in anxiety followed by improvement in depressed mood and cognitive function **[182]**.

Interestingly, chlorimipramine, which is a nonspecific dual NE/5-HT reuptake inhibitor, presented a mixed profile: subacute high doses increased climbing scores, whereas the chronic administration of low doses had no significant effect on climbing behavior, but increased swimming when the behaviors were recorded for a longer period (i.e., 15 min instead of 5 min, results not shown).

Thus, in keeping with previous reports, the present study shows that antidepressants with different mechanisms of action differentially affect climbing and swimming in the FST (for a review see **[178]**). Moreover, the different behavioral effects induced by antidepressants with distinct molecular targets may be selectively associated with the specific therapeutic efficacy of each drug on different symptoms of depression. This finding confers a potential predictive value to such behavioral patterns that may be exploited for designing novel and more efficient antidepressant drugs **[182]**.

Furthermore, it is noteworthy that the efficacy of chronic antidepressant treatments on RLA rats, as well as the lack of antidepressant effects on RHA rats, are consistent with two important lines of clinical evidence: (i) the delay (at least two weeks) necessary to have the first therapeutic effects following antidepressant treatments, and (ii) the lack of major effects on mood in normal (i.e., non depressed) subjects **[183, 184]**.

Therefore, the present study adds experimental support to the hypothesis that RLA and RHA rats may represent a valid preclinical model of, respectively, vulnerability and resistance to stress-induced depression.

3. Study II: Involvement of dopamine in the different patterns of sexual behavior of Roman high and low avoidance rats: behavioral characterization, pharmacological assays and intracerebral microdialysis study.

3.1 Introduction

RLA and RHA rats display different coping styles and behavioral profiles in experimental paradigms used to assess emotionality/fearfulness, as well as in tests used to evaluate the responsiveness to natural or drug rewards,. These differences may be due, at least in part, to distinct line-related functional properties of brain dopaminergic transmission **[87, 115, 185-187]**, although serotonin and other neurotransmitters cannot be ruled out **[187-190]**. In line with the above studies, differential dose-related responses between the inbred RLA and RHA rat strains in terms of motility, stereotypy, and yawning induced by the D1/D2 mixed dopamine (DA) receptor agonist apomorphine have been observed **[191]**. Moreover, the pro-yawning and pro-erectile effects of apomorphine and the pro-erectile effect of PD 168,077, a selective D4 receptor agonist, are more pronounced in outbred RLA rats than in their RHA counterparts and in Sprague Dawley (SD) rats **[192]**. It is noteworthy in this context that dopamine facilitates both the anticipatory (i.e., motivational and rewarding) and the consummatory (i.e., penile erection and copulatory performance) aspects of sexual behavior in experimental animals and in humans, by acting in the medial pre-optic area and hypothalamic nuclei **[156-163]**and in mesolimbic brain areas (i.e., ventral tegmental area and nucleus accumbens) **[164, 165, 167-169]**.

On the bases of the above premises, we hypothesized that RHA and RLA rats might show a different behavioral profile also when tested in a classical experimental paradigm aimed at characterizing copulatory behavior with a receptive (ovariectomized and oestrogen–progesterone-primed) female rat.

In order to verify this hypothesis, we performed:
a) an extensive **characterization of sexual behavior** of male RHA and RLA rats exposed to a sexually receptive female rat in a series of classic copulatory tests;
b) pharmacological assays with the DA agonist apomorphine and DA antagonist haloperidol, given alone or in combination, to evaluate the

differential effects of DA transmission on the copulatory performance of RHA and RLA rats;

c) intracerebral microdyalisis in the nucleus accumbens shell (AcbSh) during sexual behavior of sexually naïve and sexually experienced RHA and RLA rats to evaluate the release of DA in this brain area that is critically involved in the anticipatory and consummatory phases of sexual behavior.

3.2 Materials and methods

3.2.1 Animals

Outbred Roman high- (RHA) and low-avoidance (RLA) male rats (350–375 g at the beginning of the experiments) were from the colony established in 1998 at the University of Cagliari, Italy. Ovariectomized Sprague Dowley (SD) female rats (250–330 g at the beginning of the experiments) were obtained from Charles River (Como, Italy) for the behavioral and pharmacological assays, whereas for the microdialysis study they were obtained from Harlan Nossan (Correzzana, Italy).

Animals were housed 4 per cage (38 cm × 60 cm × 20 cm) and were acclimated to the housing facilities of the Department of Biomedical Sciences of the University of Cagliari for at least 10 days before the beginning of the experiments under controlled environmental conditions (24 °C, 60% humidity, reversed 12 h light/dark cycle with lights off from 08:00 to 20:00 h) and with free access to water and standard laboratory food. To limit the stress due to manipulation during the experiments, each animal was daily handled for approximately 1-2 min; in addition, contact with the animal house maintenance personnel was limited to a single attendant, and bedding in the home cages was not changed in the two days preceding the test. All the procedures were performed in accordance with the guidelines and protocols approved by the European Union (EU Council 86/609; D.L. 27.01. 1992, no.116) and by the Ethical Committee for Animal Care and Use of the University of Cagliari.

3.2.2 Experimental groups

***(a) Behavioral characterization**:* 15 male rats from each line were used (15 SD male rats were also used as a reference strain, but the results are not presented in this section, for the detailed study see **[193]**). All the animals, never exposed to a sexually receptive female, underwent five consecutive 60 min copulation tests at 3-day intervals.

***(b) Pharmacolgical assays**:* 45 male rats from each line were used (45 SD male rats were also used as a reference strain, but the results are not presented in this section, for the detailed study see **[194]**). All the animals, never exposed to a sexually receptive female, underwent five consecutive 45 min copulation tests at 3-day intervals.
Five days after these preliminary copulation tests, sexually experienced male rats that satisfied the criterion of at least one ejaculation reached in each of the last two tests were randomly divided into three groups of 12 animals for each line/strain, the first group was used to study the effect of apomorphine, the second the effect of haloperidol and the third the effect of haloperidol + apomorphine on copulatory behavior.

***(c) Intracerebral microdialysis study**:* 12 animals for each experimental group were used. The groups were divided into sexually naïve and sexually experienced for each line. Sexually naïve rats were adult male RHA and RLA rats never exposed to a sexually receptive female, whereas sexually experienced rats were previously exposed to five consecutive classical copulation tests at three-day intervals. Two days after these tests, sexually experienced male rats that satisfied the criterion of at least one ejaculation reached in each of the last two tests, and all the sexually naïve animals, were included in the microdialysis studies.

3.2.3 Copulatory behavior (studies a, b, c)

Briefly, male rats from each line/strain were moved during the dark phase of the cycle to a room lit by a dim red light where they were housed individually in a mating cage (45 cm × 30 cm × 24 cm) for 120 min. At the end of this habituation period a stimulus female rat (different for each

test) was introduced in the male's home cage. Estrous was induced in ovariectomized female rats by subcutaneous injections of estradiol benzoate (200 μg/rat in 0.2 ml of peanut oil) and progesterone (0.5 mg/rat in 0.2 ml of peanut oil) 48 and 6 h before the behavioral tests, respectively. In each test, the following measures of copulatory behavior were recorded:

- mount and intromission latency (ML and IL, the time elapsed since the introduction of the female into the experimental cage until the first mount and/or the first intromission, respectively);
- mount and intromission frequency (MF and IF, the number of mounts and intromissions in a series of copulatory activity (henceforth named series, with the first series beginning with the first mount/intromission and ending with the first mount/intromission after the first ejaculation, the second one beginning with the first mount/intromission after the first ejaculation and ending with the first mount/intromission after the second ejaculation, and so on), respectively;
- ejaculation latency (EL, timed from the first intromission until ejaculation in a series);
- ejaculation frequency (EF, the number of ejaculations in the 60 min of testing);
- post-ejaculatory interval (PEI, the time from ejaculation until the next intromission).

In addition, the following parameters were calculated: (1) copulatory efficacy (CE, number of intromissions recorded during a given series divided by the sum of mounts and intromissions in the same series) and (2) the inter-intromission interval (III, the ratio between the EL of a given series and the number of intromissions in that series) (see **[195]** and references therein). Unless otherwise stated, these parameters refer to the first series.

3.2.4 Drug administration (study b)

Apomorphine was dissolved in saline and administered subcutaneously (SC) in a volume of 0.2 ml/200g body weight, 10 min before the introduction of the receptive female in the mating cage. Haloperidol was dissolved with a drop of acetic acid diluted with distilled water at a pH=5.0 and administered intraperitoneally (IP) in a volume of 1ml/ 200g of body weight, 30 min before the introduction of the receptive female in the mating cage. When haloperidol was given in combination with apomorphine, the drug was administered 20 min before apomorphine. Controls received the same volume(s) of the appropriate vehicle(s).

3.2.5 Intracerebral microdialysis in the AcbSh during sexual behavior (study c)

The day before the microdialysis experiment, naïve or sexually experienced RHA and RLA rats were stereotaxically implanted (Stoelting Co., Wood Dale, IL, USA), under isoflurane anaesthesia (1.5–2%) (Harvard Apparatus, Holliston, MA, USA), with a microdialysis probe with a U-shaped dialysis membrane (approximately 2 mm of free surface for dialysis), prepared as previously described **[162]**, and aimed unilaterally at the AcbSh (coordinates: 1.8 mm anterior and 0.8 mm lateral to bregma, and 7.8 mm ventral to dura) **[196]**. The day of the experiment, during the dark phase of the cycle, the rats were transferred to a mating cage (45 cm × 30 cm × 24 cm) which contained another small Plexiglas cage (15 cm × 15 cm × 15 cm) with 25 holes (Ø 2 mm) in each vertical wall to allow for visual, olfactory and acoustic interaction. After a 2 h habituation period, the microdialysis probe was connected via polyethylene tubing to a CMA/100 microinfusion pump (Harvard Apparatus, Holliston, MA, USA) and perfused with Ringer's solution, containing 147 mM NaCl, 3 mM KCl and 1.2 mM CaCl2, pH 6.5, at a constant flow rate of 2.5 μL/min. After a 2 h equilibration period, dialysates were collected throughout the experiment every 15 min in aliquots of 37.5 μL for the determination of dopamine concentrations, as described in **Fig.1**.

Collection of dialysates every 15 min in aliquots of 37,5 µL

1) Habituation period (2h);

✓ collection of 4 dialysate aliquots to determine the basal concentration of dopamine in the AcbSh;

2) Introduction of the female (inacessible): in these conditions male rats that can see, hear and smell but not touch the female, show non contact erections (30 min);

✓ collection of 2 dialysate aliquots to determine the concentrations of dopamine in the AcbSh during the **anticipatory phase**, which reflects the **motivational aspects** of sexual behavior;

3) Sexual interaction and Copulation (75 min);

✓ collection of 5 dialysate aliquots to determine dopamine concentrations in the AcbSh during the **consummatory phase**, which reflects the **motivational and performing aspects** of sexual behavior.

4) Female removal (15 min)

✓An additonal dialysate aliquot was collected after removing the female from the mating cage.

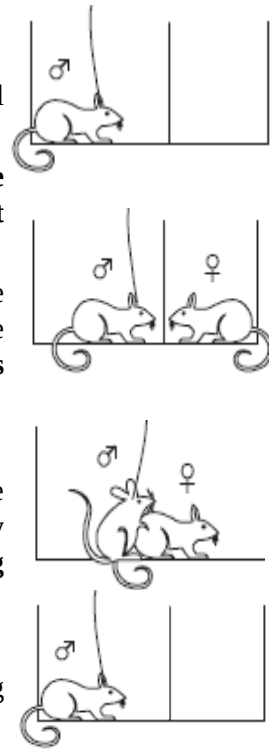

Fig.1 Intracerebral microdialysis during sexual behavior

All behavioral parameters of sexual activity were recorded throughout the experiment. In addition, non contact erections (pheromone-mediated penile erections that male rats show in the presence of an inaccessible sexually receptive female) were recorded.

Dopamine concentrations in 20 µL of dialysates were measured by high pressure liquid cromatography (HPLC) coupled to eletrochemical detection (for details see **[197]** .

3.2.6 Data analysis

(a) Behavioral characterization Line related differences in the frequency of mounts, intromissions and ejaculations along copulation tests 1 to 5 were evaluated with the chi-square (χ^2) test (not shown here). Differences

in behavioral patterns between lines during the first sexual experience (first series of the first copulation test) of naïve male rats were assessed by one-way ANOVA. An overall assessment of sexual behavior during the first series across tests 1 to 5 was performed by using the trapezoidal rule to calculate the AUCs in plots of each behavioral measure vs. test number for every rat. The average AUCs for each line/strain were compared by using one-way ANOVA. The comparison between the first series of copulation tests 1 to 5 was performed by repeated measures ANOVA.

(b) Pharmacolgical assays Differences between lines in the copulatory parameters of the first series of copulatory activity after treatment were analyzed for each treatment group (i.e., apomorphine, haloperidol and haloperidol + apomorphine) by two-way ANOVA with repeated measures over treatment (main effects: "line" and "treatment"). Differences in the control values of copulatory parameters in the first series of copulatory activity recorded from RLA and RHA rats treated with vehicle(s) (drug dose = 0) were analyzed by two-way ANOVA (between subjects factors: "control condition" and "line").

(c) Intracerebral microdialysis study Statistical analyses of biochemical (dopamine concentration in the AcbSh) and behavioral (non contact erections, ML, IL and EL and MF, IF and EF) data were conducted in each experimental subject. An overall analysis of the data obtained from each rat during the microdialysis experiments was first performed by calculating the AUCs of the plots of dopamine concentrations and the number of non contact erections, mounts, intromissions and ejaculations versus time and then comparing the obtained values by two-way ANOVA with the rat line and the sexual experience as between subjects factors. A more detailed analysis of each set of biochemical and behavioral data was then conducted by two way ANOVAs with the rat line or the sexual experience as between subject factors and time as within subject factor. An additional analysis was conducted on the values of the behavioral parameters obtained in the first series of copulatory activity (i.e., from the first mount/intromission to the first mount/intromission after the first

ejaculation) of naïve and sexually experienced male RHA and RLA rats during the microdialysis experiments. In this case, two way ANOVAs with the rat line and the sexual experience as between subjects factors were conducted on the following parameters: ML, IL, EL, MF, IF, EF, PEI, III and CE.

In all the studies when ANOVAs revealed statistically significant main effects and/or interactions, post hoc pair wise contrasts were performed with the Bonferroni's test.

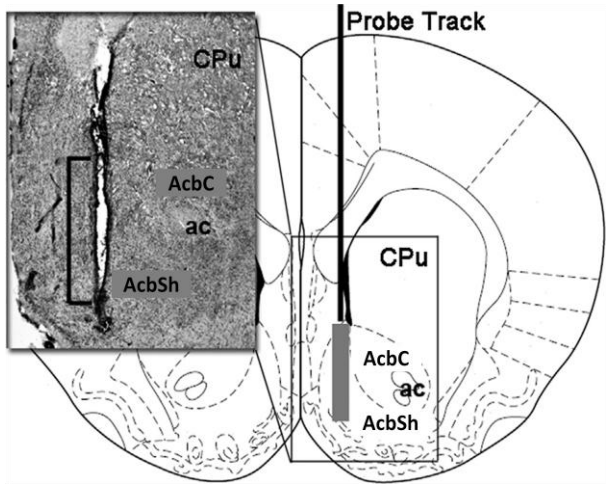

Fig. 2 Schematic representation of a coronal section of the rat brain showing the track of the microdialysis probe in the AcbSh. Insert: the square bracket in the microphotograph indicates the active portion of the dialyzing membrane. Abbreviations: ac=anterior commissure; Cpu = caudate-putamen; AcbSh = nucleus accumbens shell; AcbC = nucleus accumbens core.

3.3 Results

3.3.1 Behavioral characterization (a)

Percentage of rats engaged in sexual activity Male RHA rats engaged in sexual behavior with a receptive female much better than RLA rats since the first test, in which more than 80% of RHA rats showed mounts and intromissions and reached ejaculation in the first series as compared with only 40-50% of RLA rats. Although less marked, such differences between RHA and RLA rats were still present in the second and third copulation tests but were no longer statistically significant in the fourth and fifth tests. Additionally, while almost 100% of RHA rats reached ejaculation in the first series already in the second test, RLA rats improved their performance along the tests but did not achieve the levels of RHA rats, reaching a maximum of ~80% in the last two tests (results not shown here, see Sanna et al., 2014a).

Sexual behavior in naïve male rats One-way ANOVA analysis of the copulatory parameters measured in the first series of test 1, shows significant differences in the pattern of copulatory activity between the two Roman lines (**Fig. 3**). Thus, post hoc contrasts revealed significantly shorter ML and IL (both $P<0.05$) and higher MF ($p<0.05$) and IF ($p<0.001$) in RHA rats compared with their RLA counterparts. Conversely, no difference was found in the EL of the two lines. Considering that intromissions are preliminaries to reach ejaculation, the lack of differences in the EL combined with the higher IF shown by RHA rats explain why the III was longer in RHA vs RLA rats ($P<0.001$). Another important difference was the shorter PEI displayed by RHA compared with RLA rats ($P<0.05$).

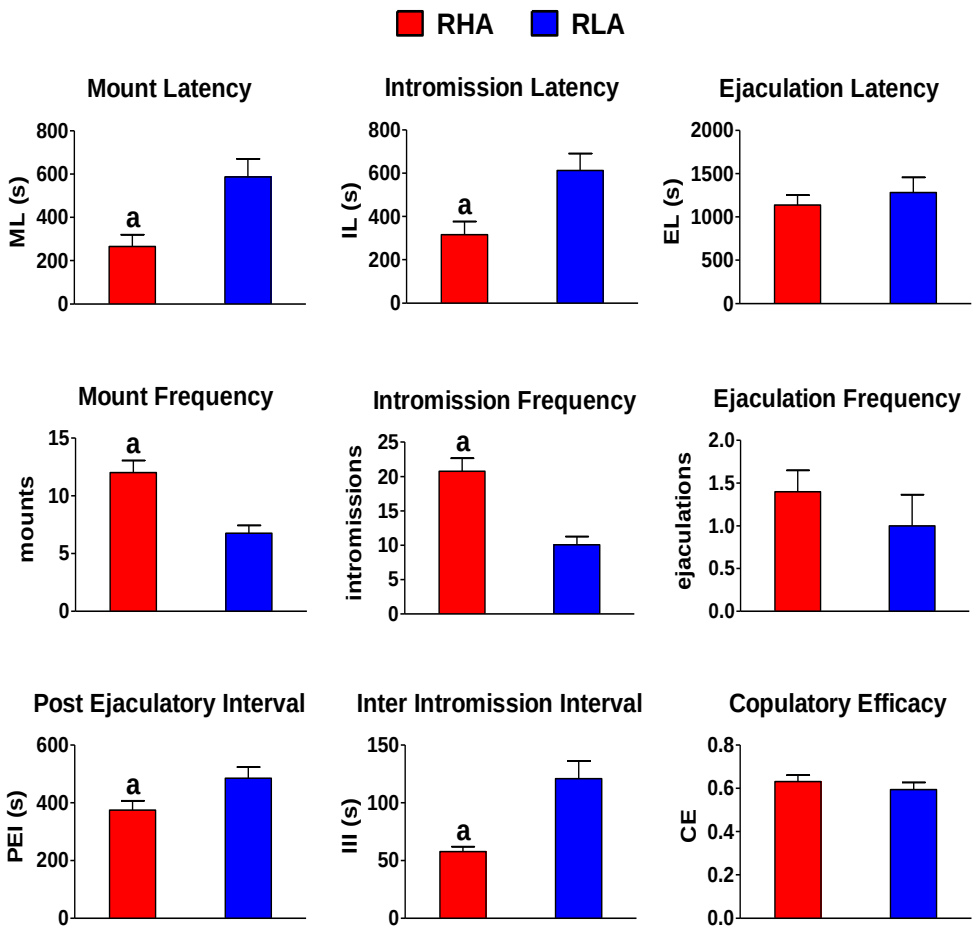

Fig. 3 Sexual behavior of male RHA and RLA rats in the first series of the copulation test 1** Columns and bars indicate the means ± SEM of 15 rats per line. a: P<0.05 vs RLA (one-way ANOVA followed by Bonferroni's post hoc contrast). Animals that did not mount/intromit/ejaculate within a given time were assigned full range values for latencies (900 s if the animal did not mount or intromit within 15 min after the presentation of the female; 1800 s if the animal did not reach ejaculation within 30 min from the first intromission and 600 s if the male did not intromit within 10 min after the last ejaculation). ****** except for ejaculation frequency that is calculated as the number of ejaculations recorded in the entire test 1.

Differential effects of sexual experience Assessment of the first series of each copulatory test indicates that the behavioral patterns of RHA and RLA rats changes differentially across tests 1 to 5. Thus, compared with test 1, such changes were more robust in test 2, some still occurred in test 3, and very minor or no changes were found in tests 4 and 5, suggesting that a stable copulatory pattern was reached after three consecutive tests: in both RHA and RLA rats there was a tendency towards a reduction of the latencies (ML, IL and EL) and the frequency of mounts (that can be considered as failed intromissions), whereas EF increased, PEI and III decreased, and IF remained unchanged, leading to an increment of the CE. Despite the above mentioned common trend, one-way ANOVA analysis of the AUCs of the curves shown in **Fig. 4** revealed significant line-related differences for the first series of copulatory parameters along the five tests. These results were further confirmed by two-way ANOVA for repeated measures. Moreover, post hoc contrasts showed that the ML of RLA rats was persistently longer than that of RHA rats in all five copulation tests ($P<0.05$). Notably, ML is the only parameter in this study that can be considered as an index of sexual motivation (i.e., the longer the ML, the lower the motivation).

In summary, male RHA rats displayed a more robust sexual motivation and a better copulatory performance than RLA rats, in keeping with their different behavioral profile and proactive coping style. These differences were more pronounced during the first exposure to a sexually receptive female but persisted, albeit attenuated, after repeated copulation tests.

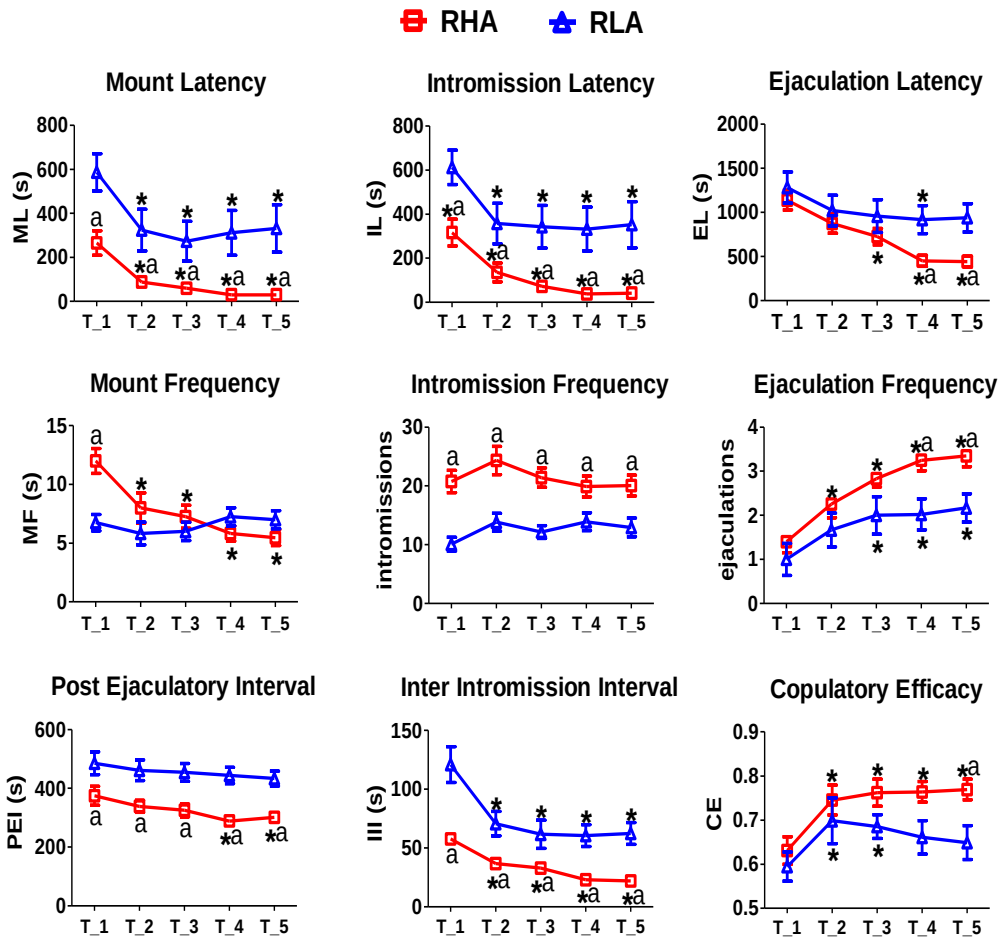

Fig. 4 Sexual behavior of male RHA and RLA rats in the first series of copulation test 1, 2, 3, 4, 5** . Shown are the means±SEM of 15 rats per line. a: P<0.05 vs RLA; *: P <0.05 vs the within line value in copulation test 1 (two-way ANOVA for repeated measures followed by Bonferroni's post hoc contrasts). ** except for ejaculation frequency that is calculated as the number of ejaculations recorded in each entire copulation test.

3.3.2 (b) Pharmacological assays (apomorphine and haloperidol)

Effects of apomorphine on copulatory behavior of sexually experienced RLA and RHA rats **Fig. 5** shows that apomorphine (0.02 and 0.08 mg/kg s.c.) facilitated copulatory behavior of both RLA and RHA rats in a dose-dependent manner, as indicated by the decrease of the EL and the increase of the EF, as compared with the respective vehicle-treated groups. Importantly, the threshold apomorphine dose was four-fold higher for RHA rats (0.08 mg/kg) than RLA rats (0.02 mg/kg).

Effects of haloperidol on copulatory behavior of sexually experienced RLA and RHA rats As shown in **Fig. 6**, haloperidol (0.1 and 0.2 mg/kg i.p.) had only minor effects on sexually experienced RHA rats, but impaired copulatory behavior of sexually experienced RLA rats. Thus, in RLA rats, the dose of 0.2 mg/kg significantly increased ML, IL, EL, III and PEI values compared with the respective vehicle-treated group (all P values<0.05). Conversely, no change was observed in the copulatory parameters of RHA rats treated with either 0.1 mg/kg or 0.2 mg/kg, except for the IF and CE values, which decreased in a dose-dependent manner compared to the control condition (haloperidol = 0), as observed also in RLA rats.

Effects of haloperidol + apomorphine on copulatory behavior of sexually experienced RLA and RHA rats As shown in **Fig. 7**, haloperidol (0.1 and 0.2 mg/kg IP) given 20 min before apomorphine (0.08 mg/kg SC), differentially antagonized in a dose-dependent manner the facilitatory effect of apomorphine on copulatory behavior in sexually experienced rats of both lines. The effect of haloperidol was particularly evident in RLA rats, in which it abolished all the changes induced by apomorphine already at the dose of 0.1 mg/kg, and, at the dose of 0.2 mg/kg, it induced changes that were similar to those observed when it was given alone. In contrast, in apomorphine-treated RHA rats, the effect of haloperidol was very small, albeit significant, (increased MF, decreased CE and longer PEI compared to vehicle + apomorphine-treated rats).

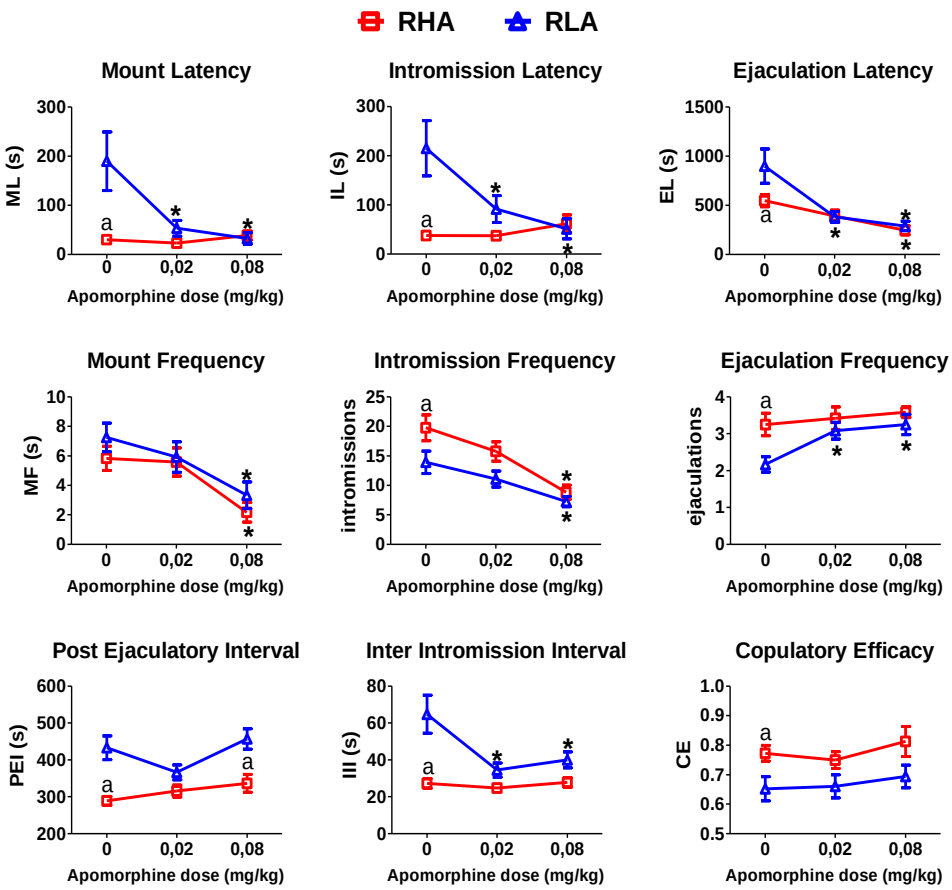

Fig. 5 Effects of apomorphine on copulatory behavior of sexually experienced RHA and RLA rats Shown are the means ± SEM of 12 rats per group. a: P<0.05 vs RLA; *****: P<0.05 vs the respective vehicle treated-group (apomorphine = 0)

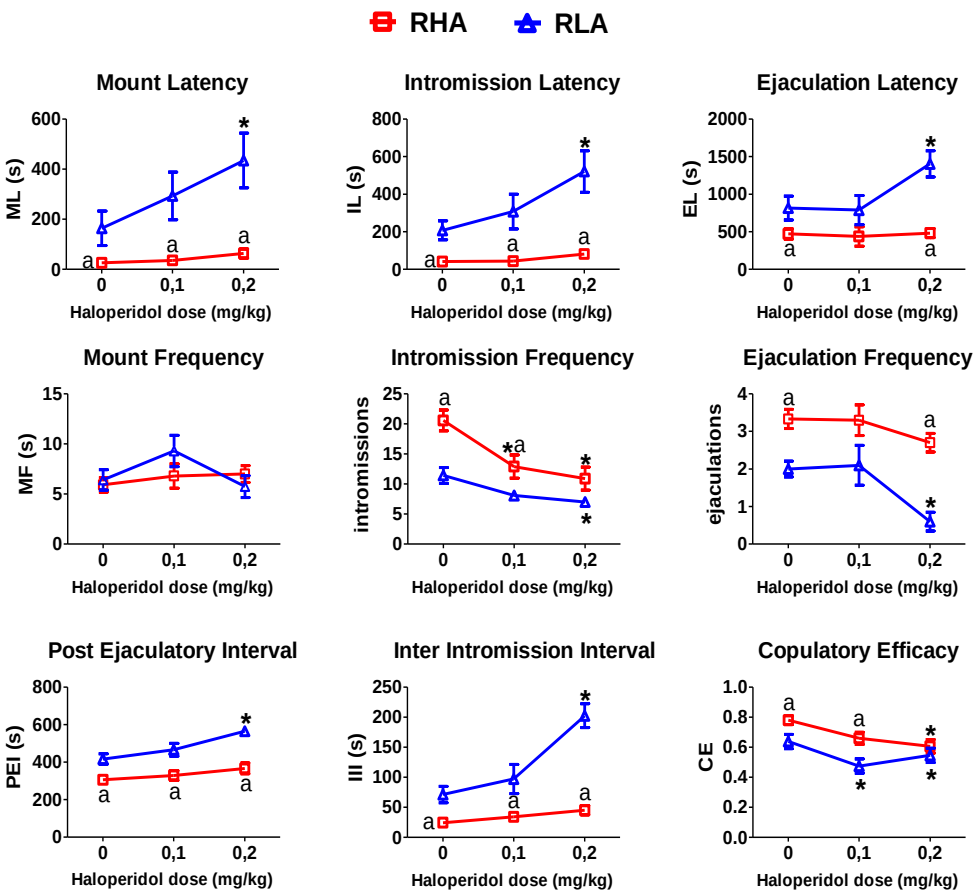

Fig. 6 Effects of haloperidol on copulatory behavior of sexually experienced RHA and RLA rats Shown are the means ± SEM of 12 rats per group. a: P<0.05 vs RLA; *****: P<0.05 vs the corresponding group treated with vehicle alone (haloperidol = 0).

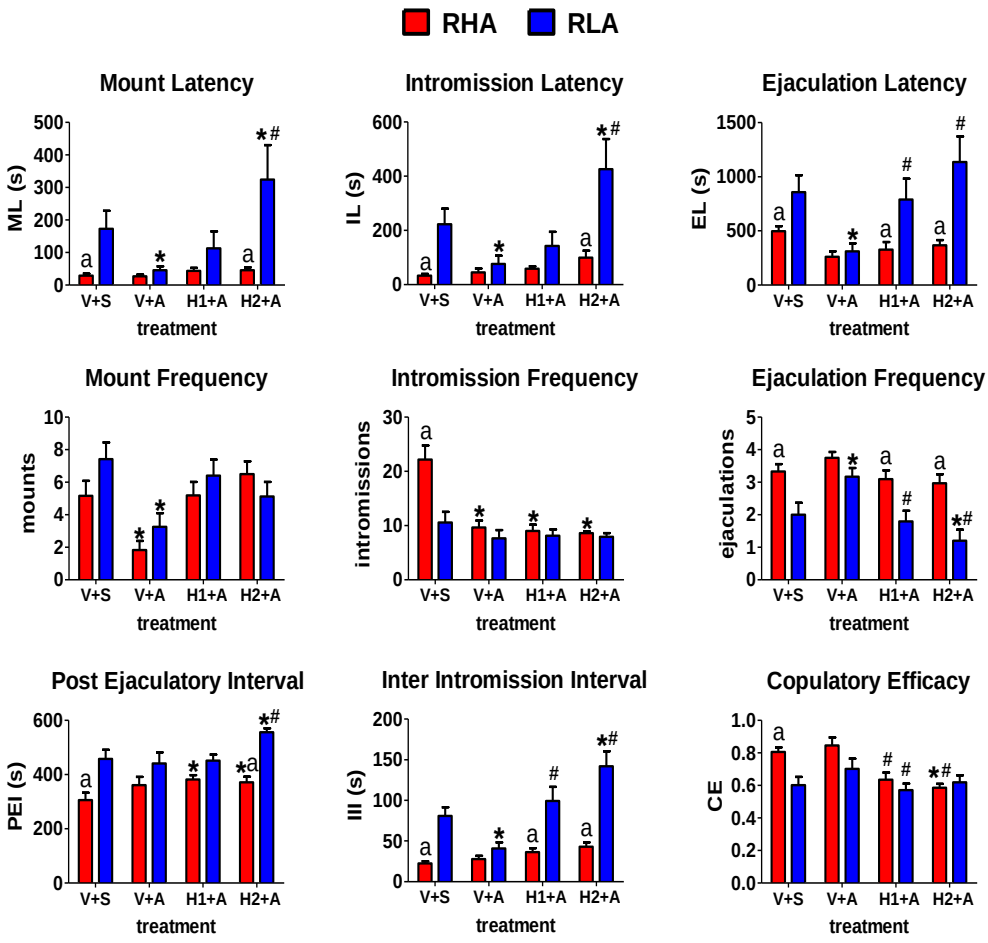

Fig. 7 Effects of haloperidol on apomorphine-induced changes in copulatory behavior of sexually experienced RHA and RLA rats Columns and bars indicate the means ± SEM of 12 rats per group. V: vehicle V+S: vehicle + saline; A: Apomorphine (0.08 mg/kg, s.c.); H1: haloperidol 0.1 mg/kg, i.p.; H2: haloperidol 0.2 mg/kg, i.p. Haloperidol (H1 or H2) or vehicle alone (V) were given 20 min before apomorphine. a: $P<0.05$ vs RLA; *: $P<0.05$ vs the line-matched vehicle-treated (V+S) group; #: $P<0.05$ (H1+A) or (H2+A) vs (V+A).

Both apomorphine and haloperidol were more effective in sexually experienced RLA than RHA rats, suggesting a greater responsiveness of RLA rats to both the stimulation and the blockade of dopamine receptors.

3.3.3 Intracerebral microdialysis studies (c)

Dopamine concentrations in the AcbSh of sexually naïve RHA and RLA rats (**Fig. 8** upper panel) Two main findings emerged from the brain microdialysis experiments in sexually naïve Roman rats. Firstly, the basal concentrations of extracellular DA of RHA and RLA rats were statistically indistinguishable. Secondly, the overall DA content during the anticipatory and consummatory phases of sexual activity was significantly higher in naïve RHA than RLA rats ($P<0.001$ and $P<0.05$, respectively). Interestingly, in RHA rats, the extracellular DA concentrations already started to increase upon the introduction of the inaccessible female (47.5% above basal values, $P<0.01$ in the second 15 min fraction), whereas in RLA rats the first significant increase in extracellular DA concentrations (51.3% above the basal values) occurred only during copulation. These results are consistent with a more robust motivation of RHA rats compared with their RLA counterparts.

Dopamine concentrations in the AcbSh of sexually experienced RHA and RLA rats (**Fig. 8** bottom panel) Increases in extracellular DA also occurred in sexually experienced RHA and RLA rats with significant line-related differences in the time course. Notably, the basal DA concentrations were statistically indistinguishable both across animal line and sexual experience, suggesting that sexual experience *per se* does not alter the basal dopamine output in the AcbSh. Most important, in sexually experienced RLA rats, the overall dopamine content was significantly larger during copulation than in their naïve counterparts ($P<0.05$), whereas no such difference was observed between sexually experienced and naïve RHA rats. In RHA rats, however, sexual experience is associated with an earlier increment in DA output upon the introduction of the inaccessible female as compared with the sexually naïve group (57.2% after the first 15 min), and also an anticipation of the increase in DA output during copulation, as reflected by the peak value at 30 min of copulation (147.4% above basal values, compared with 184.2% recorded after 60 min of copulation in the sexually naïve RHA group).

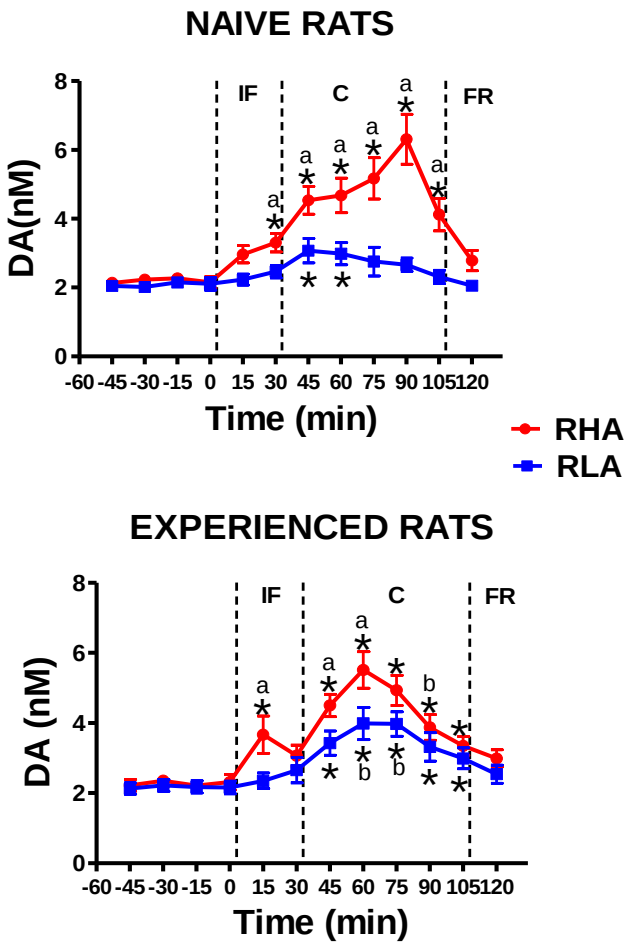

Fig. 8 Dopamine concentrations in the AcbSh dialysates from sexually naïve (upper panel) and sexually experienced (bottom panel) RHA and RLA rats during sexual activity Shown are the means ± SEM of 12 (naïve) or 10 (experienced) rats per group. *: P<0.05 vs the basal values of the same line; a: P<0.05 vs the corresponding value of the RLA group; b: P<0.05 vs the time-matched value of the line-matched sexually naïve group. IF: inacessible female; C: copulation; FR: female removal.

Effect of sexual experience on the copulatory patterns of RHA and RLA rats (**Fig. 9**) The differences observed in dopamine output in both sexually naïve and experienced rats occurred concomitantly with changes in several sexual parameters recorded during the two main phases of the experiment: female inaccessible and copulation. Thus, statiscal analysis revealed that both sexually naïve and experienced RHA rats displayed more frequent non contact erections, mounts, intromissions and ejaculations than their respective naïve and experienced RLA rats. In keeping with the results regarding the accumbal dopamine output of RHA rats, sexual experience caused changes in the time course rather than in the frequency of the recorded behavioral measures. On the other hand, in RLA rats, sexual experience led to a general improvement of the performing aspects and, less markedly, of the appetitive aspects of sexual activity.

Thus, RHA and RLA rats, either when exposed to an inaccessible female or during copulation, show different patterns of sexual activity associated with differential changes in the concentrations of extracellular dopamine in the AcbSh. Although more pronounced in sexually naïve rats, the differences in mesolimbic dopamine release persist after acquisition of a stable baseline of sexual activity.

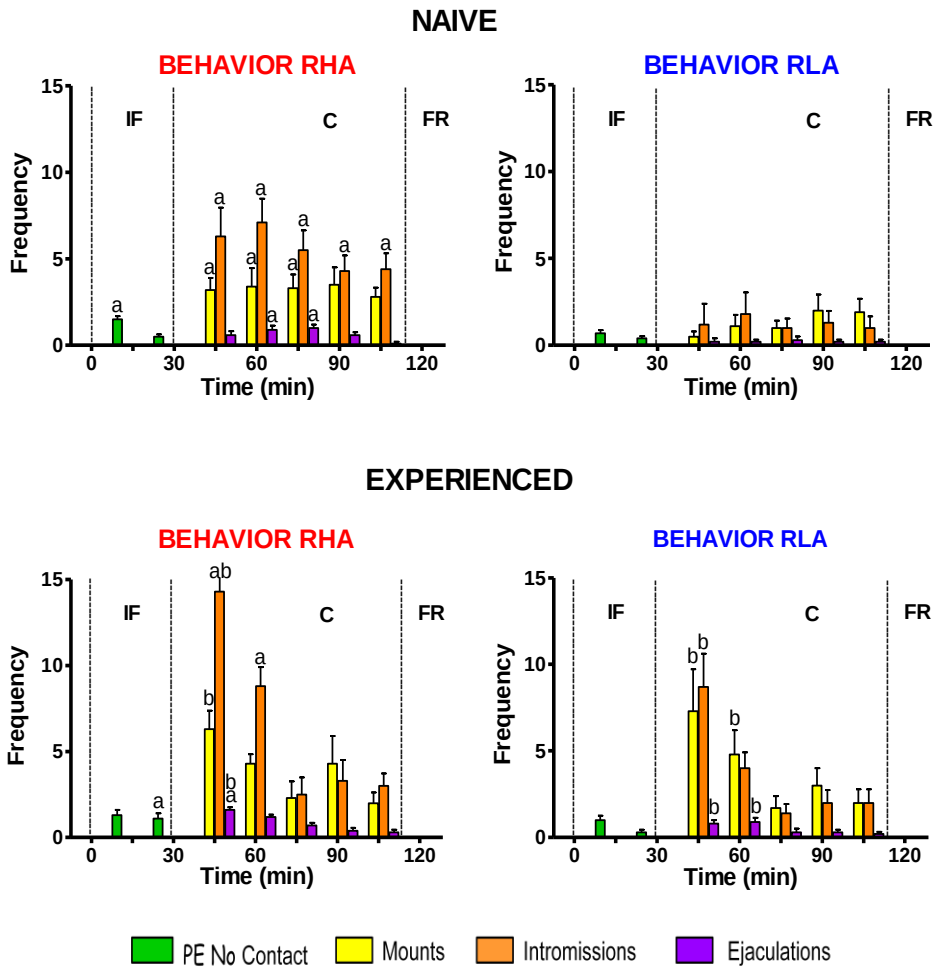

Fig. 9 Behavioral patterns of sexually naïve (upper panel) and sexually experienced (bottom panel) RHA (left side) and RLA (right side) rats during sexual activity Columns and bars indicate the means ± SEM of 12 (naïve) or 10 (experienced) rats per group. a: P<0.05 vs the corresponding values of the RLA group; b: P<0.05 vs the time-matched values of the sexually naïve rats of the same line. IF: inacessible female; C: copulation; FR: female removal.

3.4 Discussion

In **study a**, we performed the first systematic characterization of the sexual behavior of male RHA and RLA rats (compared, in the study by Sanna et al., with SD rats used as a genetically heterogeneous reference strain). The main finding emerging from this study is that RHA rats, which are impulsive, novelty seekers and proactive copers, show higher levels of sexual motivation and a better copulatory performance than their RLA counterparts, which are hyperemotional, anxious and reactive copers. These line-related differences are more marked when the animals are exposed to a receptive female for the first time and tend to decrease with repeated copulation tests. A plausible explanation for the attenuation of such differences is that RLA rats may engage more readily in sexual activity when the effect of novelty is overcome by repeated sexual experience. Nonetheless, RLA rats never reach the levels of their RHA counterparts, either in terms of percentage of animals engaging in sexual activity or in terms of individual behavioral parameters. Moreover, some differences (e.g., in copulatory efficacy) remain significant until the last test. This finding suggests that, beyond the inhibitory effect of novelty, which can be considered as a stressor, additional factors may play a relevant role in the behavioral differences between the Roman lines.

Concerning the appetitive aspects of sexual behavior, in **study a** the only parameter that can be considered as an index of sexual motivation (with a negative correlation) is the mount latency ML. Thus, the longer latencies shown by RLA vs RHA rats in all five copulation tests indicate a lower level of sexual motivation, which was subsequently confirmed using more selective behavioral measures in **study c**.

The behavioral differences in sexual motivation and performance between RHA and RLA rats were confirmed in **Study b,** which showed for the first time that the copulatory pattern of these two lines is differentially facilitated by apomorphine and impaired by haloperidol, in keeping with the well established facilitatory effect of central DA on sexual behavior **[159-162, 164, 168, 169]**. Thus, in RHA rats, apomorphine induced

changes in only a few copulatory parameters and usually at the highest dose tested, whereas low doses of apomorphine were sufficient to induce a general enhancement of the copulatory performance and sexual motivation of RLA rats, so that their sexual behavior approached that of RHA rats. Accordingly, in RHA rats, both doses of haloperidol induced only minor changes in most copulatory parameters, whereas in RLA rats this treatment further impaired the copulatory performance and sexual motivation when given alone, and completely abolished the facilitatory effect of apomorphine when given in combination. Thus, both apomorphine and haloperidol were more effective in sexually experienced RLA than RHA rats, suggesting that RLA rats are more responsive to both the stimulation and the blockade of dopamine receptors than RHA rats. Taken together, the above findings suggest that the Roman lines differ in the functional tone of their mesolimbic dopaminergic neurotransmission, in particular that involving D2-like receptors.

The possible involvement of differences in D2-like receptor-mediated neurotransmission in the distinct copulatory patterns and in the differential effects of apomorphine and haloperidol in RHA and RLA rats is supported by two recent findings: (i) the D2-like receptor-mediated pro-erectile effect of apomorphine and of the D4 receptor agonist PD 168,077 is more pronounced in RLA than RHA and SD rats **[192]**; (ii) a lower D2 (auto)receptor availability (measured by dopamine receptor binding and mRNA assays) has been found in the substantia nigra/ventral tegmental area, caudate putamen and nucleus accumbens of RHA vs. RLA rats **[185]**. Considering the inhibitory effect on DA release played by D2 autoreceptors, their lower availability in mesolimbic areas of RHA rats may result in a more robust mesolimbic dopaminergic tone as compared with their RLA counterparts, which may explain, at least in part, many behavioral differences between the two lines, including those in sexual behavior.

Therefore, on the bases of the above mentioned studies and of previous reports showing a greater increase in the extracellular concentrations of DA in the AcbSh of RHA vs RLA rats upon the administration of

psychostimulants **[87, 180, 187]**, **study c** was designed to evaluate the DA output in dialysates from AcbSh of sexually naïve and sexually experienced RHA and RLA rats during the anticipatory and consummatory phases of sexual behavior.

As expected, the results of **study c** demonstrate that the distinct patterns of sexual behavior of RHA and RLA rats are associated with differential changes in the concentrations of extracellular dopamine in the AcbSh, with RHA rats showing a greater increase in DA output compared with their RLA counterparts. Although more evident in sexually naïve rats, these differences in mesolimbic dopaminergic activity persist after acquisition of a stable baseline level of sexual activity. Interestingly, as observed for behavioral patterns, sexual experience has differential effects in the two lines. Thus, in RLA rats, sexual experience determines a significant increment in the overall DA content compared with the naïve condition, whereas, in the RHA line, the accumbal DA output increases earlier in sexually experienced than in sexually naïve rats. Moreover, while in **study a** we used a classical paradigm of copulation test in which the female was always accessible to the male and the only parameter used to assess the sexual motivation was the mount latency, in **study c** we introduced an anticipatory phase in which the female was inaccessible in order to better investigate the appetitive aspects of sexual activity. Thus, the larger number of non contact erections displayed by RHA vs RLA rats in the anticipatory phase associated with time-matched increments of accumbal DA output, confirm the higher levels of sexual motivation of RHA rats and the role of DA in the appetitive aspects of sexual behavior. In this regard, considerable experimental evidence indicates that in the mesolimbic brain areas, which include the VTA and the Acb, dopamine plays a key role in the control of the appetitive aspects of sexual activity (i.e., sexual arousal and motivation) in laboratory animals and in humans **[164, 165, 167-169]**. However, other brain areas are also involved in the dopaminergic control of sexual behavior, such as the medial preoptic area and paraventricular nucleus of the hypothalamus in which dopamine not only drives the consummatory aspects of sexual behavior such as penile erection, copulation and ejaculation (i.e., sexual performance), but also

participates in the control of sexual motivation via interaction with D2-like receptors **[156, 158, 159, 166, 168, 169, 198]**. Whether line-related differences like those described above also occur in the dopaminergic neurons innervating the medial preoptic area and the paraventricular nucleus of the hypothalamus remains unknown and warrants further investigation.

4. Study III: Differential effects of social isolation on inbred Roman high-(RHA-I) and low-(RLA-I) avoidance rats

4.1 Introduction

Clinical and preclinical studies demonstrate that the exposure to adverse experiences in early-life deeply affects brain development and may contribute to the occurrence of psychiatric disorders, such as depression, schizophrenia, and substance abuse in genetically predisposed individuals **[170-172]**. 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 **[199]**.

Post-weaning social isolation of rodents is an experimental paradigm that models some of the behavioral consequences of adverse early-life experiences in humans **[170-172, 200]**. Several reports indicate that rearing rodents in persistent social isolation from weaning produces long-lasting behavioral alterations, such as excessive reactivity to handling, aggressive and anxious behaviors, hyperactivity, neophobia, impaired sensorimotor gating and cognitive rigidity (**[172]** and references therein). The behavioral repertoire displayed by isolated rats has been defined as "isolation-induced stress syndrome" **[201, 202]** and is accompanied by neurochemical and morphological alterations, such as reduced prefrontal cortical volume, decreased cortical and hippocampal synaptic plasticity, hyperfunction of mesolimbic dopaminergic systems, and other dysfunctions in the monoaminergic systems **[172]**. Collectively, these abnormalities resemble core features of schizophrenia and provide translational relevance to the social isolation model. Hence, the use of psychogenetically selected lines/strains of rats with phenotypic traits resembling cardinal symptoms of schizophrenia, may be a valid experimental approach to study the complex interaction between genetic and environmental factors (such as adverse early-life experiences) in the development of the schizophrenic disorder.

The Roman high avoidance rats, either outbred (RHA) or inbred (RHA-I) represent one such model, inasmuch as they show: (i) enhanced impulsive

behaviors **[99, 100, 125]**, (ii) relative deficits in latent inhibition threshold [126], as well as in PPI and spatial working and reference memory **[127, 128]**, (iii) enhanced locomotion and mesolimbic dopaminergic sensitization in response to the repeated administration of psychostimulants **[115, 120, 121]**, (iv) augmented mesocortical dopaminergic response to stress **[21]**, and (v) increased stereotypic response to the dopamine agonist apomorphine **[191, 192]**. Conversely, RLA/RLA-I rats display higher levels of anxiety/ fearfulness **[86, 88, 105, 203-206]**, no sensitization upon chronic administration of psychostimulants **[115, 120, 121]**, a normal PPI and better performances in learning/memory tasks **[127, 128]**.

Therefore, the aim of this study was to evaluate the impact of post-weaning social isolation on anxiety-related behaviors in the Elevated Zero Maze and on locomotor activity in a new environment (motility cages) in the Roman inbred strains.

4.2 Materials and methods

4.2.1 Animals

A total number of 76 male inbred rats (36 RHA-I and 40 RLA-I) from the colony of the Autonomous University of Barcelona were used. At postnatal day 20-22 the animals were randomly housed in pairs (12 RHA-I rats and 16 RLA-I rats) or isolated in individual cages (24 rats of each strain). All the rats were maintained in the same room, with a 12 h light-dark cycle (lights on at 08:00 a.m.), in a temperature (22 ± 2°C) and humidity (50–70%) controlled environment. Standard laboratory rat chow and water were available *ad libitum*. The Elevated Zero Maze experiments were performed at 16 weeks of isolation, whereas the Locomotor Activity was assessed two weeks later (18 weeks of isolation). All the experimental procedures were performed in the housing facilities

of the Department of Psychiatry and Forensic Medicine of the Autonomous University of Barcelona and were approved by the committee of Ethics of the Autonomous University of Barcelona in accordance with the European Community Council Directive (86/609/EEC) regarding the care and use of animals for experimental procedures.

4.2.2 Elevated Zero Maze

The Elevated Zero Maze (EZM) is a circular corridor elevated above the floor and provided with two open quadrants and two closed quadrants. It is a variation of the elevated plus maze (EPM), which is a cross-shaped maze with two open arms and two closed arms. Both mazes are used to assess anxiety-like behavior in small laboratory animals (rats/mice), since they are based on two conflicting innate tendencies of the animal: exploring a novel environment and avoiding elevated and open spaces (that constitute situations of predator risk). When anxious, the animal tends to prefer enclosed dark spaces, which represent the security, to open brightly lit spaces. In this context, anxiety related behavior is measured by the degree to which the animal avoids the open spaces. The advantage of the EZM versus the EPM is the lack of an ambiguous central area.
The EZM used for this study was made of black plywood (105 cm diameter and 10 cm width) and was placed 65 cm above the ground level. The closed quadrants were delimited by 40 cm high walls. The test started by introducing the animal in a closed quadrant of the maze with the snout oriented towards the walls and lasted 5 min.

4.2.3 Behavioral measurements

The following behaviors were quantified by a single well-trained observer:

- latency to enter in an open space (the time elapsed between the introduction of the animal in the maze and the first entry in an open quadrant);

- time spent in open space (the sum of each time spent in open quadrants);
- entries in open space (the number of entries in the open quadrants);
- head dipping (i.e., when the animal sticks out the maze to look down and the observer cannot see its nose and eyes; it can be considered as a measure of exploration and risk-propensity);
- SAP (Stretched Attend Posture: a forward elongation of the body with a subsequent coming back; it reflects an approach-avoiding conflict);
- crossings (each time in which the animal crosses the virtual lines that divide the circle in 4 quadrants and 8 hemiquadrants).

The trials were videotaped and the behavioral measures were recorded outside the test room in order to avoid any interference with the performance of the animals. The videotaped behavioral activity was assessed at a later time.

4.2.4 Locomotor activity

In each session, three animals were tested simultaneously using one rat for each experimental condition and changing the activity cage assigned to each experimental group according to a counterbalanced schedule. The test started by placing the animal in the center of a plexiglass activity cage (40x40x40) equipped with photocell beams and interfaced by a computer software (PanLab) that recorded the horizontal locomotor activity, measured as photocell beam breaks, and divided the 30 minutes of the test in 6 intervals of 5 minutes.

4.2.5 Data analysis

Elevated Zero Maze For each behavioral parameter, two-way ANOVA with strain (RHA-I and RLA-I) and treatment (isolation or control) as between-groups factors was performed. Whenever appropriate (i.e., P for the main factors and/or their interaction <0,05), ANOVA was followed by

post-hoc pair wise contrasts with the Student's t-test. Further analysis with one-way ANOVAs for each strain was used to compare the two groups for some of the behavioral parameters.

Locomotor activity The time course of the locomotor activity was evaluated by repeated measures ANOVA with the time intervals (6 intervals of 5 minutes each) as a within-groups factor and strain and treatment as between-groups factors. Further analyses with repeated measures ANOVA for each strain and ANOVAs for each interval were performed. One-way ANOVAs for each strain was used to evaluate the effect of treatment on the total distance travelled.

4.3 Results

4.3.1 Elevated Zero Maze

Latency to enter in open space (**Fig. 1**) The latency to enter in the open space of the control RHA-I rats was significantly shorter than that of their RLA-I counterparts. Two-way ANOVA with strain and treatment as between-group factors revealed a significant “strain” x “treatment” interaction ($F(1,72) = 4.00$; $P = 0.049$) but post-hoc contrasts did not reveal significant differences due to the treatment. However, analyzing both strains separately with ANOVAs, we found a significant effect of isolation in RHA-I rats ($F(1,34) = 5.05$; $P<0.031$) indicating that isolation-reared RHA-I rats delayed their entry to the open spaces of the maze. Conversely, no differences between treatments were found in RLA-I rats.

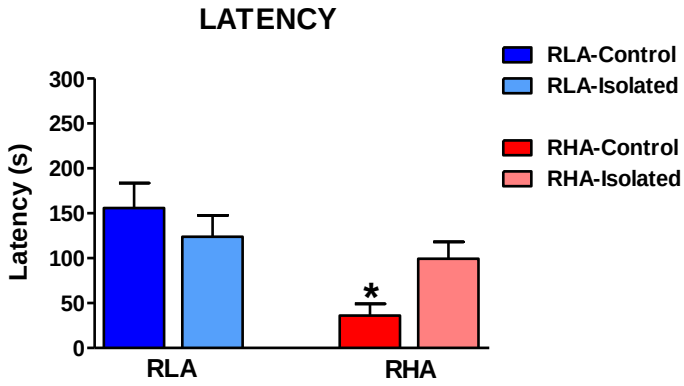

Fig.1 Latency to enter in open space Columns and bars indicate the means ± SEM of 48 isolated (24 per line) and 28 group-housed (16 RLA; 12 RHA) rats. *: P<0.05 vs RLA-control.

Time spent in open space (**Fig. 2**) Control RHA-I rats spent significantly more time in the open space compared with RLA-I controls. However, after prolonged social isolation, the time spent in open space by RHA rats was significantly reduced (P<0.001) becoming similar to that of RLA rats. On the contrary, no significant differences were found between isolated and group-housed RLA-I rats.

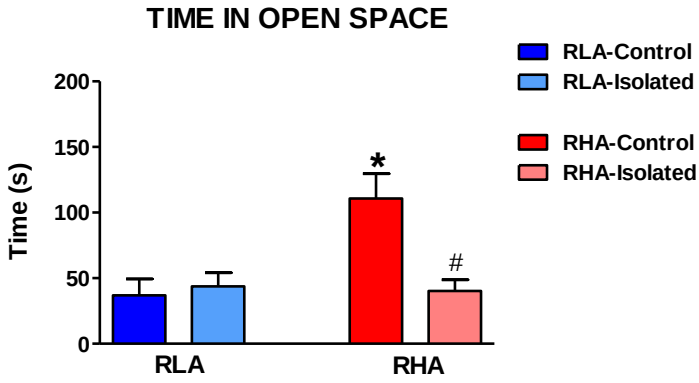

Fig.2 Time spent in open space Columns and bars indicate the means ± SEM of 48 isolated (24 per line) and 28 group-housed (16 RLA; 12 RHA) rats. *: P<0.05 vs RLA-control; #: P<0.001 vs RHA-control.

Entries in open space (**Fig 3**) The number of entries in open space was significantly larger in RHA-I vs RLA-I group-housed rats. Analyzing both strains separately, a significant reduction in the number of entries in open space was observed in RHA-I isolated rats ($F(1,34) = 4.17$; $P = 0.05$), whereas isolation did not affect the entries in open space of RLA-I isolated rats .

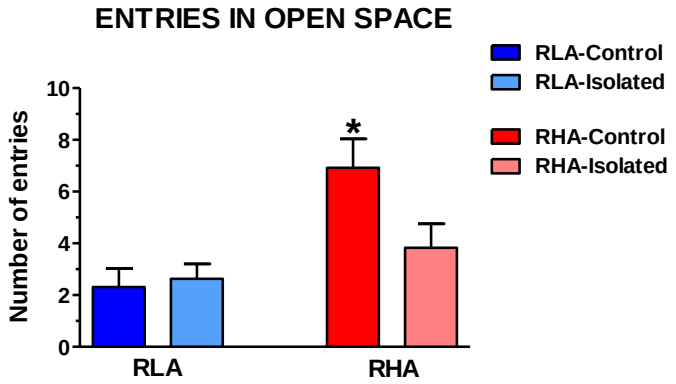

Fig.3 Entries in open space Columns and bars indicate the means ± SEM of 48 isolated (24 per line) and 28 group-housed (16 RLA; 12 RHA) rats. *: P<0.05 vs RLA-control.

Head dipping (**Fig 4**) In keeping with their high levels of impulsivity, the frequency of head dips (considered as an index of exploration and risk-propensity) displayed by RHA-I controls was significantly higher than that of their RLA-I counterparts. Moreover, isolation significantly reduced the number of head dips only in RHA-I rats.

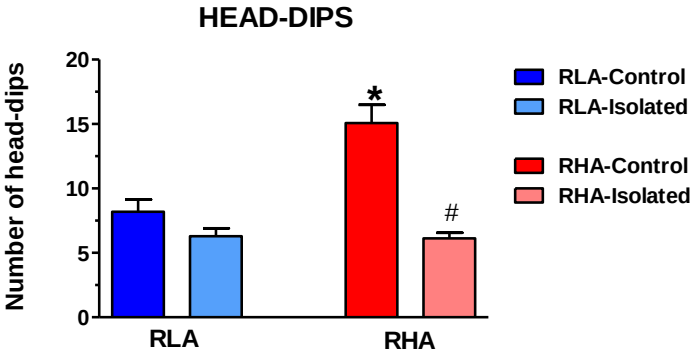

Fig. 4 Head dipping Columns and bars indicate the means ± SEM of 48 isolated (24 per line) and 28 group-housed (16 RLA; 12 RHA) rats. *: P<0.05 vs RLA-control; #: P<0.001 vs RHA-control.

SAPs (**Fig 5**) No strain- or treatment-dependent effects were detected in the frequency of SAPs, probably because of the high variability of the experimental groups.

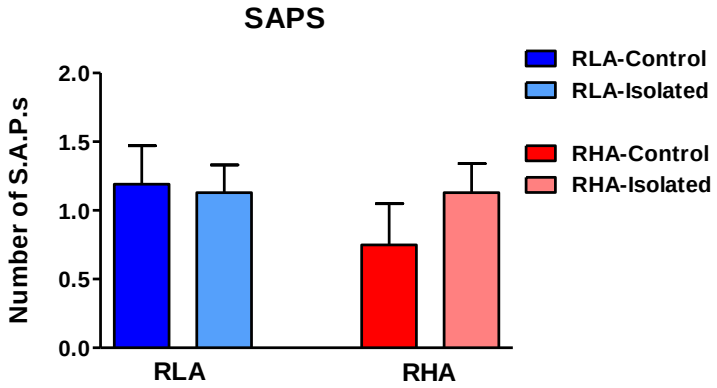

Fig.5 Stretched Attend Postures Columns and bars indicate the means ± SEM of 48 isolated (24 per line) and 28 group-housed (16 RLA; 12 RHA) rats.

Crossings (**Fig 6**) Statistical evaluation of the frequency of line crossings by means of two-way ANOVA revealed a significant "strain" effect ($F(1, 72) = 10.27$; $P = 0.002$) and post hoc contrasts showed that the frequency of line crossings was significantly higher in RHA-I controls than in their RLA-I counterparts. However, no significant effect of isolation was revealed by independent ANOVAs for either strain.

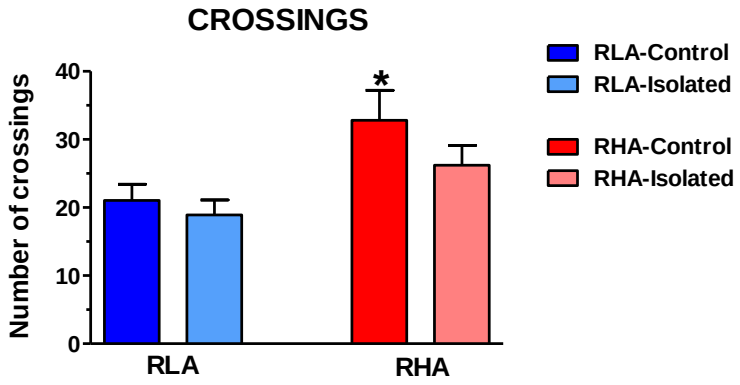

Fig.6 Crossings Columns and bars indicate the means ± SEM of 48 isolated (24 per line) and 28 group-housed (16 RLA; 12 RHA) rats. *: $P<0.05$ vs RLA-control.

4.3.2 Locomotor Activity

Repeated measures ANOVA, with strain and treatment as between-groups factors and six time intervals (of 5 minutes each) as within-groups factor, revealed a significant "strain" x "interval" interaction ($F(5,360) = 2.73$ $P = 0.020$) (**Fig.7**). Both main effects were also significant ($F(1, 72) = 7.21$ $P = 0.009$ for strain and $F(1, 72) = 4.58$ $p = 0.036$ for treatment) but not the "strain" x "treatment" interaction. Independent repeated measures ANOVAs for each strain revealed a significant effect of the isolation in RHA-I rats ($F(1, 32) = 8.89$ $P = 0.005$), indicating that RHA-I rats reared in isolation travelled longer distances during the 30-min session (**Fig. 8**). Moreover, independent ANOVAs for each interval in RHA-I rats showed significant differences in 4 out of 6 intervals (1^{st}, 3^{rd}, 5^{th} and 6^{th}), indicating an increment of the locomotor activity in isolated vs control RHA-I rats ($Fs(1, 34) \geq 4.34$ $p \leq 0.045$) (**Fig.7**).

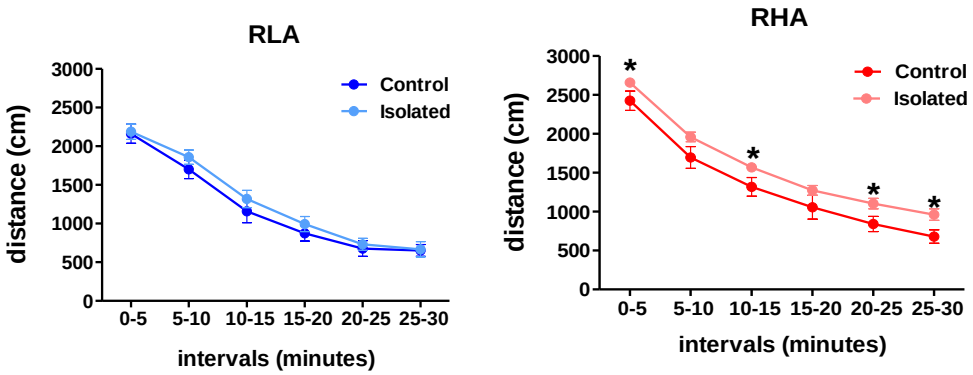

Fig. 7 Time course of locomotor activity. Shown are the means ± S.E.M. of the distances travelled in each 5-minutes interval by RLA-I (left-hand panel) and RHA-I (right-hand panel) control and isolated rats. *:P<0.045 vs the line-matched control group.

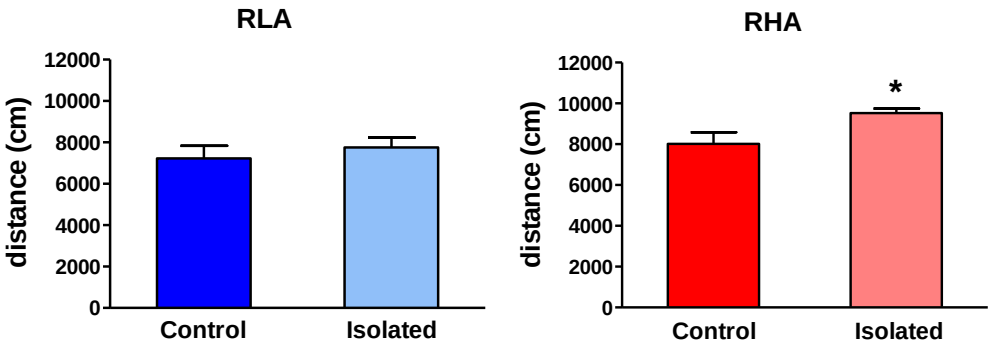

Fig.8 Total distance travelled Columns and bars indicate the mean ± S.E.M. of the total distance travelled by RLA-I (left-hand panel) and RHA-I (right-hand panel) control and isolated rats. *: P< 0.05 vs the line-matched control group.

4.4 Discussion

The results of the EZM experiments showed that social isolation significantly reduced the time spent in open space (TOS) and the frequency of head-dipping (HD) in RHA-I, but not in RLA-I rats. The reduction in these parameters is strongly influenced by the levels of anxiety. In particular, TOS is the most important measure to evaluate the levels of anxiety since if the animal spends much time in the open quadrants it means that the (innate) desire to explore novel environments overcomes the (innate) fear of open and elevated areas. Conversely, when fear prevails, the animal prefers to remain in the closed quadrants with a consequent reduction of TOS. Under basal conditions, RHA rats, which display high levels of novelty/sensation seeking, spend much more TOS compared with the hyperemotional RLA rats. However, after prolonged SI, the TOS of RHA rats is markedly reduced and becomes similar to that of RLA rats (either controls or isolated, since there is no difference between the two groups in RLA rats). Head dipping is considered a

dangerous behavior that reflects the risk propensity of the animal. In keeping with their high levels of impulsivity, RHA controls display a significantly higher HD frequency than RLA controls. Notably, social isolation makes RHA rats more cautious as indicated by the significant reduction in HD frequency of isolated versus control rats. Also the reduction in the number of entries (EOS) and the increased latency to entry in the open space displayed by the isolated RHA rats are indexes of increased anxiety. However, the number of EOS is not as important as the TOS because a high number of EOS does not necessarily imply that the animal spends much time in the open space, but may reflect a continuous going out and coming back. Similarly, the number of crossings captures locomotor activity rather than anxiety. Collectively, these results show that post-weaning social isolation increments the anxiety-related behaviors in the EZM paradigm only in RHA-I rats.

In order to test whether the results obtained with the EZM were due to non-specific effects on locomotor activity in a new environment, we used motility cages to valuate more directly this behavioral measure. The results obtained show that post-weaning social isolation increases locomotor activity only in RHA-I rats. The hyperactivity in a new environment displayed by isolated RHA-I rats argues against the possibility that their behavior in the EZM (i.e., increased latency to enter in open space, reduced TOS and EOS) may be due to a non-specific impairment of the locomotor activity.

The results obtained in the Elevated Zero Maze and the assessment of Locomotor Activity in a new environment are consistent with the large number of previous studies in the Roman strains/lines showing that, under basal conditions, RHA-I/RHA rats are less anxious and more prone to explore and eventually risk than RLA-I/RLA rats. Moreover, compared with their RLA-I/RLA counterparts, RHA-I/RHA rats display higher levels of horizontal activity in a new environment, in keeping with their marked profile of sensation/novelty seekers.
In addition, this study shows that the isolation-rearing procedure significantly increases the levels of anxiety in the EZM and the novelty-

induced locomotor activity only in RHA-I rats, suggesting a higher vulnerability of this strain (compared with its RLA-I counterpart) to some effects of social isolation. In this context, it is interesting to note that RHA-I rats reared in isolation displayed an excessive reaction to handling which, combined with the increased anxiety and the hyperactivity, contributes to delineate the behavioral features of the so-called "isolation stress-induced syndrome".

These results are in line with previous studies describing the same effects of social isolation (**[199]** and references therein) and with our pilot experiments using inbred Roman rats showing behavioral alterations resembling core features of schizophrenia, such as PPI deficits and cognitive impairments in long term spatial reference memory that are displayed exclusively by isolated RHA-I rats (**Oliveras et al., submitted**).

In conclusion, these preliminary data add experimental support to the view that the Roman strains may be used as a valid genetic model to investigate the neural underpinnings of the vulnerability (RHA-I) and the resistance (RLA-I) to develop schizophrenia-relevant symptoms upon isolation rearing.

5. General discussions

5.1 Brief summary of results

Study I: In RLA rats, which display robust depression-like behaviors in the forced swim test (FST), chronic treatments with low doses of antidepressants, that were ineffective when given subacutely, decreased immobility and also increased climbing (desipramine) or swimming (fluoxetine). Conversely, neither subacute nor chronic antidepressant treatments affected the behavior of RHA rats in the FST.

Study II: Compared with their RLA counterparts, RHA rats showed higher levels of sexual motivation and a better copulatory performance, associated with a greater release of DA in the AcbSh. These line-related differences were attenuated but not abolished by sexual experience. Moreover, RLA rats were more responsive to both, the facilitatory effect of apomorphine and the inhibitory effect of haloperidol on sexual behavior.

Study III: In RHA-I rats, the isolation-rearing procedure significantly increased the levels of anxiety in the EZM as indicated by a reduction in the number of entries and time spent in the open space, decreased head dipping, and increased latency to enter in open space, and increased the novelty-induced locomotor activity. In contrast, isolation-rearing failed to produce significant changes in the behavior of RLA-I rats.

5.2.1 Roman rats as genetic animal models of psychiatric disorders

Ideally, in order to have translational valence, an animal model should mimic the human condition of interest with respect to its etiology/biological basis (***construct*** validity), symptomatology (***face*** validity) and treatment (***predictive*** validity) **[65]**.

The etiological validation of a model, that is, of its construct validity, is a fundamental aspect of scientific investigation. This implies a basic knowledge of the etiology of the condition we want to model. Unfortunately, the etiologies of psychiatric disorders are still poorly understood. Hence, construct validity in this context is generally limited to hypotheses regarding a plausible etiology. Indeed, the purpose of the development of animal models is often to enable the identification of the etiology of the disease **[207]**.

5.2.1 Vulnerability (RLA) and resistance (RHA) to stress-induced depression

It is well established that depression is a highly heritable disorder, with genetic factors comprising roughly 50% of the risk for depression. Genetic factors contribute to determine the animal's responses to stress, which is one of the most important environmental risk factors for depression, and they influence also the response to antidepressant treatments **[208]**. Most rodent models of depression are based on the exposure to stress of normal, healthy animals, thereby ignoring the genetic-based vulnerability.

A valid approach to study the complex interaction between genetic predisposition and environmental factors in the etiopathogenesis of depression may be represented by the selective breeding of animals with marked differences in depression-like phenotypes **[209-211]**. For instance, selective breeding has been applied to the forced swim test to select animals for high (SwHi) or low (SwLo) levels of swimming activity. The Swim Low Active line shows an innate low swimming activity that is increased after antidepressant administration **[212]**. A similar innate behavior is displayed also by the Flinders Sensitive line **[74]** and the Wistar Kyoto rats **[213]** (both selected for modeling conditions different from depression), that have been proposed as genetic models of depression.

In this context, the results obtained in **study I** are in line with the findings derived from other genetically selected lines or strains of rats. Moreover, the passive strategy adopted by RLA rats in the FST can be defined as a heritable trait, since it was consistently reproduced along the six generations of Roman rats used for these studies.

Notably, **study I** also shows the predictive validity of the Roman Low Avoidance rats as a model of stress-induced depression-like phenotype, since RLA rats positively respond to chronic antidepressant drugs that are clinically effective. Conversely, RHA rats do not develop depression-like behavior in the forced swim test and, consequently, are not responsive to subacute or chronic antidepressant treatments. The results obtained in RHA rats are as important as those reported in RLA rats, since they suggest that the phenotypic traits of RHA rats may be involved in their resistance to display stress-induced depression-like behaviour.

Notably, RHA and RLA rats markedly differ in their capability to cope with stressful events, which is considered to play an important role in determining vulnerability to stress-induced depression and post-traumatic stress disorders **[72, 214, 215]**. Thus, RLA rats show a reactive coping associated with an elevated HPA axis reactivity which may explain, at least in part, their vulnerability to display stress-induced depression-like behaviour. In contrast, the proactive coping displayed by RHA rats associated with low-intensity HPA axis responses may account for their resistance to stress-induced depression **[21, 86, 88, 181, 216, 217]**.

5.2.2 The role of dopamine

Considerable experimental evidence supports the role of the central dopaminergic system in depression and other stress-related psychiatric disorders **[218, 219]**. In this context, it is interesting to note that the coping style of the Roman lines/strains of rats is related to their prefrontal dopaminergic function. Thus, when exposed to aversive stimuli, RHA, but not RLA, rats show a marked increase in dopamine output in the prefrontal cortex **[21, 114]**. This finding is in line with the view that the

mesocortical dopaminergic projection is critically involved in the concerted execution of the motor activities that define a coping strategy **[112, 113]**.

Such differences in dopamine transmission may also be involved in the lower levels of immobility and the more robust climbing activity displayed under baseline conditions by RHA rats as compared with their RLA counterparts. Thus, compounds that enhance dopaminergic transmission are known to increase climbing in the forced swim test **[179]**, whereas a mesocortical dopaminergic hypofunction may be involved in the psychomotor retardation observed in some depressed patients **[19]**.

The dopamine-related differences between the two lines are also extended to the mesolimbic transmission. Thus, the responsiveness of the mesolimbic dopaminergic system to natural and drug rewards is significantly higher in RHA than in RLA rats, as demonstrated by several studies in inbred **[220]** and outbred Roman rats **[87, 115, 116, 180, 221]**, and by the results obtained in **study II**.

5.2.3 Processing of rewarding stimuli in depression and other psychiatric disorders

Study II shows that the Roman lines also differ in sexual behavior, one of the main sources of natural reward, with RHA rats showing higher sexual motivation and better copulatory performance than RLA rats. These findings were obtained using classical copulation tests as well as in the presence of an inaccessible receptive female, where RHA rats show more non contact erections than RLA rats. Since non contact erections are considered as an index of sexual arousal, their higher occurrence in RHA vs RLA rats is an additional confirmation of the greater sexual motivation displayed by RHA rats.

It is noteworthy in this context that depression is typically linked to motivational anhedonia (i.e., the loss of interest in once pleasurable

activities, including sex) which has been consistently correlated with impaired mesolimbic dopaminergic signalling **[138, 139]**.

Notably, the distinct sexual behavioral patterns of RHA and RLA rats, both, in the presence of an inacessible female and during copulation, are related with differential changes in the concentrations of extracellular dopamine in the nucleus accumbens shell.

Thus, the larger increases in extracellular dopamine that occur in the shell of sexually naïve and experienced RHA versus RLA rats during sexual activity support the role of mesolimbic dopamine in sexual behavior **[164-168]** and are consistent with the results of previous studies showing that a higher dopamine release occurs in NAcSh of RHA versus RLA rats upon the administration of drugs that increase mesolimbic dopaminergic transmission **[87, 180, 185, 222]**.

Importantly, both RLA and RHA rats seem to display a dysfunction in processing rewarding stimuli, which is a key feature of some psychiatric conditions: on the one hand, RLA rats show a lack of motivation resembling the anhedonia observed in many depressed patients. On the other hand, RHA rats display an excessive activation of the mesolimbic dopaminergic system that promotes incentive learning, which is considered to be critically involved in substance abuse disorders.

5.2.4 Early life stress and psychiatric disorders: the contribution of Roman rats

One of the predisposing factors for the development of psychiatric disorders is the exposure to adverse experiences in early-life **[172, 199]**. Stress, including early life stress, may interact with genetically determined vulnerabilities to modify behavioral phenotypes **[208]**.

Accordingly, in **study III** we found that the hypoemotional and novelty seekers RHA-I rats became anxious and fearful after prolonged isolation rearing (IR), whereas such early life stress did not affect the behavior of

RLA-I rats. Likewise, the IR-induced hyperactivity is observed only in RHA-I rats, suggesting a higher vulnerability of this strain (compared with its RLA-I counterpart) to some effects of social isolation. Interestingly, the results of study III, combined with other promising results obtained in the Spanish colony of Roman inbred rats (see discussion of study III), suggest that post weaning social isolation may contribute to the occurrence of schizophrenia-like symptoms in the genetically predisposed RHA rats.

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. In this context, it is noteworthy that neonatal handling reduces most behavioral and physiological signs of emotionality/anxiety in RLA/Verh rats, including an improvement in the performance in the two-way active avoidance test, while environmental enrichment increases their novelty seeking, saccharin and ethanol intake, and sensitivity to amphetamine. Additionally, both treatments seem to have a sort of "protective" effect against some age-related deficits **[123, 206, 223-226]**.

5.3 Future directions

Interestingly, recent western blot and immunohistochemistry studies performed in collaboration with Quartu and colleagues (in preparation) have shown that, under baseline conditions, the expression of BDNF and its receptor trkB is significantly lower in the hippocampus of outbred male RLA rats compared with their RHA counterparts. 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 **[35]**. Accordingly, chronic administration of corticosterone reduces BDNF expression in the rat hippocampus **[46]**, suggesting that

the effects of stress on hippocampal neurons are mediated by glucocorticoids.
It is noteworthy in this context that a large percentage of depressed patients shows sustained high levels of glucorticoids which lead to an impairment of the inhibitory feedback on the HPA axis **[25]** with a consequent damage of hippocampal neurons due to the enhanced activity of the HPA axis **[26, 27]**. Moreover, the neuronal damage triggers a positive feedback because the reduction in the inhibitory control exerted by the hippocampus on the HPA axis leads to a further increase in circulating glucocorticoid levels and subsequent hippocampal damage **[2]**.

In keeping with findings in depressed patients, when exposed to stressful conditions RLA rats display higher levels of corticosterone with respect to their RHA counterparts, and a concomitant reduction of the inhibitory feedback on the HPA axis **[104, 106, 108]**.

The large increment in the levels of corticosterone shown by RLA rats in response to stress may induce a reduction in the expression of BDNF in the hippocampus thereby leading to a disruption of hippocampal function, impairment of the corticosterone-mediated inhibitory feedback and hyperactivation of the HPA axis. The persistence of such cascade of events may account, at least in part, for the depressive-like behavior displayed by RLA rats. Alternatively, it may be posited that the lower basal levels of BDNF and trkB in the hippocampus of RLA rats as compared with that of RHA rats may sensitize the hippocampal neurons to the negative effects exerted by glucocorticoids.

Hence, experiments are in progress in our laboratory aimed at assessing the expression of neutrophic factors and their receptors, as well as of pre- and post-synaptic proteins in the hippocampus and other brain areas of the Roman lines in response to acute and chronic stress, with and without combined antidepressant treatments.

Moreover, neurotrophic factors and synaptic proteins are known to play a pivotal role in learning and memory. Therefore, since learning processes are critically involved in the modifications in copulatory performance observed in sexually experienced animals as compared with their sexually

naïve counterparts, future studies will be devoted to the characterization of the plastic changes that occur during the acquisition of sexual experience in brain areas involved in learning and memory, such as the hippocampus and other limbic areas that are a part of the so called "reward circuit", including the ventral tegmental area, the amygdala, the nucleus accumbens and the medial prefrontal cortex.

6. Conclusions

In conclusion, the results of these studies have shown that the Roman lines/strains of rats may provide a valid experimental approach to study:

- alterations in the neural substrates and mechanisms involved in the individual vulnerability and resistance to stress-induced depression, with the aim of identifying potential biomarkers for an early diagnosis of depression, and molecular targets for new antidepressant treatments;
- the neurophysiology of sexual function (in its appetitive and consummatory aspects), in order to better understand the mechanisms underlying the psychopathological and pharmacologic causes of sexual dysfunctions;
- the ways in which early-life adverse (or positive) experiences may interact with the genetically determined vulnerability to develop psychiatric disorders.

The pathogenesis of depression, as well as the mechanisms of action of antidepressants, but also the learning processes involved in the acquisition of sexual experience, and the consolidation of memories of either aversive or rewarding experiences, involve the ability of the nervous system to reorganize its structure, function and connections in response to internal and external stimuli. Such ability is defined as ***neuroplasticity***.

RHA and RLA rats, which differ markedly in their responses to stress (including early life stress) and to rewarding stimuli (including sexual activity), may therefore be used to investigate the neuroplastic changes (i.e., expression of neurotrophic factors, intracellular signaling cascades, epigenetic modifications, etc.) induced by environmental factors that occur during sexual learning, and during the development and therapy of psychiatric disorders.

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9. Publications

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