Università di Foggia

Department of Clinical and Experimental Medicine

PhD in

"Translational Medicine and Management of Sanitary Systems"

(XXXV Cycle)

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Early-age stress exposure and psychopathology development: vulnerability mechanisms and resilience

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Academic Years: 2019-2022

A mio Nonno Domenico

e al suo Amore per la Vita

Table of contents

1. Introduction

1.1 Stress: general concepts

Stress is a general term that describes the altered physiological response of a human or animal organism to any physical, emotional or mental request [1]. It is a state where homeostasis is threatened and the organism activates processes to restore it [2]. Stress could be acute or chronic; the term "acute" is a condition for a short interval of time, while "chronic" is a long-lasting form and induces a maladaptive response with harmful effects on the body [3]. Indeed, scientific evidence showed that the activated mechanisms in response to chronic stress participate in the development of illnesses like psychosis with relative symptoms, such as cognitive deficits, social dysfunctions, inflammation and positive symptoms (hallucinations, delusions and repetitive movements) [4]. Furthermore, it was observed that psychotic patients usually experience anxiety and depression, especially after exposure to stress in the neurodevelopmental phase (Fig. 1).

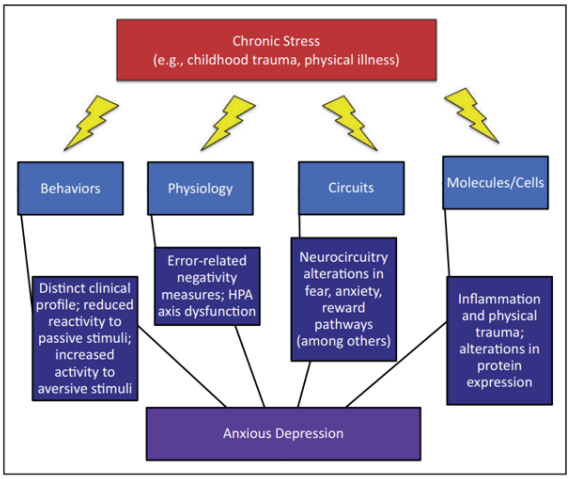

Fig.1. **Schematic representation of chronic stress effects.** Chronic stress may induce the anxious depression by altering the behavior, the HPA axis function, the neuronal circuits and the protein expression (from [5]).

However, the mechanisms underlying this comorbidity are still unclear [6]. In particular, in the stress response, the autonomic nervous system interacts with other vital centers in the central nervous system (CNS), and tissues/organs in the periphery to mobilize a successful adaptive response against the imposed stressors. In this contest, the hypothalamic-pituitary-adrenal (HPA) axis is also activated as a main neuroendocrine system [7] (Fig. 2).

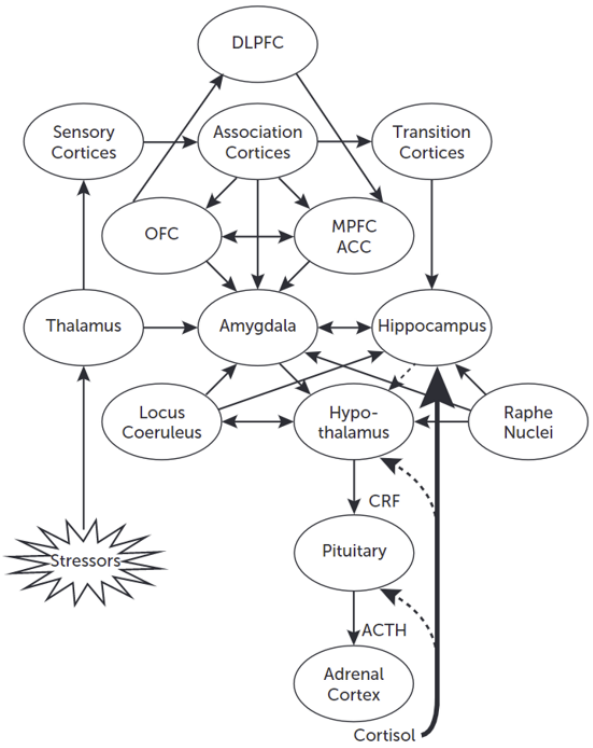

Fig.2. **Representative image of brain regions and peripheral organs involved in stress response.** Sensory receptors, after being stimulated by different kinds of stressors, send information to the thalamus, corteces, hippocampus and amygdala. Also, thalamus may convey inputs directly to the amygdala. Through multiple projections and connections from and to the amygdala, the locus coeruleus and the raphe nuclei at central levels, as well as the HPA axis at periphery, are activated. This finally results in the release of glucocorticoids, such as cortisol, from the adrenal cortex. Glucocorticoids, via a negative feedback pathway, can both inhibit the HPA axis, acting on the pituitary gland and hypothalamus, and activate the hippocampus, which, in turn, can block the axis activity (from [8]).

1.2 Role of the HPA axis in the stress response and in stress-induced mental disorders

Physiologically, through the production of mineralocorticoids and glucocorticoids, the HPA axis regulates several body functions. Indeed, it is a key player in the regulation of metabolism (such as the lipidic and glycemic pathways), the immune system, the cardiovascular functions, the sexual behavior, the mitochondrial function and energy metabolism [7]. Moreover, it also controls the reactions to stress. In particular, the hypothalamus (HYP) releases the corticotropin-releasing hormone (CRH) that induces the production of adrenocorticotropic hormone (ACTH) from the pituitary. This stimulates the adrenal gland to generate glucocorticoids, such as cortisol in humans or corticosterone (CORT) in rodents, with activation of the central sympathetic system. Under the chronic stress condition, there is a hyperactivation of the HPA axis, which consists of the overproduction of glucocorticoids, leading to an alteration of the negative feedback of this mechanism and a glucocorticoid receptors (GRs) malfunction [9]. Indeed, when the glucocorticoids are overproduced, the GRs become occupied, resulting in the desensitization of receptors. This condition is present in depressive patients, where, for example, the interaction between GRs and tropomyosin related kinase B (TrKB), receptor of brain-derived neurotrophic factor (BDNF), was seen to have an important role. BDNF influences several cellular processes as neuronal survival and synaptic plasticity; it stimulates the glutamate (GLU) release from glutamatergic neurons

through the phospholipase Cγ / inositol 1,4,5-trisphosphate /Ca^{2+} pathway. So, following the overproduction of glucocorticoids and desensitization of relative receptors, the GR-TrKB interaction decreases, resulting in the reduction of BDNF-induced glutamate release as observed in chronic restraint stress [10]. These results suggest that reduced GR expression and altered BDNF function may be involved in chronic stress-induced anxiety--and depression-like behaviors.

The overproduction of glucocorticoids implicates a reduced sensitivity of GRs also on immune cells, leading to less immunosuppression activity of glucocorticoids. Consequently, an increased secretion of pro-inflammatory molecules, as observed in patients with stress-induced mood disturbances [11].

1.3 Role of the redox balance in the stress response and in stress-induced mental disorders

In the CNS, the reactive oxygen species (ROS) regulate the neuronal fate, the signaling pathways, such as the regulation of the glutamatergic neurotransmission through the N-methyl-D-aspartate (NMDA) receptor, the neuroinflammatory response through the activation of microglia. Together with reactive nitrogen species (RNS), the ROS are the end product of oxidative phosphorylation, especially by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) (transmembrane enzyme complexes involved in the mediated release of ROS). Physiologically, the ROS are neutralized by the antioxidant system, including enzymes such as superoxide dismutase (SOD), glyoxalase, catalase

(CAT), glutathione reductase, glutathione peroxidase, Coenzyme Q10 (CoQ10) and by low-weight-molecular antioxidants like ascorbic acid, uric acid, melatonin and glutathione. The imbalance between the antioxidant defense system and excessive ROS production (which prevails in favor of the oxidants) has been linked to oxidative damage, cell degeneration, and cell function decline occur, following the oxidation of proteins and lipid peroxidation. This state is defined as *oxidative stress*. Under the chronic stress condition, after the overproduction of glucocorticoids, the mitochondria and NOXs are stimulated to generate ROS in large quantities, inducing oxidative stress. This state alters the cellular functions and stimulates an increase of GLU production, with consequent upregulation of NMDA receptor, and loss of phenotype of inhibitory neurons, resulting in reduced expression of γ-aminobutyric acid (GABA) [12]. The brain has a high lipid content and energy demand, so its phospholipids, proteins, and DNA are vulnerable to ROS action [13]. This means that oxidative stress might be dangerous for the CNS, leading to structural and functional changes in brain regions such as the hippocampus (HIPP), cortex and in amygdala (AMY), the most involved areas in depression and anxiety [14] (Fig. 3).

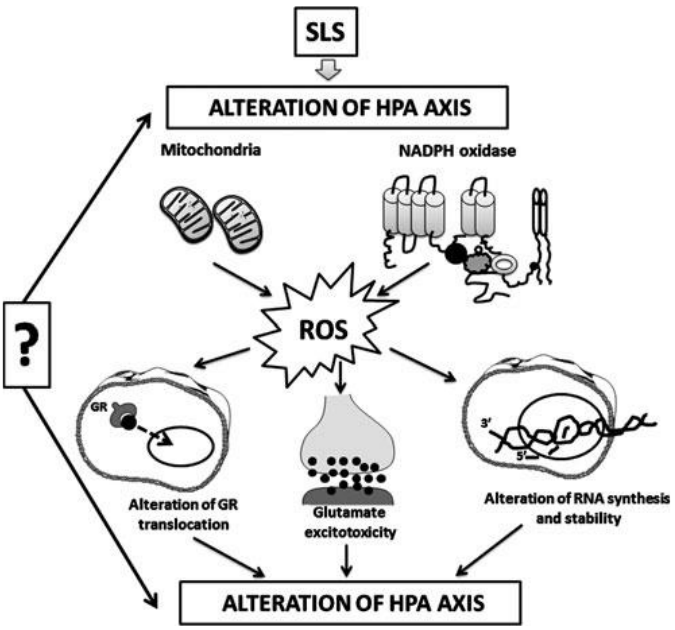

Fig.3. **Representative image of chronic stress-induced ROS overproduction.** Severe Life Stress (SLS) alters the normal functioning of the HPA axis, leading to overproduction of ROS by mitochondria and NADPH oxidases. The resulted oxidative stress induces an increase in glutamate release, as well as structural and functional modifications in brain regions (from [12]).

In this context, several studies evidenced the critical role of NOX enzymes, with overproduction of ROS, in the pathophysiology of psychiatric disorders such as schizophrenia and anxiety. Indeed, in the brain areas of schizophrenic patients, the microglia activation was detected. Moreover, animal studies showed that the NOX2 activation is essential in the development of psychosis in mice, following the administration of subanesthetic doses of ketamine that induces oxidative stress and reduces the number of parvalbumin-expressing interneurons. The NOX2 deficiency and the apocynin (an inhibitor of NADPH-oxidase) pretreatment prevented the ROS production and protected the animals from the alterations induced by ketamine. Other studies observed that the oxidative stress, induced with l-buthionine-(S,R)-sulfoximine (BSO), led to an anxiety-like behaviour in

mice. This effect was changed by apocynin, suggesting the important role of NOX enzyme activation in the evolution of anxiety. Later, it was confirmed by observation of an increased expression of NOX2 and relative subunits in HYP, AMY and cortex, following BSO treatment [15].

1.4 Role of neurochemical circuits in the stress response and in stress-induced mental disorders

In addition to this, as answer to chronic stress, the central neurochemical circuitry is important; it consists of stimulatory and inhibitory networks with multiple interaction sites. Based on reciprocal reverberatory neural connections, the hypothalamic CRH and arginine-vasopressin neurons with catecholaminergic neurons play a key role. The CRH neurons receive excitatory inputs from serotonin (5-HT), noradrenaline (NA), Neuropeptide Y and inhibitory inputs from GABA. In particular, 5-HT induces the release of CRH. However, under the chronic stress condition, the tryptophan pyrrolase activates in the liver, so the tryptophan is metabolized through the tryptophan-kynurenine pathway, decreasing the level of turnover and release of 5-HT. In this case, the 5-HT receptor is altered and could also be inhibited by glucocorticoids. Indeed its function is decreased in hipp, and this alteration, through the neuronal circuits, is also reflected in the AMY, resulting in anxious and depressive behaviour [16]. Moreover, the CRH receptors were identified in several extra-hypothalamic regions of the brain, including areas of the limbic system. In chronic stress, the

CRH receptors are desensitized, and the negative feedback mechanism is altered, resulting in a continuous release of ACTH and production of glucocorticoids from the adrenal cortex. CRH also activates locus coeruleus to promote the stress response, increasing NA. The last mentioned activates the AMY (principal brain locus for mood behavior) and increases long-term storage of aversively charged emotional memories in the HIPP and striatum [17].

1.5 Role of the endocrine modulators in the stress response and in stress-induced mental disorders

Together with the HPA axis, other endocrine modulators participate to the development of anxiety and depression following stress. As an example, oxytocin is a neuropeptide synthesized in the paraventricular and supraoptic nuclei of the HYP and found in the posterior pituitary, limbic areas including the HIPP, the AMY, striatum, HYP, nucleus accumbens (NACC), and other brain regions. Also, it is involved in lactation process and in social behavior. Oxytocin prevents stress-mediated activity in the HPA axis by performing a vital role in response to stress. Indeed, scientific evidence showed that the levels of oxytocin increased in the plasma of psychiatric patients, as a compensatory response to anxiety and depression state, in the face of malfunctioning oxytocin receptors, due to psychiatric disorders-related genetic variations [18]. The oxytocin receptors are distributed throughout the brain: in the AMY, ventromedial HYP, NACC and cortex. Whereas, other studies demonstrated that oxytocin amount decreased in

anxious and depressive patients [19]. Indeed, the oxytocin administration improved the state of subjects [20]. The role of prolactin was described in response to emotional stimulus, along with oxytocin. This pituitary hormone is essential in lactation in females. Also, it is a neuromodulator in both males and females by increasing the social behaviors, approach, learning and reward [21]. Other scientific achievements showed that the prolactin levels decreased in the plasma of patients with stress-induced anxiety and depression, as well as related immunosuppressive activity [22]. The prolactin release is regulated by melatonin, a hormone released by the pineal gland during the night, and associated with the control of the sleep–wake cycle that affects both the oxytocin and prolactin responses to stress [23]. In mood diseases such as depression, circadian rhythm disturbance was evidenced, probably due to the increase of glucocorticoids, with consequent less melatonin production. This data was confirmed by the administration of exogenous melatonin, which improved the condition in depressive patients [24]. Another hormone, altered in stress-induced disorders, is ghrelin. However, its mechanism of action has not been definitely clarified so far and available data are actually contrasting. Indeed, some lines of evidence reported that, during stress exposure, ghrelin level increased, mediating neuroendocrine and behavioral responses to different stressors, whereas other studies showed its decrease. A possible reason for this discordance may be the method of analysis used [25]. Ghrelin is produced in the HYP and from

enteroendocrine cells of the gastrointestinal tract. Moreover, it is involved in the regulation of food intake and in neurological functions [26].

Neuropeptide Y (NPY) is also important among other neuropeptides involved in stress response. It is distributed in the brain regions as limbic structures and striatum. It plays a crucial role in neurogenesis, neuroprotection, feeding regulation, neuronal excitability, energy homeostasis, emotions, and stress adaptation and in this context, the studies revealed that NPY, in depression and anxiety disorders, increases to relieve the CRH and NA tone. In addition, Neuropeptide S (NPS) also participates in response to stress. It has a similar physiological role of NPY and is produced in the region between Barrington's nucleus and the locus coeruleus as well as in the AMY. NPS has an anxiolytic effect by activating the HPA axis. Clinical studies demonstrated a link between NPS gene receptor polymorphism, which alters the activity of NPS, and mood disorders [19]. Moreover, it was demonstrated that in stress-induced anxiety and depression, there are also alterations at the peripheral level, such as 5-HT and kynurenine concentrations. In particular the gut microbiota is linked to the HPA axis and has a role in tryptophan metabolism and serotonergic system. Therefore, a chronic HPA axis hyperactivation, following stress exposure, could alter the gut microbiota and involve it in different diseases as depression and anxiety [27].

1.6 Impact of stress exposure during neurodevelopmental phases

Some mentioned alterations are even more evident if exposure to chronic stress occurs during the neurodevelopmental phase. Indeed, the period from infancy to adolescence phase is critical for the formation and development of the brain. In particular, during postnatal brain development, the cortical gray matter expansion/regression and the growth of white matter are important for the advancement of neuronal connectivity and maturation of neurobehavioral functions. Adverse experiences in this phase of life could interfere with the maturation of neuronal circuits, leading to the development of pathological alterations in specific brain regions (such as PFC, HIPP and AMY) involved in the stress response and in the regulation of emotions and behaviours and finally resulting in both a significant vulnerability to stress exposure and stress-related disorder development [28]. Whereas, following exposure to stress in early age, individuals do not always manifest diseases in adulthood; if the subject is vulnerable or resilient to stress-related disorders depends on the interaction between genetic factors and environment, epigenetic programming of stress response mediators. In this context also prenatal stress (PNS) is important. Indeed, several clinical studies evinced that offspring of mothers that were exposed to adverse events during pregnancy, frequently suffer neuropsychiatric disorders in later life [29]. In particular, children born from mothers with high anxiety symptoms during pregnancy, showed high rates of emotional problems at childhood, and others reported depressive symptoms at adolescence or in the adult

phase. The stress hormone probably crosses the placenta and alters the development of the brain, HPA axis, and limbic brain regions, preparing the individual to manifest a possible disorder in later life [30]. In rodents, following stress exposure, the CORT levels increase in pregnant dams and it passes over the placenta where it reaches the fetus. The mother's organism activates mechanisms to protect the fetus from stress, as well as enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) in the placenta. This enzyme inactivates CORT to minimize the fetal exposure to glucocorticoids, but the effect is not entirely blocked. The expression and activity of 11β-HSD2 depends from mother's genetic background. However, the alterations of the HPA axis are only one of the consequences following PNS exposure, because also the immune system is changed. Indeed the levels of pro- and anti-inflammatory cytokines are modified in the placenta and in offspring. Moreover, there is a link between stress, glucocorticoids function and neuroinflammation, in particular, the cytokines can cross the brain-blood barrier and stimulate the HPA axis to produce glucocorticoids [31] (Fig. 4).

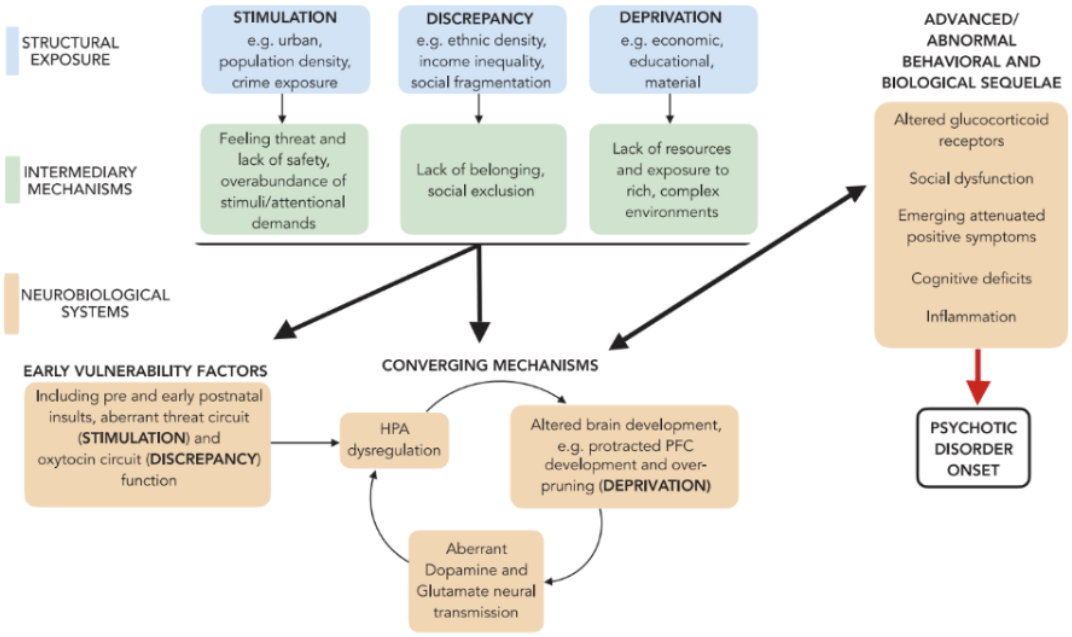

Fig.4. **Representative scheme of the effects of chronic stress exposure during the prenatal and early postnatal periods**. The chronic exposure to stressful events during the neurodevelopmental phase leads to alterations in the HPA axis functioning, neural transmission, and brain development. This finally results in cognitive and social dysfunctions, inflammation, and impaired glucocorticoid receptor function, possibly contributing to the development of mental disorders, such as psychosis, at adulthood (from [4]).

Also, studies in animals and in vitro, using microarray analysis, evidenced that chronic CORT exposure of the fetal brain alters the epigenetic mechanisms (DNA methilation and miRNA expression) possibly linked to the risk of mental illness later in life [32].

1.7 Animal models of chronic stress-induced mental disorders: the rat social isolation

The processes underlying the stress-related alterations are still unclear and the animal models of mental disorders, following chronic stress, are essential to understand these mechanisms. To this aim, several research groups applied different tools of chronic stress on the rodents such as foot shock, underwater

trauma, restraint stress, exposure of rodents to the predator or predator's odorants (for example, soiled cat litter), social defeat where animals are exposed to a trained aggressor conspecific, tail suspension and social isolation. These stresses lasted a long time and modified the mental, behavioral and emotional functions in animals [33]. Among those mentioned, the social isolation is widely used and induces behavioral and neurobiological changes in animals, resembling observed in psychotic patients: enhanced presynaptic dopaminergic and serotoninergic function in the NACC, reduced dopaminergic function in the AMY, decreased serotoninergic function in the PFC and HIPP [34]-[35]-[36]. Normally, the rats live in a gregarious environment and the social isolation, after weaning, provides a stressful condition for them. Indeed, a group of researchers observed, by performing behavioral tests, when the animals were reared under isolation. They showed a pattern of behavioral alterations, similar to the psychotic subjects: aggression, anxiety, fear, hyperactivity to novel environments, cognitive impairment, alteration in spatial learning and prepulse inhibition of acoustic startle, hypoalgesia, increased sucrose consumption. Moreover, by neurochemical analyses, the levels of dopamine (DA), 5-HT, NA, GABA and GLU resulted modified in the brain areas. In particular, some groups found an increase of DA turnover in the NACC and AMY with up-regulation of D2 receptor while others evidenced a down-regulation of this receptor. Instead, the 5-HT turnover decreased in the NACC, as well as the 5-HT_{2A} binding in prelimbic, motor and cingulate cortices. In contrast, the 5-HT_{1A} binding was reduced in the prelimbic

cortex, but it enhanced in the HIPP. In addition, a reduction in the number of GABAergic interneurons in the HIPP of isolated rats was observed, together with increased expression of GLU receptor. But the isolation induced in rats also changes in morphology of brain, indeed the volume of PFC decreased as well as the neuronal dendritic arborisation [35]-[36]. It was evidenced that the NACC and PFC are the cerebral areas most involved in chronic psychosocial stress [37]. Indeed, a study on male rodents reported that in these regions, after seven weeks of isolation, the oxidative stress from NOX2 was detected, followed by an increase of IBA-1 (a microglial marker) [38]. Moreover, by performing time-course analysis, NOX 2 increased after two weeks in the NAAC and following four weeks in the PFC. However, the levels of some indirect markers of oxidative stress, such as the immediate-early gene and redox-sensitive transcription factor c-fos, the HIF-1a protein increased in time. In contrast, after four weeks of isolation, 8-hydroxy-2'-deoxyguanosine (8-OHdG) (a marker of oxidative stress to DNA) increased in the same brain areas (in NACC already after 2 weeks) , as like as the GLU levels correlated to alterations in NMDA receptors and to altered locomotor activity analyzed by open field test. The cognitive impairments were detected by using a novel object recognition test. In isolated rats the parvalbumin protein decreases in the NACC after two weeks and in the PFC following four weeks, this effect reflects a loss of inhibitor phenotype of GABAergic neurons, shown in psychotic subjects. These data confirmed that NACC and PFC are the cerebral areas most involved in chronic psychosocial stress [37]. After isolation

period the blood-brain barrier is also involved, indeed its disruption is visible after one week of social stress and it is accompanied by a significant increase of Interleukin 6 expression; so this precedes NOX2 elevation in the brain [39]. In response to severe life stress, like social isolation, also the HPA axis is activated as the main neuroendocrine system and increasing evidence exists for a link between the HPA axis and oxidative stress. Indeed, the impact of corticosteroids, produced by HPA axis, on NOX-dependent ROS production may be cell-type dependent. In this contest, the 8-OHdG and nitrotyrosine (an end product of NO-toxic species) levels growth in the HYP from second week of social stress [40]. It was evidenced that also neuroendocrine alterations are time-dependent. Hypothalamic corticotropin-releasing factor and plasmatic neuroendocrine ACTH increase after four weeks of isolation. In this model of psychosis, the oxidative stress induced in isolated rats also changes at a peripheral level as well as alteration of total and visceral fat amount with an increase of both [41]. It is an example of a pathogenic process observed in psychotic patients [42]. In addition to this, in the liver, the chronic psychosocial stress alters the redox state with over expression of NOX4 followed by more production of ROS and malondialdeyde (MDA) accompanied by a decreased expression of antioxidant species: CAT, glutathione also if a more activity of SOD2 as a reaction to stress was reported. The stress impacts also the insulin pathway, in particular isolated animals evidenced enhanced insulin resistance (condition evidenced in psychotic patients [43]) with increased levels of insulin and less peripheral sensitivity to this

hormone with reduced phosphorylation of insulin receptor and expression of glucose transporter 2. In the same region, the lipid metabolism was also altered. After seven weeks of isolation, carnitine palmitoyltransferase 1, a trasporter of fatty acids in mitochondria, decreased as well as a phosphorylated form of adenosine monophosphate-activated protein kinase and the peroxisome proliferator-activated receptors-α levels [44]. Chronic psychosocial stress impairs bone homeostasis, indeed in isolated rats the bone mineral density increases at level of femur, suggesting the development of a hyperostosis condition; whereas at serum level, it was observed a decrease of cathepsin K (a protein involved in bone remodeling and resorption) and an increase of C-terminal telopeptide cross-link of type1 collagen, a product of bone remodeling process [45]. Effects of social stress were also studied in female rats, where a group of researchers observed that social deprivation during adolescence alters the mitochondrial function and increases dendritic spines in the NAAC. Probably by modifying the neuronal circuits and inducing anxiety and depression disorders; indeed the dendritic spine of the NAAC is the target for the convergence of dopaminergic axons from the ventral tegmental area and glutamatergic axons from the PFC, AMY and HIPP [46]. But also in the AMY and in the HIPP of isolated animals the alterations were found. In the HIPP, after two months of social stress, there is an increase of 5-HIAA/5-HT ratio and less concentration of 5-HT; whereas in the AMY, following three months of isolation, the 5-HT levels enhance and the 5-HT turnover reduces [47]. In this context, the chronic social isolation, during

adolescence, alters in rats the Wnt pathway. In particular, Wnt factors, by blocking the phosphorylation, stabilize the β-catenin protein that, especially in the neurodevelopmental period, promotes synapse formation and function. They analyzed that five days of social isolation induced an anxious behaviour in rats and a reduction of β-catenin with long-term changes in PFC function and related neuronal circuits with increased response to cocaine in adulthood. Moreover, in clinical studies, the Wnt pathway has been shown to be involved in the progression of mental illness [48]. Among other proteins involved in response to social isolation, also BDNF and nerve growth factor are altered; they participate in brain development as well as synaptogenesis, neuron survival, specialization and migration. In the HIPP, for example, they are important for memory and learning, but after chronic social stress these proteins resulted less expressed [49].

2. Aims of the study

Since how chronic stress on organism leads to anxiety and depression, which are frequently comorbid with psychosis, is still unclear, here, we aimed to investigate the mechanisms underlying this comorbidity, by using the rat social isolation protocol, especially focusing on the exposure to chronic stress during the neurodevelopmental phase.

In particular, this study consisted of two parts: in the first one, we investigated the mechanisms linking stress-induced anxiety, depression and psychosis, from a behavioral, neurochemical and neuroendocrine point of view. Indeed, considering that social isolation for an extended period represents a state of chronic stress for animals, we used this protocol from weaning until the end of adolescence, to induce psychosis and anxiety- and depressive- like behavior in aged male rats. We also investigated the possible neurochemical, neuroendocrine alterations and peripheral serotonergic, and kynurenergic changes, which could contribute to the development of comorbidity between anxiety-, depressive- like behavior and psychosis in adulthood.

In the second part of this study, to investigate whether this behavior, in adulthood, was correlated not only to individual stress but also to possible chronic stress, experienced by mother, before the conception, we studied the adult offspring of socially isolated females. This progeny was studied from a behavioural, neurochemical and biomolecular point of view, also following exposure to stress in the adolescent period, to understand if it becomes vulnerable or resilient to a

condition of stress lived through the neurodevelopmental phase. In particular, we exposed the female rats to chronic social isolation and researched if their adult male offspring, also after the exposure to social deprivation from adolescence postnatal day (PND) 42 until adulthood (PND70), showed locomotor, cognitive, learning dysfunctions as well as alterations at a neurochemical level, in the excitatory-inhibitory balance and changes in oxidant-antioxidant species balance.

3. Materials and Methods of the first part of the study

In the first part of this study, to investigate the mechanisms linking stress-induced anxiety, depression and psychosis, from a behavioral, neurochemical and neuroendocrine point of view, we used the following methods:

3.1 Animals

Wistar male and female rats (Envigo, San Pietro al Natisone, Italy) were used in this part of the study. They were reared at constant room temperature (22 ± 1 ◦C) and relative humidity (55 ± 5 %), under a 12 h light/dark cycle (lights on from 7:00 AM to 7:00 PM). During housing, mating and experimentation, the animals had free access to water and food. The analyses were performed in accordance with the institutional guidelines of the Italian Ministry of Health (D.L. 26/2014), the Guide for the Care and Use of Laboratory Animals: Eight Edition, the Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research and the Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010, on the protection of animals used for scientific purposes. The Italian Ministry of Health approved the experimental protocol of this study (approval number 485/2019-PR and protocol n. B2EF8.22.). Furthermore, all experimental phases were accomplished in observance of ARRIVE guidelines. The animals' health status was monitored daily and any possible sign of distress during the entire study duration was avoided. All efforts were made to minimize the number of animals and their suffering.

3.2 Social isolation protocol

In order to induce chronic stress in animals, we applied the social isolation protocol and in the first part of the study, we considered the sex of animals as inclusion/exclusion criterion. In particular, it was carried out only on male rats, as previously detailed [38]. Specifically, one male and two females were put in the same cage for mating. At PND 21, a total number of 29 male pups were weaned and separated from their mothers to be housed either in group (GRP, three or four rats per cage) or in social isolation (ISO, one rat per cage). To avoid a litter effect, each mother contributed one rat to the GRP group and one rat to the ISO cohort. GRP and ISO animals were reared in the same room so that ISO rats could maintain visual, olfactory and auditory contacts with GRP subjects. This condition lasted seven weeks, until PND 70 when the rats were adults.

3.3 Behavioural tests

After seven weeks of social deprivation, in order to avoid possible carry over effects in behavioural assays, a set of animals was exposed to Forced swimming test (FST) and another set of animals was used to perform the Elevated Zero Maze test (EZM).

a) FST

To investigate if male rats had depressive-like behavior, one set of animals (ISO and GRP) performed the FST. As previously described [50], we used an apparatus consisted of two clear Perspex cylinders (70 cm height × 23 cm diameter). During

the preconditioning period, animals were placed individually in cylinders containing 30 cm of water at a constant temperature of 25 °C and forced to swim for 15 min. Then, rats were removed from the apparatus, towel-dried in a clean Plexiglas cage and then placed in their home cage. The cylinders were cleaned and the water was changed before each trial. Twenty-four hours later, each rat was tested for 5 min under identical conditions. This session (test phase) was recorded by using a video camera placed above the cylinder. An observer, blind to the experimental groups, scored the frequency time that rats spent performing the following behaviors: struggling (time spent in tentative of escaping), swimming (time spent moving around the cylinder) and immobility (time spent remaining afloat making only the necessary movements to keep its head above the water). Behavioral counts were taken at 5s intervals during the 5 min test.

b) EZM

To evaluate if social isolation induced an anxiety-like behavior in rats, another group of animals (ISO and GRP) performed the EZM. We used an annular dark gray metal platform (120 cm in diameter and elevated 60 cm from the floor), (Ugo Basile, Camerio, Varese, Italy). The platform was divided into four corridors: two opposing open and two opposing closed regions with black side walls 30 cm in height and 10 cm in width. The test began with the animal placed in the open area, facing the inside of the closed corridor. The test lasted 5 min and it was video recorded. Between trials, the maze was cleaned with 50% ethanol. In this test, an observer blind to experimental groups analyzed the time (sec) spent in the open

and closed corridors and the number of full entries in open and closed regions for each rat [51].

c) Open field (OF) test

The rats carried out OF test for the same purpose, namely the investigation of anxious attitude in animals following seven weeks of social deprivation. In this test, a blind experimenter scored the time (sec) spent by rodents in grooming activity, such as: head washing (semicircular movements over the top of the head and behind the ears), face grooming (strokes along the snout) and body grooming (body fur licking), as previously reported [52]. As apparatus, a dark plastic circular arena with 75 cm diameter was used. The animals were acclimatized to the test room for 1h before the test. After habituation, each rat was let freely explore the arena for 20 min, under dim lighting. The experimental session was videotaped by a camera fixed above the arena.

3.4 Collection of amygdala (AMY), hippocampus (HIPP) and plasma

After performing the behavioral tests, in adulthood, the rats were anesthetized with ketamine (50 mg/kg, i.p.) + xylazine (10 mg/kg, i.p.) and sacrified by decapitation. The brains were removed and cooled on ice for AMY and HIPP dissection, as indicated in Paxinos and Watson atlas. The blood samples were collected from the trunk and put in tubes containing 10% of EDTA, following centrifuged at 4000 g for 10 min at 4°C to obtain plasma.

3.5 Neurochemical analyses by high-performance liquid chromatography (HPLC)

To evaluate if chronic social isolation altered the function of noradrenergic, serotonergic, neuroendocrine systems and excitatory-inhibitory balance, during the neurodevelopmental phase, we performed HPLC in AMY, HIPP and plasma of adult rats. In particular, we measured the NA, 5-HT, 5-Hydroxyindoleacetic acid (5-HIAA), GLU and GABA levels in AMY and HIPP. Instead, in plasma we analyzed 5-HT, 5-HIAA, KYN and melatonin amount. The levels of NA, 5-HT, 5-HIAA, and KYN were quantified by HPLC coupled with an electrochemical detector. Here, the LC18 reverse phase column (Kinetex, 150mm x 3.0 mm, ODS 5 µm; Phenomenex, Castel Maggiore-Bologna, Italy) was utilized to separate the catecholamines that were detected by a thin-layer amperometric cell (Dionex, ThermoScientific, Milan, Italy) including a 5-mm diameter glassy carbon electrode, by using a working potential of 400 mV vs Pd for NA, 5-HT, 5-HIAA and 0.550 V vs. Pd for KYN and melatonin (Ultimate ECD, Dionex Scientific, Milan, Italy). An aqueous buffer (pH 3.0) composed of 75 mM NaH2PO4, 1.7 mM octane sulfonic acid, 0.3 mM EDTA, acetonitrile 10%, was used as mobile phase and an isocratic pump (Shimadzu LC-10 AD, Kyoto, Japan) worked at 0.7 ml·min-1 as flow rate [53]. Results were analyzed by Chromeleon software (version 6.80, Thermo Scientific Dionex, San Donato Milanese, Italy), normalized for total area weight and expressed as concentration/mg of tissue, in specific the 5-HT turnover was calculated as 5-HIAA/5-HT ratio. The GLU and

GABA concentrations in AMY and HIPP were quantified as previously reported [54]. Indeed, a mobile phase (pH 6.95) containing 50 mM sodium acetate with a methanol gradient increasing linearly from 2 % to 30 % (v/v) in 40 min, and an ODS-3 column (Kinetex 150 mm × 3 mm, ODS 5 μm; Phenomenex, Castel Maggiore-Bologna, Italy) with fluorescence detection, after pre-column derivatization with o-phthalaldehyde/3-mercaptopropionic acid (emission length, 460 nm; excitation length, 340 nm) [55] were used. A pump (Jasco, Tokyo, Japan) maintained a gradient flow rate at 0.5ml/min. Data acquisition and integration were performed by Borwin software (version 1.50; Jasco, Cremella, Italy) and the results were expressed as concentration/mg of tissue after being normalized for total area weight.

3.6 Enzyme-linked immunosorbent assay (ELISA)

We performed ELISA in plasma samples to investigate whether seven weeks of isolation had modified the expression of endocrine modulators other than melatonin. Indeed, oxytocin, prolactin, ghrelin and NPS were quantified by using commercially available kits, such as: Rat oxytocin ELISA kit - EIAR-OXT-1 (RayBiotech Life, Peachtree Corners, GA, USA), Rat prolactin ELISA kit - SEA846Ra (Cloud-CloneCorp., Katy, TX 77494, USA), Rat ghrelin ELISA kit – MBS731169S (MyBioSource, San Diego, CA 92195–3308, USA) and Rat Neuropeptide S ELISA kit – MBS1605136 (MyBioSource, San Diego, CA 92195–3308, USA). The analyses were carried out in accordance with the

manufacturer's instructions. Moreover, standards and samples were run in duplicate to avoid intra-assay variations.

3.7 Statistical analyses

In order to carry out statistical analysis, the GraphPad 9.0 software for Windows (GraphPad Software, San Diego, CA) was used. We performed Student's t-test with Welch's correction where required. For all tests, a p-value < 0.05 was considered statistically significant.

4. Materials and Methods of the second part of the study

In the second part of this study, to investigate whether the anxiety- and depressive-like behavior, in adulthood, was induced not only by individual stress but also by possible chronic stress, suffered by mother, before the conception, and if this progeny becomes vulnerable or resilient to a condition of chronic stress in adolescence, we used the following materials and methods:

4.1 Animals

As in the previous part of the study, the adult Wistar male and female rats (Envigo, San Pietro al Natisone, Italy) were used. They were reared in the same conditions as the animals used in the first part of the project and the analyses were performed in accordance with the institutional guidelines as described in paragraph 3.1. The experimental protocol used was the same as the study's first part, with approval number 485/2019-PR and protocol n. B2EF8.22.

4.2 Social isolation protocol

In the second part of the project, we applied social isolation protocol on female offspring from PND21 until PND70. GRP and ISO females were coupled at the end of this period. The male pups of GRP females were weaned at PND 21 and reared in GRP (GRP/GRP) state. Instead, the male offspring of ISO females were weaned and housed in GRP (ISO/GRP) or ISO condition (ISO/ISO) until adolescence (PND42). At this time phase, a cohort of GRP/GRP animals

continued to be reared in GRP state until the adult period (PND 70) (GRP/GRP/GRP), another subset of GRP/GRP animals was put in ISO condition (GRP/GRP/ISO) until the same time point. Whereas a subset of ISO/GRP rats continued to be housed in GRP conditions (ISO/GRP/GRP) until PND70, while another cohort of ISO/GRP animals was put in ISO conditions (ISO/GRP/ISO) until the same time point. Lastly, the ISO/ISO rats prolonged to stay in ISO condition (ISO/ISO/ISO) until PND 70. At this time point, the animals performed behavioural tests: OF, novel object recognition (NOR) and passive avoidance (PA). After the behavioural analyses, the animals were sacrificed to collect PFC for neurochemical and biomolecular investigations. The experimental protocol is demonstrated in Fig. 5.

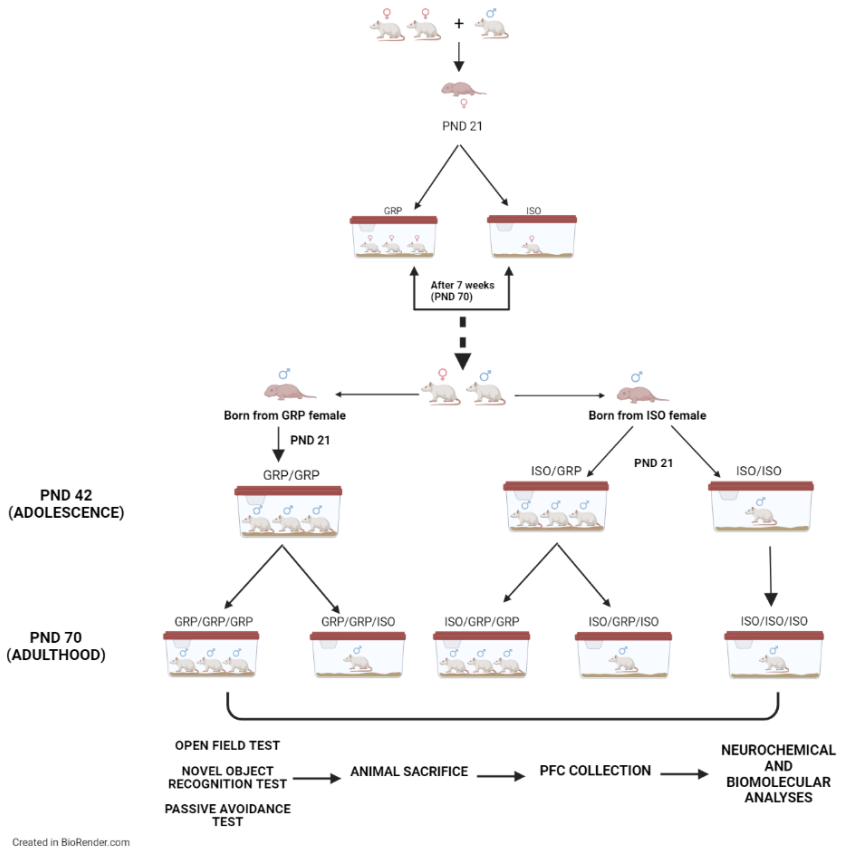

Fig.5 Graphical representation of the experimental protocol.

For the sake of clarity, in Fig. 12 A-C, Fig. 13 A, Fig. 14 A, Fig. 15 A-B, Fig. 16 A-B, Fig. 17 A-D, Fig. 18 A-B, results are shown in graphs by pointing out female rearing condition (GRP or ISO) on x axis and offspring rearing condition in white (GRP/GRP) and black (GRP/ISO) bars.

4.3 Behavioural tests:

a) OF

The OF test was performed to investigate if offspring of socially isolated females showed locomotor deficits as well as anxiety-like behaviour at PND70, also following exposure to stress from adolescence (PND42) until adulthood. It was performed as already described in paragraph 3.3c and analyzed by video-tracking motion analysis system (ANY-maze version 7.0, San Diego Instrument, San Diego, CA). The following parameters were automatically scored: distance travelled (expressed in meters), the number of center entries, time (sec) spent in the center and wall of the arena together with freezing (frequency (number) and time (sec)).

b) NOR test

The same set of rats performed NOR test at PND70, to evaluate if male offspring of socially isolated females had deficits of discrimination ability in adulthood, following exposure to stress in the neurodevelopmental phase. The NOR test was carried out as previously described [56]. Briefly, the experimental protocol

consisted of a training and a test trials, intervalled by an intersession period of 1 min, under dim lighting. In the training trial (T1), rats were left free to explore two identical objects, placed in the center of the experimental arena for 3 min. One min later, the test phase (T2, 3 min) started, and during this trial, one of the familiar objects was replaced by a novel object. The test sessions were video recorded and an operator blind to the experimental groups, measured the time (sec) the animal spent exploring familiar and novel objects (exploratory activity). After each trial, to avoid the presence of olfactory cues that could invalidate the subsequent test, the objects and arena were cleaned with a 50% ethanol solution and dried.

c) PA test

The PA task was carried out in another set of male offspring at PND70. This test was performed to investigate if the preconceptional stress of females had induced an alteration in the learning and memory of adult progeny, also after exposure to stress in the neurodevelopmental phase. In this behavioural experiment, the rat learns to avoid entering a place where it has previously received pain. The latency time (sec) to cross in the punished zone is an index to evaluate memory acquisition in the animal. As previously reported [57], a rectangular apparatus (48x20x22(h) cm) sectioned in two chambers divided by an automatic guillotine door (Ugo Basile, Camerio, Varese, Italy) was used. One compartment was dark and another one had a light (100% as intensity) on the top. Each chamber had a grid floor through which a footshock was delivered. The behavioural test lasted two days.

In the first day (training trial) the rat was put in the illuminated part, facing to dark space and the door was opened. When the animal entered in dark chamber with four legs, the guillotine door was closed and a footshock (0,6 mA for 2 sec) was released. The latency time to enter was recorded. The cut-off time was set at 300 sec. After 15 sec the rat was placed in its home cage and on the second day (24h later) it carried out the test, where footshock wasn't applied and only the latency time to cross in the dark compartment was recorded. After each trial, the apparatus was cleaned with a 50% ethanol solution to avoid inter assay bias.

4.4 Collection of prefrontal cortex (PFC)

The rats were sacrificed at the end of behavioural tests, as delineated in paragraph 3.4. The brains were removed and dissected to collect PFC, one of the most involved areas in memory and learning. Brain tissue was stored at -80°C until the execution of neurochemical and neuromolecular analyses.

4.5 HPLC

To the same aim and by using HPLC with identical parameters mentioned above (paragraph 3.5), we quantified NA, 5-HT, GLU and GABA levels in the PFC of male offspring of socially isolated females.

4.6 Reactive oxygen species (ROS) quantification

The ROS levels were measured to investigate if the preconceptional stress of females and the social isolation through the adolescent phase induced an oxidative stress in PFC of adult male offspring. ROS amount was quantified, as previously described, by using the fluorogenic dye 2'-7'dichlorofluorescein diacetate (Sigma Aldrich, 573 Milano, Italy) [58]. Specifically, the tissue was homogenized in PBS 1X (pH = 7.4), and after the fluorogenic dye (final concentration of 5 mM) was added. The samples were incubated at 37°C for 15 min and then centrifuged (10 min, 4°C, 12.500 rpm); to resuspend the pellet, five ml of PBS 1X were used, and after, it was put on ice for 10 min. Following this, another incubation was carried out at 37°C for 1h; and then the samples were analysed by using a fluorometer (Filter Max F5, Multi-Mode Microplate Reader, Thermo Fisher Scientific, USA, excitation length 475 nm, 579 emission length 535 nm). The results were expressed as µmol DCF/mg of tissue.

4.7 ELISA

To the same aim, namely if adult male offspring of chronic stressed females, following social stress, showed oxidative stress, we also analyzed the amount of pro- and anti- oxidant species in the PFC. In particular, the NOX1, CoQ10 and SOD1 levels were measured, as described in paragraph 3.6, by using the following commercially available kits: Rat NOX1 ELISA kit- MBS2508743, Rat CoQ10

ELISA kit- MBS9364193, Rat SOD1 ELISA kit- MBS2514900 (MyBioSource, San Diego, CA 92195-3308, USA).

4.8 Western Blotting

This technique was performed to understand if the NADPH enzymes and other proteins, linked to production of ROS, were altered in PFC of aged male offspring from isolated females, also following the exposure to stress in the neurodevelopmental phase. In particular, the expression of NOX2 and nuclear factor kappa B (NF-Kb) was evaluated. To extract the total proteins from samples, a Halt™ Protease and Phosphatase Inhibitor Single-Use Cocktail, EDTA-Free (Thermo Fisher Scientific, USA) was added to tissue at 4°C for 10 minutes; after this, the samples were sonicated, stored on ice for 30 min. and centrifuged at 12000 rpm for 10 min at 4 °C. Protein concentrations were measured by Pierce™ BCA protein assay kit (Thermo Fisher Scientific, USA) in the Multiskan™ FC Microplate spectrophotometer (Thermo Fisher Scientific, USA) by using light with wavelengths 570 nm. Then, 50 µg of denatured samples were loaded in the wells of 4–15% Mini-PROTEAN™ TGX Stain-Free™ Protein Gels, to separate proteins by electrophoresis. The proteins were transferred onto Nitrocellulose membrane (Bio-Rad, Italy) using Pierce™ Power Blotter (Thermo Fisher Scientific, USA). Nonspecific binding sites on the membranes were blocked by 5% skimmed milk in PBS1x containing 0.1% Tween 20 for 1 h at room temperature. After, the membranes were overnight incubated at 4°C in diluted

primary antibody solutions to Rabbit monoclonal Anti-NOX2 (ab80508; 1:1000, Abcam, UK), Rabbit polyclonal Anti-NF-kB (ab16502; 1:1000, Abcam, UK), and Mouse monoclonal Anti-β-actin (ab8226; 1:1000, Abcam, UK). Proteins were then labeled with corresponding horseradish peroxidase-conjugated secondary antibodies to Goat anti-rabbit immunoglobulin G (ab6721; 1:5000, Abcam, UK) or goat anti-mouse immunoglobulin G (ab205719; 1:5000, Abcam, UK) for 1 h at room temperature. The Clarity™ Western ECL Substrate was used to catalyze the proteins detection, which were visualized by using a ChemiDoc™ XRS+ and Bio-Rad Image Lab™ software version 5.2.1 (Bio-Rad, Italy). The densitometric intensity of protein bands was measured by using ImageJ software version 1.52 a (National Institutes of Health, USA) and the relative protein levels were normalized to housekeeping protein bands (β-actin).

4.9 Statistical analyses

In order to carry out statistical analysis, the GraphPad 9.0 software for Windows (GraphPad Software, San Diego, CA) was used. We performed Student's t-test with Welch's correction where required and Two-Way ANOVA with Tukey's multiple comparisons test. For all tests, a p-value < 0.05 was considered statistically significant.

5. Results of the first part of the study

In the first part of this study, the analyses of the investigation of the mechanisms, linking stress-induced anxiety, depression and psychosis, showed the following results:

5.1 Development of a depressive-like behaviour in adult rats following seven weeks of social isolation

FST was performed in both GRP and ISO rats in order to evaluate if social isolation could induce a depressive-like behaviour in adulthood. The social deprivation determined a significant increase of immobility frequency (Fig. 6A, Student's t-test, $p < 0,05$), accompanied by a decrease of swimming frequency (Fig. 6C, Student's t-test, $p < 0,05$). The two groups showed no significant difference in struggling frequency (Fig. 6B, Student's t-test, $p > 0.05$).

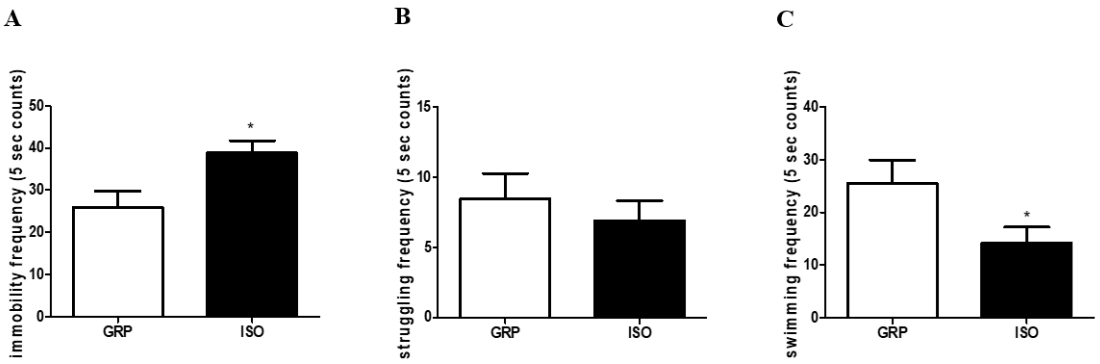

Fig. 6. Depressive-like behaviour in rats exposed to social isolation. (A) Immobility frequency (5 sec counts) of rats reared in group (GRP, n=9) and in isolation (ISO, n=9) conditions. Student's t-test, *p<0.05; (B) Struggling frequency (5 sec counts) of rats reared in group (GRP, n=9) and in isolation (ISO, n=9) conditions. Student's t-test, p>0.05; (C) Swimming frequency (5 sec counts) of grouped (GRP, n=9) and isolated (ISO, n=9) rats. Student's t-test, *p<0.05;

5.2 Development of an anxiety-like behaviour in adult rats following seven weeks of social isolation

EZM and OF were carried out in both GRP and ISO animals to investigate whether social isolation could induce an anxiety-like behaviour in adulthood. The chronic social stress led to a significant decrease in the time spent in open corridors (Fig. 7A, Student's t-test with Welch's correction, $p < 0,05$), accompanied by an increase in the time spent in closed regions (Fig. 7B, Student's t-test with Welch's correction, $p < 0,05$). Furthermore, in the OF test, the ISO animals evidenced increased grooming time respect to the GRP group (Fig. 7C, Student's t-test, $p < 0,001$).

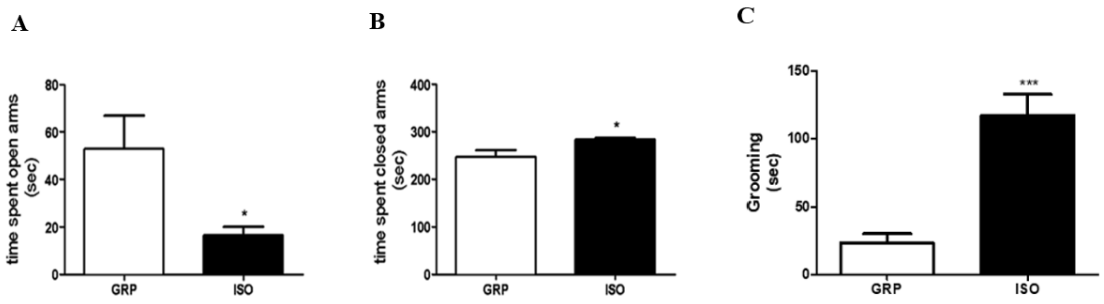

Fig. 7. Anxiety-like behaviour in rats exposed to social isolation. (A) Time (sec) spent in open corridors by grouped (GRP, n=8) and isolated (ISO, n=8) rats. Student's t-test with Welch's correction, *p<0.05; (B) Time (sec) spent in closed regions by rats reared in group (GRP, n=8) and in isolation (ISO, n=8) conditions. Student's t-test with Welch's correction, *p<0.05; (C) Time (sec) spent for grooming by grouped (GRP, n=6) and isolated (ISO, n=7) animals. Student's t-test, ***p<0.001;

5.3 Neurochemical effects of social deprivation in brain regions of adult rats modulating depressive- and anxiety-like behaviour

We measured the NA, 5-HT and its turnover, GLU, and GABA levels in AMY and HIPP to assess whether seven weeks of social isolation altered the noradrenergic, serotonergic systems and excitatory-inhibitory balance. In particular, in AMY of isolated animals, a significant decrease of NA and 5-HT was detected (Fig. 8A-B, Student's t-test, $p < 0.05$), it was accompanied by an increase of 5-HT turnover (Fig. 8C, Student's t-test, $p < 0,01$). Regarding the excitatory-inhibitory balance in AMY, an enhancement of GLU levels (Fig. 8D, Student's t-test, $p < 0,01$) and a reduction of GABA amount (Fig. 8E, Student's t-test, $p<0,05$) were observed in ISO compared to GRP.

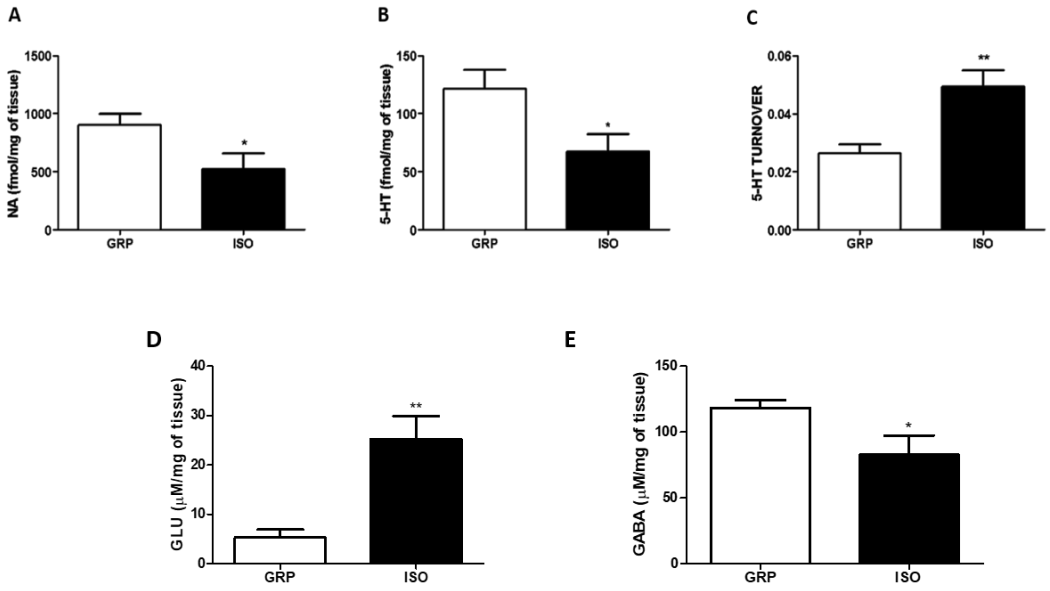

Fig. 8. Neurochemical effects of social isolation in AMY. (A) NA tissue levels (fmol/mg of tissue) in the AMY of grouped (GRP, n=6) and isolated (ISO, n=5) animals. Student's t-test, *p<0.05; (B) 5-HT tissue levels (fmol/mg of tissue) in the AMY of rats reared in group (GRP, n=7) and in isolation (ISO, n=6) conditions. Student's t-test, *p<0.05; (C) 5-HT turnover in the AMY of grouped (GRP, n=6) and isolated (ISO, n=5) rodents. Student's t-test, **p<0.01; (D) GLU tissue levels (µmol/mg of tissue) in the AMY of grouped (GRP, n=6) and isolated (ISO, n=5) animals. Student's t-test, **p<0.01; (E) GABA tissue levels (µmol/mg of tissue) in the AMY of rats reared in group (GRP, n=6) and in isolation (ISO, n=6) conditions. Student's t-test, *p<0.05;

Instead, no significant differences in the NA and 5-HT levels in HIPP, as well as 5-HT turnover, were found between control and isolated animals (Fig. 9A-B-C). However, a significant increase of GLU (Fig. 9D, Student's t-test, $p < 0,01$) and a reduction of GABA (Fig. 9E, Student's t-test, $p<0,05$) quantities were detected after seven weeks of isolation respect to control.

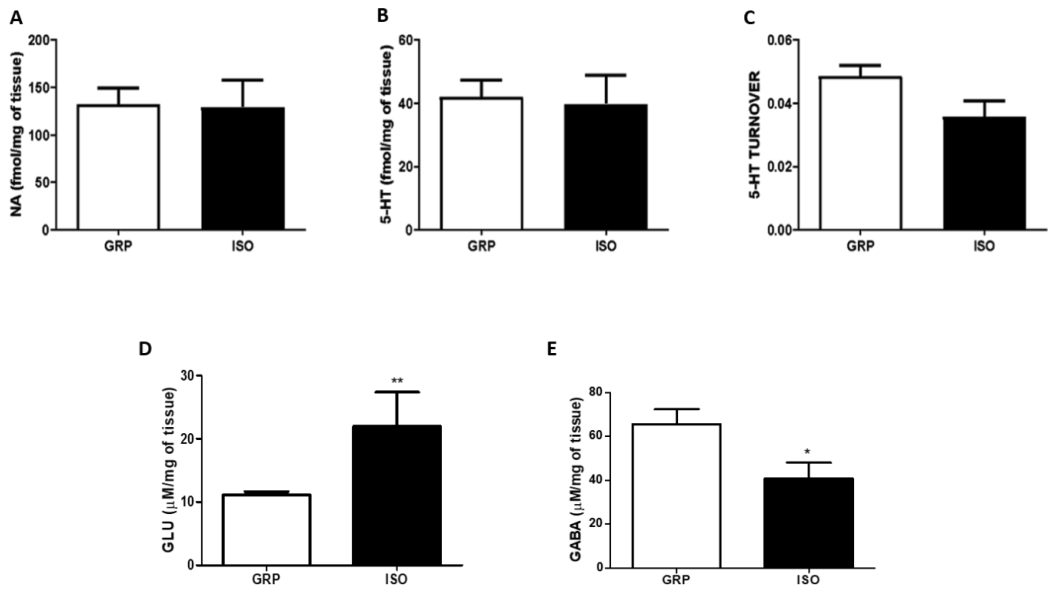

Fig. 9. Neurochemical effects of social isolation in HIPP. (A) NA tissue levels (fmol/mg of tissue) in the HIPP of grouped (GRP, n=6) and isolated (ISO, n=5) animals. Student's t-test, $p>0.05$; (B) 5-HT tissue levels (fmol/mg of tissue) in the HIPP of rats reared in group (GRP, n=6) and in isolation (ISO, n=5) conditions. Student's t-test, $p>0.05$; (C) 5-HT turnover in the HIPP of grouped (GRP, n=6) and isolated (ISO, n=5) rodents. Student's t-test, $p>0.05$; (D) GLU tissue levels (µmol/mg of tissue) in the HIPP of grouped (GRP, n=6) and isolated (ISO, n=6) animals. Student's t-test, $**p<0.01$; (E) GABA tissue levels (µmol/mg of tissue) in the HIPP of rats reared in group (GRP, n=6) and in isolation (ISO, n=6) conditions. Student's t-test, $*p<0.05$;

5.4 Effects of social isolation on peripheral serotonergic and kynurenergic pathways

The 5-HT and KYN amounts, as well as the 5-HT turnover, were measured in plasma to determine if social stress, during neurodevelopmental phase, altered the serotonergic and kynurenergic pathways, at the peripheral level, resulting in

anxiety- and depressive- like behaviour in adulthood. In particular, the isolated animals showed a significant reduction of plasmatic 5-HT level compared to grouped rats (Fig. 10A, Student's t-test, $p < 0.05$), but no significant differences in 5-HT turnover were found in the same animals compared to control (Fig. 10B, Student's t-test, $p > 0.05$). Furthermore, after seven weeks of isolation, the plasmatic KYN levels were enhanced concerning animals reared in grouped conditions (Fig. 10C, Student's t-test, $p < 0.01$).

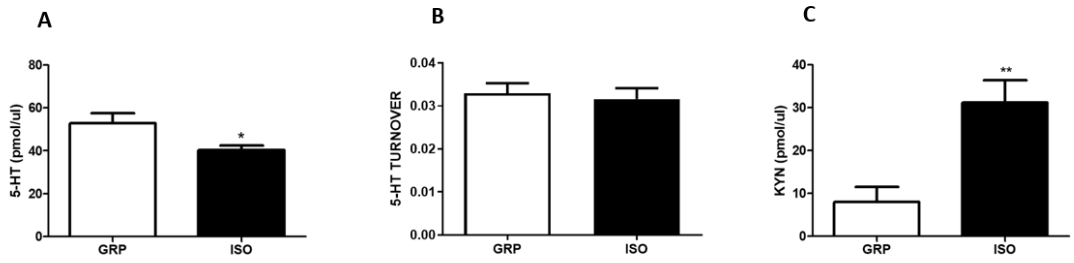

Fig. 10. Effects of social isolation on serotonergic and kynrenergic pathways. (A) 5-HT levels (pmol/µl) in the plasma of grouped (GRP, n=5) and isolated (ISO, n=5) animals. Student's t-test, *p<0.05; (B) 5-HT turnover in the plasma of grouped (GRP, n=5) and isolated (ISO, n=5) rodents. Student's t-test, p>0.05; (C) KYN levels (pmol/µl) in the plasma of rats reared in group (GRP, n=5) and in isolation (ISO, n=5) states. Student's t-test, **p<0.01;

5.5 Effects of social isolation on neuroendocrine modulators of anxiety- and depressive-like behaviour

In order to evaluate the effects of social deprivation on neuroendocrine factors involved in response to stress and in development of anxiety- and depressive- like behaviour, the plasmatic levels of oxytocin, prolactin, ghrelin, melatonin and NPS

were measured by ELISA. The analyses evidenced a significant decrease of oxytocin (Fig. 11A, Student's t-test with Welch's correction, $p < 0.05$), prolactin (Fig. 11B, Student's t-test, $p < 0.05$), ghrelin (Fig. 11C, Student's t-test with Welch's correction, $p < 0.05$) and melatonin (Fig. 11D, Student's t-test with Welch's correction, $p < 0.05$) in isolated rats compared to control. In comparison, no changes were found in plasmatic levels of NPS between the two experimental groups (Fig. 11E, Student's t-test, $p > 0.05$).

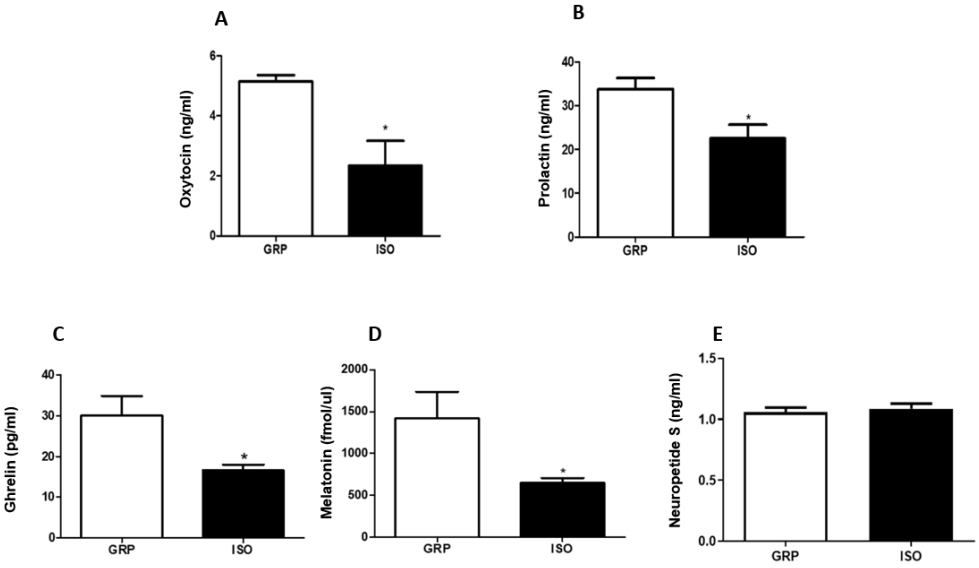

Fig. 11. Effects of social isolation on neuroendocrine modulators. (A) Oxytocin levels (ng/ml) in plasma of grouped (GRP, n=5) and isolated (ISO, n=7) animals. Student's t-test with Welch's correction, *p<0.05; (B) Prolactin levels (ng/ml) in plasma of grouped (GRP, n=5) and isolated (ISO, n=5) rodents. Student's t-test, *p<0.05; (C) Ghrelin levels (pg/ml) in plasma of rats reared in group (GRP, n=5) and in isolation (ISO, n=5) states. Student's t-test with Welch's correction, *p<0.05; (D) Melatonin levels (fmol/µl) in plasma of rats reared in group (GRP, n=5) and in isolation (ISO, n=5) conditions. Student's t-test with Welch's correction, *p<0.05; (E) NPS levels (ng/ml) in plasma of grouped rats (GRP, n=5) and isolated animals (ISO, n=5), Student's t-test, p>0.05;

6. Results of the second part of the study

In the second part of this study, the analyses about the investigation if the anxiety- and depressive-like behavior, in adulthood, could be correlated not only to individual stress but also to a possible chronic stress, suffered by mother, before conception and if this progeny could be vulnerable or resilient to stress in adolescence, showed the following results:

6.1 Effects of stress exposure on young-adult offspring's locomotor activity and anxiety-like behaviour

The OF was carried out on adult rats in order to evaluate the effects of the preconceptional stress on the offspring's locomotor activity, also following exposure to stress in the adolescent phase. The results showed no significant differences in distance travelled (Fig. 12A, Two-Way ANOVA followed by Tukey's post hoc test, $p > 0.05$), time spent in the center (Fig. 12B, Two-Way ANOVA followed by Tukey's post hoc test, $p > 0.05$), and in time spent in the wall (Fig. 12C, Two-Way ANOVA followed by Tukey's post hoc test, $p > 0.05$) between offspring of grouped and isolated females, after exposure to stress in the neurodevelopmental period. Moreover, also when the male litters maintained the same condition as the mother (GRP or ISO) until the adult phase, no significant changes were observed in the distance travelled (Fig. 12D, Student's t-test, $p > 0.05$), in time spent in the center (Fig. 12E, Student's t-test, $p > 0.05$) and in time spent in the wall (Fig. 12F, Student's t-test, $p > 0.05$).

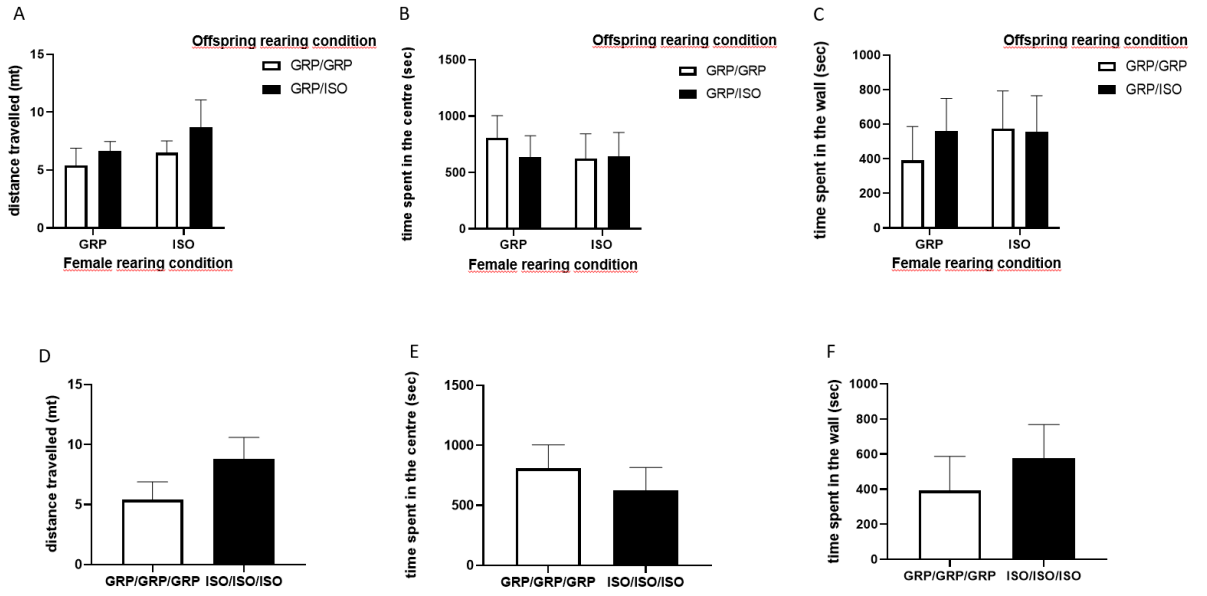

Fig. 12. Effects of the preconceptional stress on the young-adult offspring's locomotor activity, also following chronic exposure to social isolation during adolescence. (A) Distance travelled (mt) in open field test by offspring (reared in group, GRP/GRP, n=8 until adulthood or put in isolation from PND 42 to PND 70, GRP/ISO, n=9) of grouped females and by offspring (housed in group, GRP/GRP, n=7 until adulthood or put in isolation from PND 42 to PND 70, GRP/ISO, n=8) of isolated females. Two-way ANOVA followed by Tukey's post hoc test, $p>0.05$; (B) Time spent in the center (sec) in open field test by offspring (housed in group, GRP/GRP, n=9 until adulthood or put in isolation from PND 42 to PND 70, GRP/ISO, n=10) of grouped females and by offspring (reared in group, GRP/GRP, n=8 until adulthood or put in isolation from PND 42 to PND 70, GRP/ISO, n=8) of isolated females. Two-way ANOVA followed by Tukey's post hoc test, $p>0.05$; (C) Time spent in the wall (sec) in open field test by offspring (reared in group, GRP/GRP, n=9 until adulthood or put in isolation from PND 42 to PND 70, GRP/ISO, n=10) of grouped females and by offspring (housed in group, GRP/GRP, n=8 until adulthood or put in isolation from PND 42 to PND 70, GRP/ISO, n=8) of isolated females. Two-way ANOVA followed by Tukey's post hoc test, $p>0.05$; (D) Distance travelled (mt) in open field test by offspring (reared in group, GRP/GRP/GRP, n= 8, until adulthood) of grouped females and by offspring (reared in isolation, ISO/ISO/ISO, n= 10, until adulthood) of isolated.

Student's t-test, p>0.05; (E) Time spent in the center (sec) in open field test by offspring (reared in group, GRP/GRP/GRP, n= 9, until adulthood) of grouped females and by offspring (reared in isolation, ISO/ISO/ISO, n=10, until adulthood) of isolated. Student's t-test, p>0.05; (F) Time spent in the wall (sec) in open field test by offspring (reared in group, GRP/GRP/GRP, n=9, until adulthood) of grouped females and by offspring (reared in isolation, ISO/ISO/ISO, n=10, until adulthood) of isolated females. Student's t-test, p>0.05;

6.2 Effects of stress exposure on the young-adult offspring's discrimination ability

The NOR test was carried out on adult animals to assess if the preconceptional stress, experienced by the female, altered the discrimination ability in her offspring, also following exposure to chronic stress in the adolescent phase. The data evidenced a significant increase of time spent in exploratory activity on the novel object compared to the familiar one by grouped offspring of GRP females (Fig. 13A, Two-Way ANOVA followed by Tukey's post hoc test, $p < 0.0001$). The same result was also found after exposure to stress during adolescence (Fig. 13A, Two-Way ANOVA followed by Tukey's post hoc test, $p < 0.05$). Whereas, for offspring of isolated females, no significant changes were detected in exploratory activity between two objects, also following social stress in the neurodevelopmental phase (Fig. 13A, Two-Way ANOVA followed by Tukey's post hoc test, $p > 0.05$). Furthermore, when the male litters maintained the grouped condition of the mother until adult phase, evidenced a significant increase of exploratory activity on the novel object versus to the familiar one (Fig. 13B, Two-

Way ANOVA followed by Tukey's post hoc test, $p < 0.01$); on the other hand, if the litter remain in isolation condition, as well as mother, no significant differences were observed in exploratory activity between two objects (Fig. 13B, Two-Way ANOVA followed by Tukey's post hoc test, $p > 0.05$).

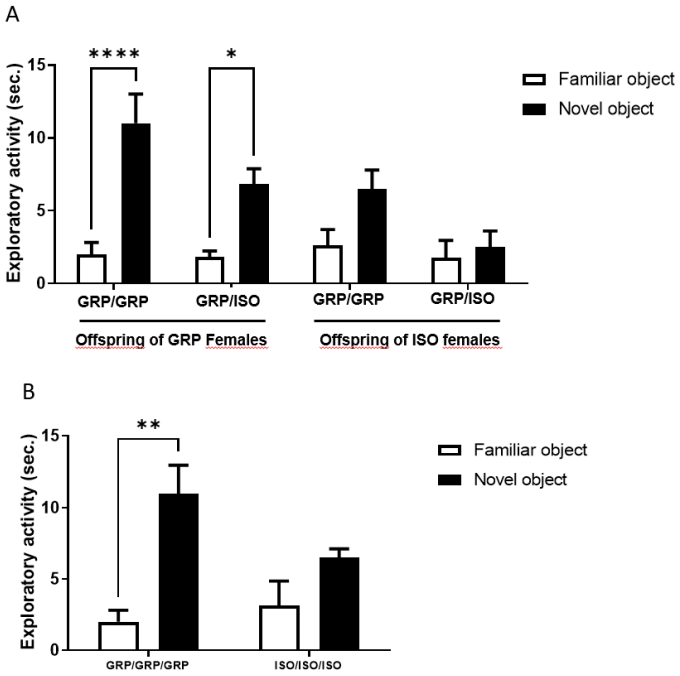

Fig. 13. Effects of the preconceptional stress on the young-adult offspring's discrimination ability, also following chronic exposure to social isolation during adolescence. (A) Exploratory activity (sec) on familiar and novel objects by offspring (reared in group, GRP/GRP, n= 6 until adulthood, Two-way ANOVA followed by Tukey's post hoc test, ****$p < 0,0001$, or put in isolation, GRP/ISO, n=6 from PND 42 to PND 70, Two-way ANOVA followed by Tukey's post hoc test, *$p < 0,05$) of grouped females and by offspring (reared in group, GRP/GRP, n= 8 until adulthood or put in isolation, GRP/ISO, n= 8 from PND 42 to PND 70, Two-way ANOVA followed by Tukey's post hoc test, $p>0.05$) of isolated females.; (B) Exploratory activity (sec) on familiar and novel objects by offspring (reared in group, GRP/GRP/GRP, n= 6 until adulthood, Two-way ANOVA followed by Tukey's post hoc test, **$p < 0,01$) of grouped

females and by offspring (reared in isolation, ISO/ISO/ISO, n= 8 until PND 70, Two-way ANOVA followed by Tukey's post hoc test, $p>0.05$) of isolated females.

6.3 Effects of stress exposure on the young-adult offspring's learning and memory

The passive task was accomplished on the adult litter to investigate whether the preconceptional stress of females impacted on the learning and memory processes in progeny, also after chronic exposure to stress in the adolescent phase. In this test, a significant reduction of latency time (sec) was observed in the offspring (housed both in group and put in isolation from adolescence to adulthood) of isolated females compared to that (reared in grouped state until PND70) of grouped (Fig. 14A, Two-Way ANOVA followed by Tukey's post hoc test, $p < 0.05$). Moreover, when the male litters preserve the isolated condition of mother, from weaning to adulthood, they showed a significant decrease in latency time (sec) compared to control (Fig. 14B, Student's t-test with Welch's correction, $p < 0.01$).

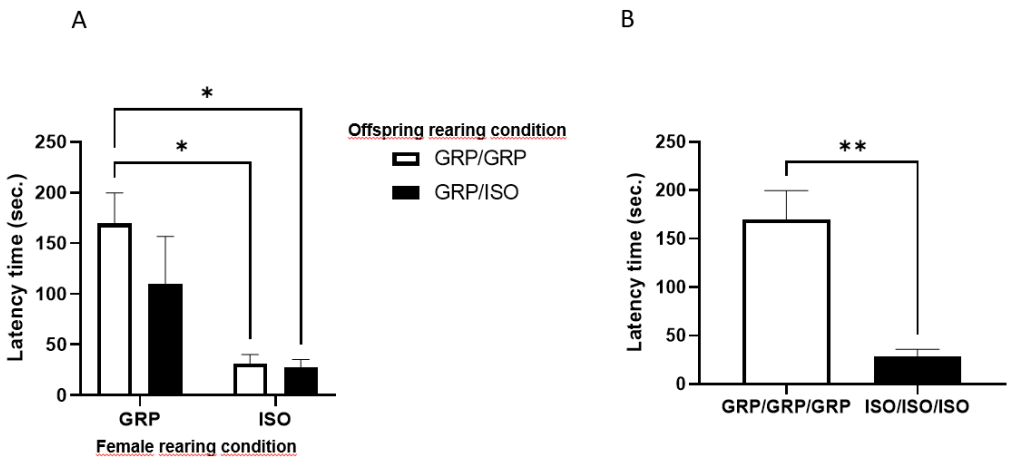

Fig. 14. Effects of the preconceptional stress on the young-adult offspring's learning and memory, also following chronic exposure to social isolation during adolescence. (A) Latency time (sec) for offspring (reared in group, GRP/GRP, n= 6 until adulthood or put in isolation, GRP/ISO, n=7 from PND 42 to PND 70) of grouped females and for offspring (reared in group, GRP/GRP, n= 7 until adulthood or put in isolation, GRP/ISO, n= 6 from PND 42 to PND 70) of isolated females, Two-Way ANOVA followed Tukey's post hoc test, *$p < 0.05$; (B) Latency time (sec) for offspring (reared in group, GRP/GRP/GRP, n=6 until adulthood) of grouped females and for isolated litter (of isolated mothers) until PND 70, ISO/ISO/ISO, n=12, Student's t-test with Welch's correction, **$p<0.01$;

6.4 Effects of stress exposure on the offspring's cortical serotonergic and noradrenergic systems

The HPLC was carried out to order to assess if the female's preconception of social stress altered the serotonergic and noradrenergic systems in the PFC of progeny, also following exposure to chronic stress during the neurodevelopmental period. The results evidenced a significant enhancement of the NA levels in the offspring (housed in grouped condition until adulthood) of isolated females

compared to grouped (Fig. 15A, Two-Way ANOVA followed by Tukey's post hoc test, $p < 0.001$). The same result was found between two offspring (GRP/ISO), of both females, also after the social stress exposure in adolescence (Fig. 15A, Two-Way ANOVA followed by Tukey's post hoc test, $p < 0.001$). Instead, no significant changes of the 5-HT levels were evidenced between the offspring of two females, following exposure to chronic stress in the neurodevelopmental period (Fig. 15B, Two-Way ANOVA followed by Tukey's post hoc test, $p > 0.05$). Moreover, when the male litters maintain the condition of isolation or grouping, like their own mother, from weaning to adulthood, no significant differences in the NA amount were observed between the groups (Fig. 15C, Student's t-test with Welch's correction, $p > 0.05$). On the other hand, a significant reduction of the 5-HT level was detected in the PFC of isolated offspring of ISO females compared to a grouped litter of grouped (Fig. 15D, Student's t-test with Welch's correction, $p < 0.05$).

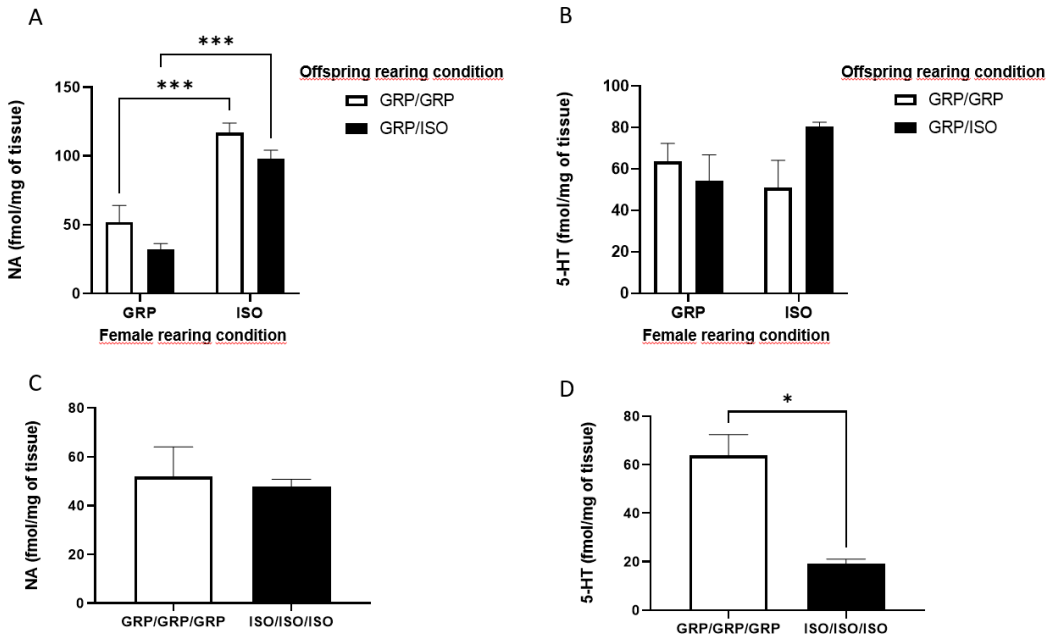

Fig. 15. Effects of the preconceptional stress on the serotonergic and noradrenergic systems in the PFC of offspring, also following exposure to chronic stress in adolescence. (A) NA tissue levels (fmol/mg of tissue) in the PFC of offspring (reared in group, GRP/GRP, n= 4 until adulthood or put in isolation, GRP/ISO, n=4 from PND 42 to PND 70) of grouped females and of offspring (reared in group, GRP/GRP, n= 4 until adulthood or put in isolation, GRP/ISO, n= 4 from PND 42 to PND 70) of isolated, Two-Way ANOVA followed Tukey's post hoc test, ***$p < 0.001$; (B) 5-HT tissue levels (fmol/mg of tissue) in the PFC of offspring (reared in group, GRP/GRP, n=4 until adulthood or put in isolation, GRP/ISO, n=4 from PND 42 to PND 70) of grouped females and of offspring (reared in group, GRP/GRP, n=4 until adulthood or put in isolation, GRP/ISO, n=4 from PND 42 to PND 70) of isolated females, Two-Way ANOVA followed Tukey's post hoc test, $p > 0.05$; (C) NA tissue levels (fmol/mg of tissue) in the PFC of offspring (reared in group, GRP/GRP/GRP, n=4 until adulthood) of grouped females and of isolated litter (of isolated females) until PND 70, ISO/ISO/ISO, n=4, Student's t-test with Welch's correction, $p>0.05$; (D) 5-HT tissue levels (fmol/mg of tissue) in the PFC of offspring (reared in group, GRP/GRP/GRP, n=4 until adulthood) of grouped females and of

isolated litter (of isolated females) until PND 70, ISO/ISO/ISO, n=4, Student's t-test with Welch's correction, *p<0.05;

6.5 Effects of stress exposure on the offspring's cortical excitatory-inhibitory balance

The HPLC was also performed to evaluate a possible alteration of excitatory-inhibitory balance in the PFC of adult progeny of socially isolated females, also following exposure to chronic stress during the neurodevelopmental period. In particular, no significant differences were observed in the GLU levels between the two offspring (of GRP and ISO females), following the social stress exposure in the neurodevelopmental phase (Fig. 16A, Two-Way ANOVA followed by Tukey's post hoc test, $p > 0.05$). On the other hand, a significant decrease of the GABA quantity was observed in the PFC of offspring (reared in isolated state from PND 42 to PND 70) of GRP females in comparison with control (Fig. 16B, Two-Way ANOVA, followed by Tukey's post hoc test $p < 0.05$); but no significant changes were detected in the offspring of isolated female, if put in isolation condition during neurodevelopmental period, compared to grouped condition (Fig. 16B, Two-Way ANOVA followed by Tukey's post hoc test, $p > 0.05$). Additionally, when the male litters maintain the condition of isolation or grouping, like their own mother, from weaning to adulthood, no significant changes were observed in the GLU levels between the groups (Fig. 16C, Student's t-test, $p > 0.05$). Instead, a significant reduction of the GABA quantity was

detected in the PFC of isolated offspring of ISO females compared to the control (Fig. 16D, Student's t-test, $p < 0.05$).

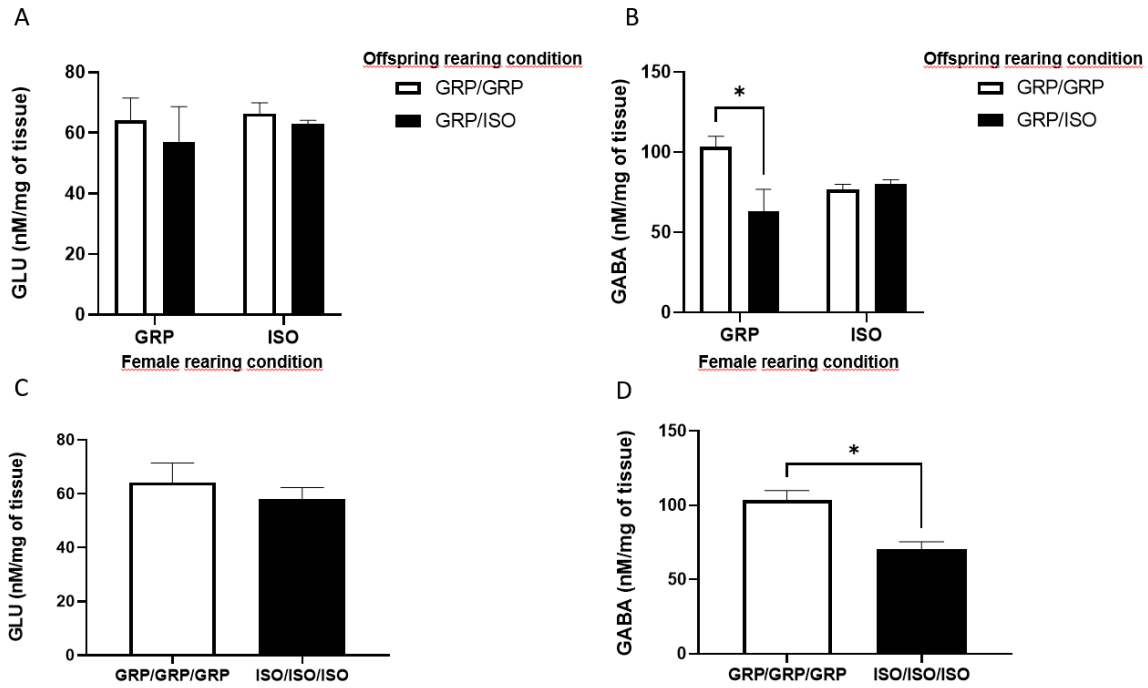

Fig. 16. Effects of the preconceptional stress on excitatory-inhibitory balance in the PFC of offspring, also following exposure to chronic stress in adolescence. (A) GLU tissue levels (nM/mg of tissue) in the PFC of offspring (reared in group, GRP/GRP, n=4 until adulthood or put in isolation, GRP/ISO, n=4 from PND 42 to PND 70) of grouped females and of offspring (reared in group, GRP/GRP, n= 4 until adulthood or put in isolation, GRP/ISO, n= 4 from PND 42 to PND 70) of isolated females, Two-Way ANOVA followed Tukey's post hoc test, $p > 0.05$; (B) GABA tissue levels (nM/mg of tissue) in the PFC of offspring (reared in group, GRP/GRP, n=4 until adulthood or put in isolation, GRP/ISO, n=4 from PND 42 to PND 70) of grouped females and of offspring (reared in group, GRP/GRP, n=4 until adulthood or put in isolation, GRP/ISO, n=4 from PND 42 to PND 70) of isolated, Two-Way ANOVA followed Tukey's post hoc test, $*p < 0.05$; (C) GLU tissue levels (nM/mg of tissue) in the PFC of offspring (reared in group, GRP/GRP/GRP, n=4 until adulthood) of grouped females and of isolated litter (of isolated mothers) until PND 70, ISO/ISO/ISO, n=4, Student's t-test, $p>0.05$;

(D) GABA tissue levels (nM/mg of tissue) in the PFC of offspring (reared in group, GRP/GRP/GRP, n=4 until adulthood) of grouped females and of isolated litter (of isolated mothers) until PND 70, ISO/ISO/ISO, n=4, Student's t-test, *p<0.05;

6.6 Effects of stress exposure on the offspring's cortical ROS production

The quantity of ROS and relative producers were evaluated, by performing the ROS measurement, ELISA and WB, in the PFC of adult progeny to investigate if the exposure to social stress of females, before conception, induced an oxidative stress in adult offspring, also following exposure to chronic stress in the neurodevelopmental phase. The data showed no significant differences in the ROS amount between the two offspring (of GRP and ISO female), following the chronic exposure to social stress in the neurodevelopmental phase compared to control (Fig. 17A, Two-Way ANOVA followed by Tukey's post hoc test, $p > 0.05$). The similar results of NF-kB (Fig. 17B, Two-Way ANOVA followed by Tukey's post hoc test, $p > 0.05$) and of NOX2 (Fig. 17C, Two-Way ANOVA followed by Tukey's post hoc test, $p > 0.05$) expressions were found. Instead, a significant decrease of NOX1 levels was detected in the PFC of offspring (reared in isolated state from PND 42 to PND 70) compared to grouped one, until adulthood, of GRP females. The same results direction was found in the PFC of the grouped litter of ISO females compared to that one of grouped (Fig. 17D, Two-Way ANOVA followed by Tukey's post hoc test, $p < 0.01$). On the other hand, NOX1 increased in the offspring, after chronic social stress exposure in

adolescence, of ISO females compared to that grouped one of the same females and also versus offspring (housed in isolated conditions in neurodevelopmental period) of grouped (Fig. 17D, Two-Way ANOVA followed by Tukey's post hoc test, $p < 0.05$). But if the male litters were housed in isolated conditions, like their own mother, from weaning to adulthood, a significant increase of ROS quantity was observed in the PFC of adult rats, compared to control (Fig. 17E, Student's t-test, $p < 0.01$). On the other hand, a significant reduction of Nf-kB (Fig 17F, Student's t-test, $p < 0.001$), NOX2 (Fig. 17G, Student's t-test, $p < 0.01$) and of NOX1 (Fig. 17H, Student's t-test, $p < 0.001$) quantity was observed in the PFC of mentioned above animals, compared to GRP/GRP/GRP group.

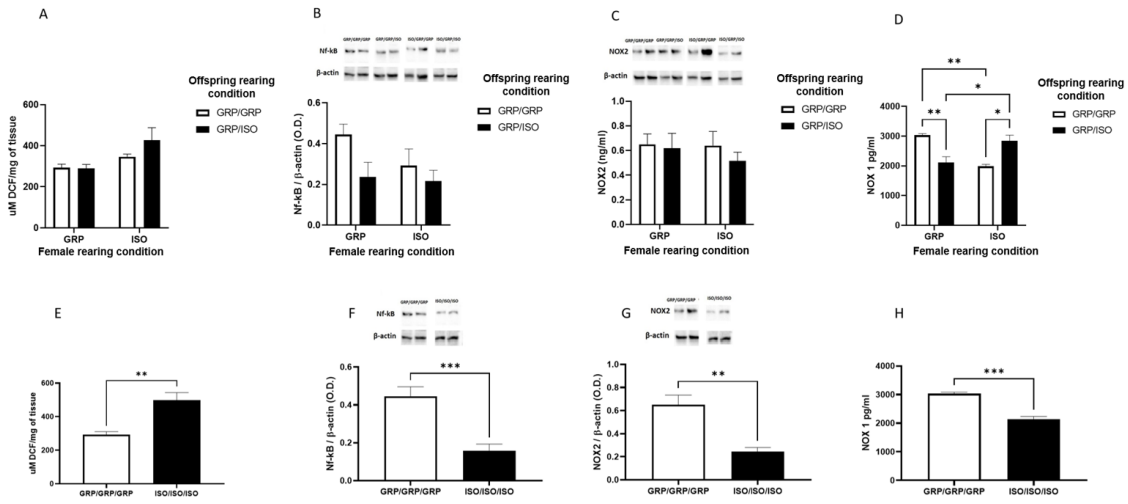

Fig. 17. Effects of the preconceptional stress on the ROS production in the PFC of offspring, also after exposure to chronic stress in the neurodevelopmental phase (A) Measure of ROS amount in the PFC of offspring (reared in group, GRP/GRP, n=4 until adulthood or put in isolation, GRP/ISO, n=4 from PND 42 to PND 70) of grouped females and of offspring (reared in group, GRP/GRP, n=4 until adulthood or put in isolation, GRP/ISO, n=4 from PND 42 to

PND 70) of isolated females, Two-Way ANOVA followed Tukey's post hoc test, $p > 0.05$; (B) Representative images of Western Blotting bands of NF-κB and β-actin housekeeping gene in the PFC of offspring of grouped and isolated females, control and after exposure to chronic stress in the neurodevelopmental phase; and quantification of the optical band density of NF-κB normalized for optical band density of β-actin housekeeping gene in the PFC of offspring (reared in group, GRP/GRP, n=6 until adulthood or put in isolation, GRP/ISO, n=5 from PND 42 to PND 70) of grouped females and of offspring (reared in group, GRP/GRP, n=6 until adulthood or put in isolation, GRP/ISO, n=5 from PND 42 to PND 70) of isolated, Two-Way ANOVA followed Tukey's post hoc test, $p > 0.05$; (C) Representative images of Western Blotting bands of NOX2 and β-actin housekeeping gene in the PFC of offspring of grouped and isolated females, control and after exposure to stress in the neurodevelopmental phase; and quantification of the optical band density of NOX2 normalized for optical band density of β-actin housekeeping gene in the PFC of offspring (reared in group, GRP/GRP, n=6 until adulthood or put in isolation, GRP/ISO, n=6 from PND 42 to PND 70) of grouped females and of offspring (reared in group, GRP/GRP, n=6 until adulthood or put in isolation, GRP/ISO, n=6 from PND 42 to PND 70) of isolated females, Two-Way ANOVA followed Tukey's post hoc test, $p > 0.05$; (D) NOX1 (pg/ml) in the PFC of offspring (reared in group, GRP/GRP, n=4 until adulthood or put in isolation, GRP/ISO, n=4 from PND 42 to PND 70) of grouped females and of offspring (reared in group, GRP/GRP, n=3 until adulthood or put in isolation, GRP/ISO, n=4 from PND 42 to PND 70) of isolated females, Two-Way ANOVA followed Tukey's post hoc test, $*p < 0.05$, $**p < 0.01$; (E) Measure of ROS amount in the PFC of offspring (reared in group, GRP/GRP/GRP, n=4 until adulthood) of grouped females and of offspring (reared in isolation, ISO/ISO/ISO, n=4 until PND 70) of isolated, Student's t-test, $**p < 0.01$; (F) Representative images of Western Blotting bands of NF-κB and β-actin housekeeping gene in the PFC of offspring which maintain the same social condition of their mothers (GRP or ISO) until adulthood; and quantification of the optical band density of NF-κB normalized for optical

band density of β-actin housekeeping gene in the PFC of offspring (reared in group, GRP/GRP/GRP, n=6 until adulthood) of grouped females and of litter (housed in isolation, ISO/ISO/ISO, n=6 until PND 70) of isolated females, Student's t-test ,***$p < 0.001$; (G) Representative images of Western Blotting bands of NOX2 and β-actin housekeeping gene in the PFC of offspring which maintain the same social condition of their mothers (GRP or ISO) until adulthood; and quantification of the optical band density of NOX2 normalized for optical band density of β-actin housekeeping gene in the PFC of offspring (reared in group, GRP/GRP/GRP, n=6 until adulthood) of grouped females and of litter (housed in isolation, ISO/ISO/ISO, n=5 until PND 70) of isolated, Student's t-test, **$p < 0.01$; (H) NOX 1 (pg/ml) in the PFC of offspring (reared in group, GRP/GRP/GRP, n=4 until adulthood) of grouped females and of litter (housed in isolation, ISO/ISO/ISO, n=4 until PND 70) of isolated females, Student's t-test, ***$p < 0.001$;

6.7 Effects of stress exposure on the offspring's antioxidant species production

SOD1 and CoQ10 were measured in the PFC of adult offspring in order to evaluate if the exposure to social stress of the mother, before conception, led to less production of these species, resulting in oxidative stress in adult rats, also following exposure to chronic social stress in adolescence. The analyses, performed by ELISA, evidenced a significant increase of the SOD1 levels in the PFC of offspring (housed in GRP condition until adulthood) of isolated females compared to that (reared in the same state) of grouped (Fig. 18A, Two-Way ANOVA followed by Tukey's post hoc test, $p < 0.05$). In addition to this, a significant enhancement of the CoQ10 amount was found in the PFC of offspring

(of GRP female), exposed to social stress in adolescence, compared to control (Fig. 18B, Two-Way ANOVA followed by Tukey's post hoc test, $p < 0.0001$). On the other hand, CoQ10 decreased in offspring, housed in isolated state from PND 42 to PND 70, of ISO females versus to that one of grouped (Fig. 18B, Two-Way ANOVA followed by Tukey's post hoc test, $p < 0.0001$). When the male litters were reared in the isolated state, like their own mother, from weaning to adulthood, the SOD1 levels were enhanced in the PFC of aged rats compared to the control (Fig. 18C, Student's t-test, $p < 0.01$). Instead, in the same region, no significant differences were observed in the CoQ10 amount between the two offspring, which were housed in the same social condition as their mothers, grouped and isolated, respectively (Fig. 18D, Student's t-test, $p > 0.05$).

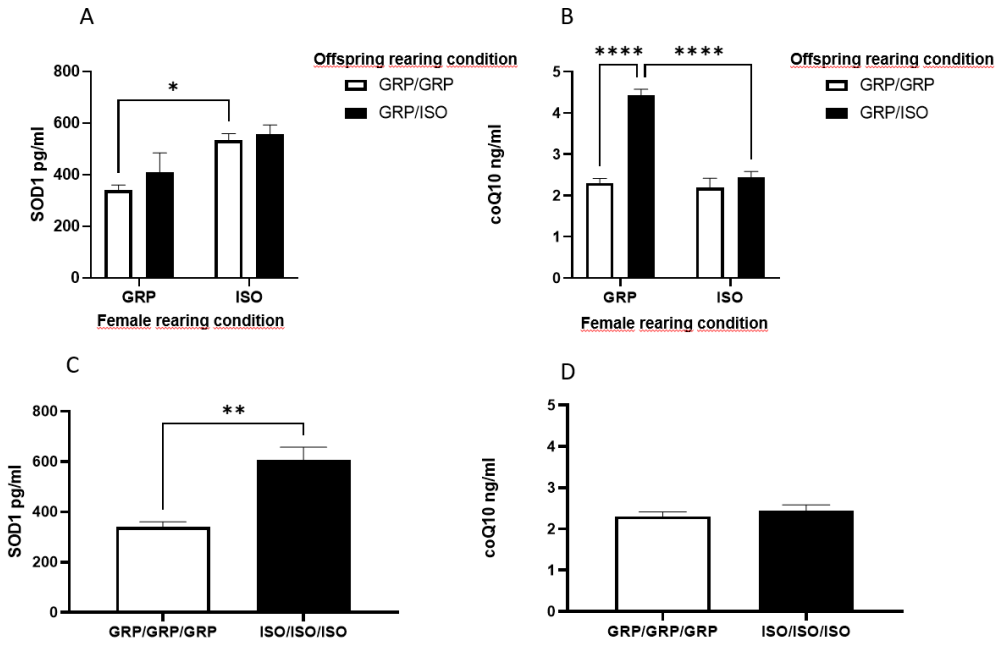

Fig. 18. Effects of the preconceptional stress on antioxidant species production in the PFC of offspring, also after exposure to chronic stress in the neurodevelopmental phase. (A) SOD1 (pg/ml) in the PFC of offspring (reared in group, GRP/GRP, n=4 until adulthood or put in isolation, GRP/ISO, n=4 from PND 42 to PND 70) of grouped females and of offspring (reared in group, GRP/GRP, n=4 until adulthood or put in isolation, GRP/ISO, n=4 from PND 42 to PND 70) of isolated, Two-Way ANOVA followed Tukey's post hoc test, *$p < 0.05$; (B) CoQ10 (ng/ml) in the PFC of offspring (reared in group, GRP/GRP, n=4 until adulthood or put in isolation, GRP/ISO, n=4 from PND 42 to PND 70) of grouped females and of offspring (reared in group, GRP/GRP, n=4 until adulthood or put in isolation, GRP/ISO, n=4 from PND 42 to PND 70) of isolated, Two-Way ANOVA followed Tukey's post hoc test,****$p < 0.0001$; (C) SOD1 (pg/ml) in the PFC of offspring (reared in group, GRP/GRP/GRP, n= 4 until adulthood) of grouped females and of isolated litter (of isolated mothers) until PND 70, ISO/ISO/ISO, n=4, Student's t-test, **$p<0.01$; (D) CoQ10 (ng/ml) in the PFC of offspring (reared in group, GRP/GRP/GRP, n=4 until adulthood) of grouped females and of isolated litter (of isolated mothers) until PND 70, ISO/ISO/ISO, n=4, Student's t-test, $p>0.05$;

7. Discussion

In the first part of the study, we evidenced that exposure to social isolation, during the neurodevelopmental phase, induced an anxiety- and depressive-like behaviour in adult rats. Indeed, scientific evidence showed that the social deprivation, from PND28 to PND72, induced, in adult rats, an aggressive and fear behaviour together with deficits in acquiring extinction learning, cognition and a decrease of time spent in the open arm of an elevated plus maze, resulting in an anxiety-like behaviour. These effects could not be reversed by periods of resocialization [59]. The anxious attitude was also shown in OF test where the increase of locomotor activity was detected after six weeks of isolation post-weaning [60]. After five weeks of isolation, other studies observed that the adult animals showed an increased immobility in the FST resulting in a depressive attitude [61]. Physiologically, the response to acute stress is important to preserve the body homeostasis. However, the response to chronic stress is dysregulated, resulting in brain alteration at structural and functional levels. Indeed, the post-mortem studies evidenced a reduced volume of PFC and HIPP with loss of neurons and synaptic plasticity in patients with stress-induced disorders such as anxiety and depression [62]. In particular, the chronic social deprivation, during the neurodevelopmental phase, represents a state of stress for animals. It alters normal brain maturation and neuronal activity, leading to the possible development of mental disorders in adulthood. Indeed, during the postnatal period, the development of brain continues with expansion/regression of grey matter (increase and refinement of synapses) and with the maturation of white matter, which consists of neuronal

myelination. Based on this, both processes lead to the maturation of neuronal connectivity and neurobehavioral functions. The maturation regards all brain areas, including regions of limbic system that control and regulate stress, mood and emotions, in particular, AMY, PFC and HIPP. Indeed, in response to stress, the limbic system and the HPA axis have a key role. Under the action of catecholamines, the HYP releases the CRH neuropeptide that stimulates the anterior pituitary gland to produce the ACTH, which acts on the adrenal gland to generate glucocorticoids that, together with catecholamines, activate the regions of the limbic system. The limbic zones work in parallel and send inputs to regulate the HPA axis and autonomic system responses to stress agents. In regard to chronic stress, an overproduction of glucocorticoids alters the negative feedback mechanism of the HPA axis. It induces the GRs desensitization in several areas, resulting in generalized change [30]. In our experimental conditions, after seven weeks of isolation, decreased levels of 5-HT and NA were detected in the AMY of isolated animals compared to grouped rats. An increase of 5-HT turnover accompanies this reduction. Such results are in accordance with previous studies that observed a reduction of noradrenergic and serotonergic transmission in the AMY of isolated rats [63], together with other analyses that found an involvement of enhancement of 5-HT turnover in the development of pathological states [64]. Instead, in the HIPP, we detected no significant differences in NA, 5-HT levels and 5-HT turnover. These results established that the catecholaminergic changes, induced by chronic social deprivation are specific for the AMY, which is

consistent with literature data. Indeed, scientific evidence demonstrated that the social experience, during the post-weaning period, is important for the development of normal behaviours and the AMY is the most involved area in the social behaviour. The patients, showing unusual social behaviours, usually, evidenced an altered activity of this brain area. The AMY is mainly implicated in processing social information and in the control of mood and emotions, particularly fear response. It is connected to the locus coeruleus, which produces NA in response to stress, and activates the HPA axis, being also connected to the paraventricular nucleus of the HYP. Reciprocal connections exist between the AMY, thalamus, cortical areas and the HIPP. Specifically, the AMY receives sensory input about anxiety-inducing stimuli from thalamus, more processed information from cortical regions and contextual input from HIPP. Following this, the AMY sends input to striatum and periaqueductal gray which promote the autonomic, skeletalmotor and endocrine responses to anxiety [63]. Animal studies showed that the post-weaning isolation leads to altered social behaviour in adult rats. This modified attitude is accompanied by a decreased volume of AMY with changes in the morphology and activity of its neurons. Indeed, they evidenced, by electrophysiological analyses, that the chronic social stress exposure decreased the glutamatergic synaptic inputs, leading to a progressive reduction of postsynaptic membrane excitability in the AMY, being less coordinated with other brain areas, and so inducing the impairments at behavioural level [65]. Our results demonstrated an enhanced level of KYN in the isolated animals, where

the central serotonergic reduction is also reflected in the periphery. Scientific evidence demonstrated that the reduction of peripheral 5-HT and increased plasmatic KYN are involved in development of stress-induced anxiety and depression. Physiologically, in response to stress, the organism accelerates the 5-HT production from tryptophan by inhibiting the alternative pathway and the final reduction of KYN amount. But under the chronic stress condition, the overproduction of glucocorticoids activates the tryptophan 2,3-dioxygenase (TDO) with the increase of the KYN quantity and reduction of 5-HT. Indeed, the conversion of TRP to KYN is controlled by two enzymes: TDO and indolamine 2,3-dioxygenase [66]. Moreover, following seven weeks of isolation, we found an increase of GLU and a decrease of GABA levels in AMY and HIPP. Indeed, it was previously shown that the glutamatergic projections from a basolateral complex of the AMY to the ventral HIPP, mediate anxiety and reduce the social behaviours [67]. In addition to this, it was reported that in isolated animals, the expression of NMDA receptor for GLU increases in the HIPP, indeed the inhibition of this receptor reduces the negative outcomes of stress. However, it also has anticonvulsant, antidepressant and anxiolytic effects. Moreover, an increase in glutamatergic signal results from reduced expression of GABAergic receptors in CNS, induced by social isolation. In general, the release of GABA increases when the 5-HT3 receptor, expressed by GABAergic neurons, is activated by 5-HT. Therefore, a reduction of 5-HT level, as well as evidenced in our experimental conditions, induces a less release of GABA. In this context, the

dysfunction of the GABA system, which results in the reduction of GABAergic neurotransmission, together with the serotonergic one, was previously correlated to anxiety disorder [68]. Other scientific studies found a correlation between less expression of PV-positive GABAergic interneurons in the HIPP and a depressive-like behaviour, as well as evidenced by increased immobility during the tail suspension test, following social deprivation in the neurodevelopmental phase [28]. Furthermore, in addition to 5-HT, the NA and other catecholamines mediate the excitatory and inhibitory neurotransmission in AMY [69]. Based above, we could deduce that chronic stress (i.e., social isolation) might alter the excitatory-inhibitory balance by modifying the catecholaminergic signaling and leading to the development of anxiety- and depressive-like behaviour. But also, the hypothalamic oxytocinergic circuits modulate the GABAergic signaling in the AMY, indeed, the endogenous oxytocin enhances the activity of the amygdalar GABAergic interneurons. In particular, the oxytocin-expressing neurons, in the paraventricular nucleus of HIPP, are projected to other brain regions like AMY. Such circuit appears to modulate the fear response. In fact, it was reported that oxytocin mediates social stress and modulates the activity of the AMY. In this context, after five weeks of social isolation, a study evidenced that the production of oxytocin in the HYP was less. However, the mRNA transcription of oxytocin receptor in AMY was also reduced, resulting in an alteration of GABAergic transmission and an anxiety-, depressive-like behaviour [70]. In addition, clinical studies showed that an enhancement of GABA and oxytocin prevents the

evolution of anxiety- and depressive-like attitude in women, during the postpartum period. Indeed, in this phase hormones, neuropeptides and neurotransmitters influence the mother's emotional and mood states [71]. These reported data are in accordance with our results, which evidenced a decrease of plasmatic oxytocin after seven weeks of isolation post-weaning; underlining that this neuropeptide is important in the regulation of sociability and also that its reduction, together with that of the GABA, is involved in the development of depressive- and anxiety-like behaviour. Moreover, the reduced oxytocin amount is also correlated to a decrease in 5-HT level. Indeed, scientific studies demonstrated that the 5-HT stimulates the hypothalamic neurons to express oxytocin and it was observed how the oxytocin is released in response to treatment with selective 5-HT reuptake inhibitors [72]. In our experimental conditions, after social deprivation post-weaning, also a decrease in plasmatic melatonin was evidenced in adult rats. This hormone is synthesized and released by the pineal gland according to a circadian cycle (it is high during the night and low in the day). The melatonin is involved in the physiological response to stress because it decreases the plasmatic CORT amount, by inhibiting the adrenocorticotropin, and increases the 5-HT level. This lead to elevated release of oxytocin with subsequent suppressing of altered behaviour, induced by stress. The pineal gland presents a large quantity of GRs and, for this reason, under the chronic stress condition, it is a target of overproduced glucocorticoids that induce the alterations, such as the reduction of melatonin release. This effect leads to increased CORT levels,

decreased 5-HT and oxytocin levels, and reduced GABAergic transmission in AMY with subsequent development of anxiety- and depressive-like behaviour [73]. Moreover, it was also reported that the melatonin treatment improves the anxiety- and depressive-like attitude, by inhibiting the oxidative stress and neuroinflammation induced by chronic stress. Indeed, the melatonin has antioxidant, anti-inflammatory and anti-apoptotic properties; it protects the glutathione peroxidase, catalase and other enzymes which can scavenge the ROS. To demonstrate this, the analyses in vitro, similar to the in vivo results, showed that the melatonin is enabled to reverse the inactivation of antioxidant enzymes, induced by H_2O_2 in animals that were exposed to stress. The exposure to chronic stress causes oxidative stress, inducing to neuroinflammation in the brain, by activating the NF-kB pathway. Indeed, NF-kB translocates in the nucleus and activates the inflammatory cytokines, as observed in humans and also in the cortex and in the HIPP of rats, after exposure to stress. Moreover, under stress, the microglia is also more activated and releases several proinflammatory cytokines that induce neurodegeneration and subsequent autophagy with apoptosis of nerve cells, loss of neurons and ultimately the development of anxiety- and depressive-like behaviours. In this context, it was reported that the melatonin treatment inhibited the NF-kB activation in the HIPP and also the proinflammatory cytokines action, resulting in an improvement of anxiety- and depressive-like behaviours, caused by the chronic stress exposure [74]. In addition, together with the decrease of plasmatic oxytocin and melatonin, we also found a reduction of

prolactin in adult rats following social deprivation post-weaning. Physiologically, the prolactin and oxytocin positively regulate each other, and are important in social recognition and learning. Indeed, their receptors are present in the HIPP and AMY. The release of prolactin from anterior pituitary is regulated by tonic DA inhibition and melatonin. Moreover, there are sex differences in pituitary prolactin release and prolactin receptor density in the brain; indeed, in females, the prolactin receptors are mainly distributed in the HIPP, AMY and HYP. Instead, in the males, these receptors are less expressed. Regarding prolactin release, the basal levels in females are double those in males. Probably, these differences could be due to estrogen in females. Indeed, the estrogen is a positive regulator of prolactin, and clinical data demonstrated that the estradiol treatment increases the plasmatic prolactin levels [21]. Furthermore, in the female, prolactin is implicated in the production of milk and adaptive changes of the brain during pregnancy. Instead, in both males and females, it is involved, by inhibiting the HPA axis action, in the specific response brain to stress stimuli, that can lead to anxiety- and depressive-like behaviours. It was reported that the social deprivation, from post-weaning period to adulthood, reduced the prolactin amount as observed in patients with stress-induced anxiety and depression [22]. Thus, our results are in accordance with previous data and we can hypothesize that social isolation induced the less production of melatonin accompanied by a reduction of oxytocin and prolactin with a final decrease function of these neuropeptides and development of anxious and depressive behaviours. This hypothesis was

confirmed by clinical studies where administration of exogenous neuropeptides improved the condition of patients with these disorders. It was also shown a decrease in the plasmatic ghrelin levels in isolated animals. The ghrelin is biochemically and functionally correlated to serotonergic and melatoninergic systems. This hormone is produced by the HYP and by a group of enteroendocrine ghrelin cells localized to the gastric mucosa. It is implicated in the metabolism processes, regulation of mood, learning and memory. Several studies showed that ghrelin has antidepressant and anxiolytic effects in animals exposed to chronic stress. Together with metabolic changes, the exposure to stress induces also an enhancement of this hormone to cope with the low-energy state and to maintain the homeostatic balance. Hence, the decreased level of ghrelin observed in this study, could appear as the loss of physiological capacity to cope the exposure to chronic stress during the neurodevelopmental phase. Indeed, this effect could be one of the social isolation-induced metabolic disturbances such as increased levels of insulin with less peripheral sensitivity to this hormone and also alteration of lipid metabolism in the liver of adult rats [44]. Also the NPS is involved in stress response. It is released in areas such as the AMY and it promotes several processes such as neurogenesis, neuroprotection, neuronal excitability, energy homeostasis and emotions. Clinical studies reported that this neuropeptide has anxiolytic effect by modulating the stress-induced outcomes; moreover, there is a link between the altered activity of the NPS receptor, caused by polymorphism of its gene and mood disorders [19]. However, in our experimental condition, no significant

differences were found in the levels of NPS between socially isolated animals and controls. In the second part of the study, we demonstrated that exposure to the social isolation of female rats, before conception, doesn't induce an anxiety-like behavior and alterations in the locomotor activity. However, it determines modifications in the learning and memory of their male offspring, independently from the grouped or isolated condition to which it exposed during the neurodevelopmental phase. Indeed, they showed a decreased latency time in the PA test, but no significant differences in the exploratory activity between a familiar and novel object in the NOR test. Previous studies evidenced that female exposure to social deprivation, during the neurodevelopmental phase, leads to stress-induced alterations in adulthood with the evolution of anxiety and depression disorder. Indeed, the isolated female rats showed an increase of ACTH and CORT as a response to stress. Also the serotonergic system was altered in the regions of the limbic system, which modulate the anxiety state. Specifically there is a hyposerotonergic tone and an increase of 5-HT turnover [75]. To confirm this data, another research group detected that the isolated female rats manifested an anxiety-like behavior in elevated plus-maze, compared to grouped animals. This anxious profile is correlated to a decreased concentration of allopregnanolone in the plasma and brain. This steroid is a metabolite of progesterone and a positive modulator of $GABA_A$ receptors, by exercising anxiolytic and antidepressant effects [76]. Our data demonstrate that the female exposure to social stress not only induces alterations in adulthood but also might determine cognitive

dysfunctions in their progeny. Indeed, the period before conception is important for uterine priming and for normal development of the placenta and fetus. Clinical studies evidenced that the exposure of females to preconceptional stress could be associated with lower birthweight of offspring. This correlation is possible because the neuroendocrine, immunological and cardiometabolic systems, involved in response to stress, are the same, which influence the prenatal health, the development of the placenta and fetus [77]. In general, several studies focused on the effects of maternal antenatal stress. In particular, it was evidenced that the exposure to stress, during pregnancy, induces the development delay, cognitive dysfunctions, mental and behavioral problems in the offspring, also later in life. This is possible because the fetus is exposed to enhanced amount of glucocorticoids that induce structural and functional changes in the brain areas during their evolution. In contrast, less is known about the effects of preconceptional stress [78]. In this regard, pregnancy health is dependent from the health status before conception. Indeed, the stress exposure, lasted within one week before conception, increased anxiety and reduced the social interaction, spatial memory, and sucrose consumption in the progeny. Besides that, the dendrites were more complex and the spines were longer in the PFC of offspring; instead enhanced levels of NA were detected in the HIPP, as well as decreased amount of 5-HT in the HYP. The preconceptional stress, especially during adolescence, impacted on HPA axis activity, with altered CORT levels, and on neuroendocrine system of progeny [79]. Following the reported data, in our

experimental conditions, together with observed cognitive changes, we also detected an increase of NA quantity in the PFC of offspring of socially isolated females, independently from the rearing conditions. At the cortical level, this increase could be explained as a response to preconceptional stress, resulting in cognitive alterations. Indeed, the NA is involved in the pathophysiology of stress-induced diseases. Scientific evidence shows that social isolation decreases the excitability of PFC neurons, resulting in emotional and cognitive impairments, which are present in patients with psychiatric diseases. NA is a neurotransmitter involved in the stress response, but an exposure to chronic stress induces the prolonged activation of a noradrenergic system that resulting in the development of psychiatric disorders. Specifically NA is produced in the locus coeruleus, where the noradrenergic neurons are projected to PFC; here the α1 and α2A adrenergic receptors are activated to regulate the neural activity. At the basal level, NA binds to α2A adrenergic receptor, but under the chronic stress condition, this neurotransmitter is produced in large quantity and binds to α1 adrenergic receptors (more expressed in the stress period), leading to functional cortical change, resulting in emotional and cognitive dysfunctions, anxiety-like behaviour and aggression [80]. Instead, in our experiment, no significant differences were detected in the cortical NA levels between the chronic isolated progeny and controls, resulting as possible compensation in the stress response. Furthermore, in the long-lasting socially isolated progeny, we detected a reduction of cortical 5-HT and GABA amount; the serotoninergic system is important in the stress

response and physiological processes. Indeed, it is a key modulator of thermoregulation. In the specific, the body temperature is regulated by the preoptic area of HYP; the mentioned area integrates thermo-sensory information from peripheral and central afferents. Indeed, it receives serotonergic innervation from the dorsal and medullary raphe nuclei and stimulates the brain areas to respond to the thermal input [81]. Instead, several studies evidenced that exposure to stress alters the serotonergic transmission, especially in the AMY, ventral striatum and PFC. In particular, during the neurodevelopmental phase, 5-HT is important in the CNS neuroplasticity, and the exposure to stress in this period could modify it. Its involvement in the development of stress-induced disorders is confirmed by the successful use of antidepressants, as the 5-HT reuptake inhibitors, in patients with stress-generated psychiatric diseases [82]. Additionally, the reduction of GABA, observed in our experiment, is in accordance with the literature. It was evidenced that the chronic stress induces a less GABA amount in the PFC, resulting in evolution of mental disorders. GABA exercises the inhibitory role and modulates the excitatory action of GLU; in particular, in the PFC, the GABAergic interneurons are in large quantity and the GABA receptors are highly expressed. In this region, the GABAergic system is important in emotional processing and so, it could be vulnerable to stress that could induce a less expression of system. Indeed, animal studies detected that the maternal separation stress, in the postnatal period, led to reduced expression of cortical $GABA_A$ receptors, inducing anxiety and depression in adulthood.

Moreover, the GABAergic neurons modulate the termination of the stress response by regulating the HPA axis, and a possible modification of this circuit participates in the development of negative effects of chronic stress. Under the chronic stress condition, at the HPA axis level, a mutation of $GABA_A$ receptors induces a reduced binding to GABA with consequent HPA axis hyperactivity, resulting in anxiogenic and depressive behaviour [83]. Furthermore, in our analyses, an increased ROS quantity was detected in the PFC of long-lasting socially isolated offspring, in accordance with previous studies that reported enhanced ROS levels in the CNS, after chronic stress exposure. Indeed, the ROS are produced especially by mitochondria and NOX enzymes. They participate in the control of neuronal fate, in the signaling pathways, such as the regulation of glutamatergic neurotransmission and the neuroinflammatory response. Moreover, there is a balance between ROS production and antioxidant enzymes, such as CAT, SOD, glutathione peroxidase, CoQ10, and low-weight-molecular antioxidants. When this balance is destroyed, the ROS are produced in large quantity, resulting in *oxidative stress* status. During chronic stress, the glucocorticoids, in large amount, stimulate the NOX enzymes to produce ROS. The brain has a high lipid content and energy demand, so it is vulnerable to the action of the ROS, resulting in alteration of the cellular functions, increase of GLU production, with consequent up-regulation of the ionotropic glutamate receptor (NMDA) and loss of phenotype of inhibitory neurons. This state leads to structural and functional changes especially in regions of the limbic system, such as cortex,

HIPP and AMY, with the development of stress-induced psychiatric disorders [12]. In addition to this, it was evidenced that the chronic social isolation stress, during the neurodevelopmental phase, induces oxidative stress not only at the central level, but also in periphery. Indeed, in the liver of adult rats, following isolation period, the NOX4 enzyme was overexpressed, resulting in overproduction of ROS with the generation of MDA (a result of lipid peroxidation induced by ROS) and accompanied by a reduced amount of antioxidant species [44]. Therefore, the enhanced amount of ROS, observed in the PFC of long-lasting isolated progeny of our experiment, could result from the association between the stress suffered by the female before conception and the condition of social isolation in which the offspring was raised during the neurodevelopmental period, a high vulnerable phase of life. In this study, another interesting thing is the reduction of NF-kB expression in the PFC of long-lasting socially isolated offspring of the females exposed to chronic stress. NF-kB is the redox-sensitive transcription factor nuclear and is situated in the cytoplasm as an inactive form. The ROS or the GLU can activate NF-kB and stimulate its translocation in the nucleus to induce the expression of genes for the production of molecules involved in the cell protection (CuZnSOD, MnSOD and Bcl-2 or Bcl-Xl) or the cell injury (COX-2, nNOS, iNOS) [84]. For this, our result contradicts previous analyses, which evidenced an increase of NF-kB expression in the PFC of rats, after twentyone days of isolation, with consequent iNOS-induced production of NO, which causes oxidative/nitrosative damage and so resulting in development

of anxiety and depression [85]. In addition, another group of research detected the same result; indeed the chronic stress induced an enhancement of NF-kB pathway activation accompanied by an increase of pro-inflammatory cytokines [86]. Instead, in another type of studies, such as on Ewing sarcomas, NF-kB, by stimulating the cell protection with the production of enzymes such as MnSOD, suppressed ROS induced by TNF alfa. Indeed, the inhibition of NF-kB pathway leads to an enhancement of ROS levels [87]. In light of this, we can hypothesize that, in our experiments, the detected NF-kB decrease may appear as a loss of the ability to repress the cortical ROS, following the long-lasting exposure to chronic isolation of progeny of preconceptional stressed females. As described above, the NF-kB pathway is modulated (activated or inhibited) by ROS, depending on the context. It can also stimulate the expression of specific genes for production of pro-oxidant species such as the NADPH oxidase enzymes. In particular, in this experimental condition, we also observed a decrease of NOX2 and NOX1 expression at the cortical level, subsequently long-lasting stress suffered by offspring of socially isolated females. So in this context, such decrease could be a protective reaction in response to ROS and linked to reduction of NF-kB. Moreover, we observed that the exposure to chronic stress of females, before conception, induced an increase of cortical NOX1 in adult offspring, following suffering the stress in adolescence. Instead, the cortical NOX1 decreased in adult offspring of normal females, after exposure to stress in adolescence. Thus, we could hypothesize that in the first case, the increase of NOX1 is the result of an

association between stress suffered by females and exposure to stress of offspring in adolescence. Specifically, we could deduce that the preconceptional stress prepares, induces effect on offspring also if it is exposed to stress later and not necessary from weaning. In the second case, the decrease of NOX1 could appear as a neuroprotective attempt. In this context, in the PFC of long-lasting stress exposed offspring of isolated females, beside to the enhancement of ROS amount, to the decrease of NF-kB with NOX1 and NOX2, we also observed an increase of SOD1 amount (an anti-oxidant enzyme, which converts superoxide anion in molecular oxygen and hydrogen peroxide (H_2O_2)). The enhancement of SOD1 expression could be a reaction to oxidative stress, following reported data, and a reaction to attenuate the NF-kB pathway that is controlled by cell redox status [88]. Instead, the levels of CoQ10 enzyme increased in the PFC of the offspring of normal females, following stress exposure in adolescence, versus to control. However, it also enhances in relation to the offspring of isolated females, reared in the same condition. This result could be a reaction to oxidative stress status caused by isolation in adolescence, but we can also hypothesize that the preconceptional stress of females, reduces the antioxidant action of CoQ10 in the offspring. Physiologically, in neuronal cells, the CoQ10 exerts its antioxidant role by donating its electron to free radicals, resulting in neutralization of these species and preservation of mitochondria and lipid membranes. It was evidenced that the CoQ10 supplementation in mice, after exposure to chronic restraint stress, induced a decrease of ROS level and inhibited the neuronal apoptosis, by

attenuating the NF-kB pathway, and so resulting in improvement of depressive state [89].

8. Conclusions

In light of our data, we can deduce that the neurodevelopmental phase (from weaning, through adolescence, until young-adulthood) is an important period for the development of the brain, from a structural and functional point of view. The exposure to chronic stress, in this vulnerable period of life, represents a threat. Indeed, it induces the morphological and functional alterations in brain areas such as those of the limbic system. Therefore, it modifies the cognitive, learning, behavioral and emotional aspects in adult individuals, resulting in the possible development of psychiatric disorders. In this context, we demonstrated that the seven weeks of isolation post-weaning induced neurochemical and neuroendocrine alterations, resulting in depressive- and anxiety-like behaviors in adult rats. This result adds to the data already reported on the effects of chronic isolation in animals. In fact, the chronic social isolation of animals induces outcomes similar to those observed in psychotic patients. It could be a handy tool for understanding the pathological links between stress and psychotic disorders. Moreover, we demonstrated that the effects of the chronic stress in adulthood, not only are linked to individual experience of stress in the neurodevelopmental phase, but could also be correlated to the stress suffered by the mother, before conception. Indeed, in presented study, the female exposure to stress, before pregnancy, had impact not only on cognitive and neurochemical aspects, but also on the redox balance of progeny, independently from its rearing conditions (in group or isolation) to which it exposed from weaning to young adulthood. This result demonstrates how the preconception healthy of the female is important for

the normal neurodevelopment of her future progeny. Therefore, the data, evidenced in this study, open new lights on understanding of the link between chronic stress and psychotic disorders, as well as anxiety and depression, and also on understanding that the alterations of the adult individual could be linked to stressed state suffered by own mother, before conception. The latter could be a time point for intervention, also pharmacologically, and prevent the harmful effects of stress in adulthood.

9. References

1. Selye, H., *A syndrome produced by diverse nocuous agents. 1936.* J Neuropsychiatry Clin Neurosci, 1998. **10**(2): p. 230-1.
2. Lu, S., F. Wei, and G. Li, *The evolution of the concept of stress and the framework of the stress system.* Cell Stress, 2021. **5**(6): p. 76-85.
3. Noushad, S., et al., *Physiological biomarkers of chronic stress: A systematic review.* Int J Health Sci (Qassim), 2021. **15**(5): p. 46-59.
4. Vargas, T., R.E. Conley, and V.A. Mittal, *Chronic stress, structural exposures and neurobiological mechanisms: A stimulation, discrepancy and deprivation model of psychosis.* Int Rev Neurobiol, 2020. **152**: p. 41-69.
5. Ross, R.A., S.L. Foster, and D.F. Ionescu, *The Role of Chronic Stress in Anxious Depression.* Chronic Stress (Thousand Oaks), 2017. **1**: p. 2470547016689472.
6. Wilson, R.S., A.R. Yung, and A.P. Morrison, *Comorbidity rates of depression and anxiety in first episode psychosis: A systematic review and meta-analysis.* Schizophr Res, 2020. **216**: p. 322-329.
7. Tsigos, C., et al., *Stress: Endocrine Physiology and Pathophysiology*, in *Endotext*, K.R. Feingold, et al., Editors. 2000: South Dartmouth (MA).
8. Tafet, G.E. and C.B. Nemeroff, *The Links Between Stress and Depression: Psychoneuroendocrinological, Genetic, and Environmental Interactions.* J Neuropsychiatry Clin Neurosci, 2016. **28**(2): p. 77-88.

9. Song, M.K., J.H. Lee, and Y.J. Kim, *Effect of chronic handling and social isolation on emotion and cognition in adolescent rats.* Physiol Behav, 2021. **237**: p. 113440.

10. Chiba, S., et al., *Chronic restraint stress causes anxiety- and depression-like behaviors, downregulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic factor in the prefrontal cortex.* Prog Neuropsychopharmacol Biol Psychiatry, 2012. **39**(1): p. 112-9.

11. Leonard, B.E. and C. Song, *Changes in the immune system in rodent models of depression.* Int J Neuropsychopharmacol, 2002. **5**(4): p. 345-56.

12. Schiavone, S., et al., *Severe life stress and oxidative stress in the brain: from animal models to human pathology.* Antioxid Redox Signal, 2013. **18**(12): p. 1475-90.

13. Salim, S., *Oxidative Stress and the Central Nervous System.* J Pharmacol Exp Ther, 2017. **360**(1): p. 201-205.

14. Moritz, B., et al., *The role of vitamin C in stress-related disorders.* J Nutr Biochem, 2020. **85**: p. 108459.

15. Sorce, S. and K.H. Krause, *NOX enzymes in the central nervous system: from signaling to disease.* Antioxid Redox Signal, 2009. **11**(10): p. 2481-504.

16. Leonard, B.E., *HPA and immune axes in stress: involvement of the serotonergic system.* Neuroimmunomodulation, 2006. **13**(5-6): p. 268-76.

17. Gold, P.W., *The organization of the stress system and its dysregulation in depressive illness.* Mol Psychiatry, 2015. **20**(1): p. 32-47.

18. Hoge, E.A., et al., *Oxytocin levels in social anxiety disorder.* CNS Neurosci Ther, 2008. **14**(3): p. 165-70.

19. Rana, T., et al., *Exploring the role of neuropeptides in depression and anxiety.* Prog Neuropsychopharmacol Biol Psychiatry, 2022. **114**: p. 110478.

20. Jones, C., et al., *Oxytocin and social functioning.* Dialogues Clin Neurosci, 2017. **19**(2): p. 193-201.

21. Donhoffner, M.E., et al., *Prosocial effects of prolactin in male rats: Social recognition, social approach and social learning.* Horm Behav, 2017. **96**: p. 122-129.

22. Esquifino, A.I., et al., *24-hour pattern of circulating prolactin and growth hormone levels and submaxillary lymph node immune responses in growing male rats subjected to social isolation.* Endocrine, 2004. **25**(1): p. 41-8.

23. Juszczak, M., *Melatonin affects the oxytocin and prolactin responses to stress in male rats.* J Physiol Pharmacol, 1998. **49**(1): p. 151-63.

24. Li, C., et al., *The Therapeutic Effect of Exogenous Melatonin on Depressive Symptoms: A Systematic Review and Meta-Analysis.* Front Psychiatry, 2022. **13**: p. 737972.

25. Huang, H.J., et al., *Ghrelin alleviates anxiety- and depression-like behaviors induced by chronic unpredictable mild stress in rodents.* Behav Brain Res, 2017. **326**: p. 33-43.

26. Menzies, J.R., et al., *Ghrelin, reward and motivation.* Endocr Dev, 2013. **25**: p. 101-11.

27. Santoro, A., et al., *Gut microbiota changes in the extreme decades of human life: a focus on centenarians.* Cell Mol Life Sci, 2018. **75**(1): p. 129-148.

28. Wang, H.L., et al., *Adolescent stress increases depression-like behaviors and alters the excitatory-inhibitory balance in aged mice.* Chin Med J (Engl), 2019. **132**(14): p. 1689-1699.

29. Van den Bergh, B.R.H., et al., *Prenatal developmental origins of behavior and mental health: The influence of maternal stress in pregnancy.* Neurosci Biobehav Rev, 2020. **117**: p. 26-64.

30. Atrooz, F., H. Liu, and S. Salim, *Stress, psychiatric disorders, molecular targets, and more.* Prog Mol Biol Transl Sci, 2019. **167**: p. 77-105.

31. Creutzberg, K.C., et al., *Long-lasting effects of prenatal stress on HPA axis and inflammation: A systematic review and multilevel meta-analysis in rodent studies.* Neurosci Biobehav Rev, 2021. **127**: p. 270-283.

32. Babenko, O., I. Kovalchuk, and G.A. Metz, *Stress-induced perinatal and transgenerational epigenetic programming of brain development and mental health.* Neurosci Biobehav Rev, 2015. **48**: p. 70-91.

33. Schoner, J., et al., *Post-traumatic stress disorder and beyond: an overview of rodent stress models.* J Cell Mol Med, 2017. **21**(10): p. 2248-2256.

34. Bakshi, V.P. and M.A. Geyer, *Ontogeny of isolation rearing-induced deficits in sensorimotor gating in rats.* Physiol Behav, 1999. **67**(3): p. 385-92.

35. Lapiz, M.D., et al., *Influence of postweaning social isolation in the rat on brain development, conditioned behavior, and neurotransmission.* Neurosci Behav Physiol, 2003. **33**(1): p. 13-29.

36. Fone, K.C. and M.V. Porkess, *Behavioural and neurochemical effects of post-weaning social isolation in rodents-relevance to developmental neuropsychiatric disorders.* Neurosci Biobehav Rev, 2008. **32**(6): p. 1087-102.

37. Schiavone, S., et al., *NADPH oxidase elevations in pyramidal neurons drive psychosocial stress-induced neuropathology.* Transl Psychiatry, 2012. **2**(5): p. e111.

38. Schiavone, S., et al., *Involvement of NOX2 in the development of behavioral and pathologic alterations in isolated rats.* Biol Psychiatry, 2009. **66**(4): p. 384-92.

39. Schiavone, S., et al., *Early Loss of Blood-Brain Barrier Integrity Precedes NOX2 Elevation in the Prefrontal Cortex of an Animal Model of Psychosis.* Mol Neurobiol, 2017. **54**(3): p. 2031-2044.

40. Colaianna, M., et al., *Neuroendocrine profile in a rat model of psychosocial stress: relation to oxidative stress.* Antioxid Redox Signal, 2013. **18**(12): p. 1385-99.

41. Schiavone, S., et al., *Visceral Fat Dysfunctions in the Rat Social Isolation Model of Psychosis.* Front Pharmacol, 2017. **8**: p. 787.

42. Thakore, J.H., et al., *Increased visceral fat distribution in drug-naive and drug-free patients with schizophrenia.* Int J Obes Relat Metab Disord, 2002. **26**(1): p. 137-41.

43. Chen, S., et al., *Relationship between insulin resistance, dyslipidaemia and positive symptom in Chinese antipsychotic-naive first-episode patients with schizophrenia.* Psychiatry Res, 2013. **210**(3): p. 825-9.

44. Bove, M., et al., *Social isolation triggers oxidative status and impairs systemic and hepatic insulin sensitivity in normoglycemic rats.* Biomed Pharmacother, 2022. **149**: p. 112820.

45. Schiavone, S., et al., *Chronic Psychosocial Stress Impairs Bone Homeostasis: A Study in the Social Isolation Reared Rat.* Front Pharmacol, 2016. **7**: p. 152.

46. Zhao, J., et al., *Protective Effects of Resveratrol on Adolescent Social Isolation-Induced Anxiety-Like Behaviors via Modulating Nucleus Accumbens Spine Plasticity and Mitochondrial Function in Female Rats.* Nutrients, 2022. **14**(21).

47. Krupina, N.A., et al., *The Levels of Monoamines and Their Metabolites in the Brain Structures of Rats Subjected to Two- and Three-Month-Long Social Isolation.* Bull Exp Biol Med, 2020. **168**(5): p. 605-609.

48. Cuesta, S., A. Funes, and A.M. Pacchioni, *Social Isolation in Male Rats During Adolescence Inhibits the Wnt/beta-Catenin Pathway in the Prefrontal Cortex and Enhances Anxiety and Cocaine-Induced Plasticity in Adulthood.* Neurosci Bull, 2020. **36**(6): p. 611-624.

49. Okudan, N. and M. Belviranli, *Long-term voluntary exercise prevents post-weaning social isolation-induced cognitive impairment in rats.* Neuroscience, 2017. **360**: p. 1-8.

50. Cryan, J.F., M.E. Page, and I. Lucki, *Differential behavioral effects of the antidepressants reboxetine, fluoxetine, and moclobemide in a modified forced swim test following chronic treatment.* Psychopharmacology (Berl), 2005. **182**(3): p. 335-44.

51. Tucker, L.B. and J.T. McCabe, *Behavior of Male and Female C57BL/6J Mice Is More Consistent with Repeated Trials in the Elevated Zero Maze than in the Elevated Plus Maze.* Front Behav Neurosci, 2017. **11**: p. 13.

52. Morgese, M.G., et al., *Lifelong Nutritional Omega-3 Deficiency Evokes Depressive-Like State Through Soluble Beta Amyloid.* Mol Neurobiol, 2017. **54**(3): p. 2079-2089.

53. Muller, P., et al., *[Endoscopic studies of gastroduodenal tolerance of 100 mg versus 500 mg acetylsalicylic acid daily: a randomized double-blind study with healthy probands].* Z Gastroenterol, 1989. **27**(8): p. 418-20.

54. Schiavone, S., et al., *Ketamine administration induces early and persistent neurochemical imbalance and altered NADPH oxidase in mice.* Prog Neuropsychopharmacol Biol Psychiatry, 2020. **96**: p. 109750.

55. Bove, M., et al., *The Visible Burrow System: A behavioral paradigm to assess sociability and social withdrawal in BTBR and C57BL/6J mice strains.* Behav Brain Res, 2018. **344**: p. 9-19.

56. Morgese, M.G., et al., *Chlorella sorokiniana Extract Improves Short-Term Memory in Rats.* Molecules, 2016. **21**(10).

57. Morgese, M.G., et al., *Modulatory activity of soluble beta amyloid on HPA axis function in rats.* Curr Pharm Des, 2014. **20**(15): p. 2539-46.

58. Morgese, M.G., et al., *N-3 PUFA Prevent Oxidative Stress in a Rat Model of Beta-Amyloid-Induced Toxicity.* Pharmaceuticals (Basel), 2021. **14**(4).

59. Burke, A.R., et al., *Impact of adolescent social experiences on behavior and neural circuits implicated in mental illnesses.* Neurosci Biobehav Rev, 2017. **76**(Pt B): p. 280-300.

60. Skelly, M.J., et al., *Adolescent social isolation increases anxiety-like behavior and ethanol intake and impairs fear extinction in adulthood: Possible role of disrupted noradrenergic signaling.* Neuropharmacology, 2015. **97**: p. 149-59.

61. Kuramochi, M. and S. Nakamura, *Effects of postnatal isolation rearing and antidepressant treatment on the density of serotonergic and noradrenergic axons and depressive behavior in rats.* Neuroscience, 2009. **163**(1): p. 448-55.

62. Duman, R.S., et al., *Synaptic plasticity and depression: new insights from stress and rapid-acting antidepressants.* Nat Med, 2016. **22**(3): p. 238-49.

63. Marazziti, D., et al., *Neurobiological correlates of social anxiety disorder: an update.* CNS Spectr, 2015. **20**(2): p. 100-11.

64. dos Santos, L., T.G. de Andrade, and F.G. Graeff, *Social separation and diazepam withdrawal increase anxiety in the elevated plus-maze and serotonin turnover in the median raphe and hippocampus.* J Psychopharmacol, 2010. **24**(5): p. 725-31.

65. Adams, T. and J.A. Rosenkranz, *Social Isolation During Postweaning Development Causes Hypoactivity of Neurons in the Medial Nucleus of the Male Rat Amygdala.* Neuropsychopharmacology, 2016. **41**(7): p. 1929-40.

66. Butler, M.I., et al., *The immune-kynurenine pathway in social anxiety disorder.* Brain Behav Immun, 2022. **99**: p. 317-326.

67. Felix-Ortiz, A.C. and K.M. Tye, *Amygdala inputs to the ventral hippocampus bidirectionally modulate social behavior.* J Neurosci, 2014. **34**(2): p. 586-95.

68. Mumtaz, F., et al., *Neurobiology and consequences of social isolation stress in animal model-A comprehensive review.* Biomed Pharmacother, 2018. **105**: p. 1205-1222.

69. Karkhanis, A.N., et al., *Chronic social isolation during adolescence augments catecholamine response to acute ethanol in the basolateral amygdala.* Synapse, 2015. **69**(8): p. 385-95.

70. Han, R.T., et al., *Long-Term Isolation Elicits Depression and Anxiety-Related Behaviors by Reducing Oxytocin-Induced GABAergic Transmission in Central Amygdala.* Front Mol Neurosci, 2018. **11**: p. 246.

71. Lonstein, J.S., et al., *Emotion and mood adaptations in the peripartum female:complementary contributions of GABA and oxytocin.* J Neuroendocrinol, 2014. **26**(10): p. 649-64.

72. Neumann, I.D. and R. Landgraf, *Balance of brain oxytocin and vasopressin: implications for anxiety, depression, and social behaviors.* Trends Neurosci, 2012. **35**(11): p. 649-59.

73. Gomaa, A.M., H.M. Galal, and A.T. Abou-Elgait, *Neuroprotective effects of melatonin administration against chronic immobilization stress in rats.* Int J Physiol Pathophysiol Pharmacol, 2017. **9**(2): p. 16-27.

74. Wang, X., et al., *Melatonin ameliorates anxiety-like behaviors induced by sleep deprivation in mice: Role of oxidative stress, neuroinflammation, autophagy and apoptosis.* Brain Res Bull, 2021. **174**: p. 161-172.

75. Lukkes, J.L., et al., *Post-weaning social isolation of female rats, anxiety-related behavior, and serotonergic systems.* Brain Res, 2012. **1443**: p. 1-17.

76. Pisu, M.G., et al., *Juvenile social isolation affects maternal care in rats: involvement of allopregnanolone.* Psychopharmacology (Berl), 2017. **234**(17): p. 2587-2596.

77. Hipwell, A.E., et al., *Preconception stress exposure from childhood to adolescence and birth outcomes: The impact of stress type, severity and consistency.* Front Reprod Health, 2022. **4**: p. 1007788.

78. Tuovinen, S., et al., *Maternal antenatal stress and mental and behavioral disorders in their children.* J Affect Disord, 2021. **278**: p. 57-65.

79. Keenan, K., et al., *Extending the developmental origins of disease model: Impact of preconception stress exposure on offspring neurodevelopment.* Dev Psychobiol, 2018. **60**(7): p. 753-764.

80. Kawanabe, S., et al., *Upregulations of alpha(1) adrenergic receptors and noradrenaline synthases in the medial prefrontal cortex are associated with emotional and cognitive dysregulation induced by post-weaning social isolation in male rats.* Neurosci Lett, 2023. **797**: p. 137071.

81. Natarajan, R., N.A. Northrop, and B.K. Yamamoto, *Protracted effects of chronic stress on serotonin-dependent thermoregulation.* Stress, 2015. **18**(6): p. 668-76.

82. Krystal, J.H. and A. Neumeister, *Noradrenergic and serotonergic mechanisms in the neurobiology of posttraumatic stress disorder and resilience.* Brain Res, 2009. **1293**: p. 13-23.

83. Fogaca, M.V. and R.S. Duman, *Cortical GABAergic Dysfunction in Stress and Depression: New Insights for Therapeutic Interventions.* Front Cell Neurosci, 2019. **13**: p. 87.

84. Filipovic, D., et al., *Oxidative and nitrosative stress pathways in the brain of socially isolated adult male rats demonstrating depressive- and anxiety-like symptoms.* Brain Struct Funct, 2017. **222**(1): p. 1-20.

85. Zlatkovic, J. and D. Filipovic, *Chronic social isolation induces NF-kappaB activation and upregulation of iNOS protein expression in rat prefrontal cortex.* Neurochem Int, 2013. **63**(3): p. 172-9.

86. Al Omran, A.J., et al., *Social isolation induces neuroinflammation and microglia overactivation, while dihydromyricetin prevents and improves them.* J Neuroinflammation, 2022. **19**(1): p. 2.

87. Djavaheri-Mergny, M., et al., *NF-kappaB activation prevents apoptotic oxidative stress via an increase of both thioredoxin and MnSOD levels in TNFalpha-treated Ewing sarcoma cells.* FEBS Lett, 2004. **578**(1-2): p. 111-5.

88. Rojo, A.I., et al., *Regulation of Cu/Zn-superoxide dismutase expression via the phosphatidylinositol 3 kinase/Akt pathway and nuclear factor-kappaB.* J Neurosci, 2004. **24**(33): p. 7324-34.

89. Salehpour, F., et al., *Near-infrared photobiomodulation combined with coenzyme Q(10) for depression in a mouse model of restraint stress: reduction in oxidative stress, neuroinflammation, and apoptosis.* Brain Res Bull, 2019. **144**: p. 213-222.

10. Appendix

PhD program research activity

Dimonte Stefania has carried out her PhD program in the Pharmacology Laboratory, Department of Clinical and Experimental Medicine, University of Foggia (Italy), from November, 2019 to December, 2022. During those three years, she has focused her research activity on preclinical pharmacology. In particular, she has performed behavioral, neurochemical and biomolecular analyses on rodent models of neuropsychiatric disorders. Moreover, during her PhD, in the context of a "Mobility Erasmus + Traineeships" program, she has participated to the experimental activities of the Groningen Institute for Evolutionary Life Sciences, University of Groningen, the Netherlands, from March 2nd to March 13th 2020. From May to July 2022, she has participated to the experimental activities of the Department of Physiology and Biochemistry, Faculty of Medicine and Surgery, University of Malta, Msida, Malta.

Training activities previous to PhD program

From March to May 2019, Dimonte Stefania has performed experimental training in pharmacological research participating to the experimental activities of the Pharmacology Laboratory, Department of Clinical and Experimental Medicine, University of Foggia (Italy).

Publications

1) Bove M, Tucci P, Dimonte S, Trabace L, Schiavone S, Morgese MG. *Postnatal Antioxidant and Anti-inflammatory Treatments Prevent Early Ketamine-Induced Cortical Dysfunctions in Adult Mice*. Front Neurosci. 2020 Nov 4;14:590088. doi: 10.3389/fnins.2020.590088. PMID: 33250707; PMCID: PMC7672215. Frontiers in Neuros.doi:103389/fnins.2020.590088

2) Morgese MG, Schiavone S, Bove M, Colia AL, Dimonte S, Tucci P, Trabace L. *N-3 PUFA Prevent Oxidative Stress in a Rat Model of Beta-Amyloid-Induced Toxicity*. Pharmaceuticals (Basel). 2021 Apr 8;14(4):339. doi: 10.3390/ph14040339. PMID: 33917814; PMCID: PMC8068120.

3) Morgese MG, Bove M, Francavilla M, Schiavone S, Dimonte S, Colia AL, Bevilacqua M, Trabace L, Tucci P. *Sublingual AKBA Exerts Antidepressant Effects in the Aβ-Treated Mouse Model*. Biomolecules. 2021 May 3;11(5):686. doi: 10.3390/biom11050686. PMID: 34063630; PMCID: PMC8170916.

4) Morgese MG, Bove M, Di Cesare Mannelli L, Schiavone S, Colia AL, Dimonte S, Mhillaj E, Sikora V, Tucci P, Ghelardini C, Trabace L. *Precision Medicine in Alzheimer's Disease: Investigating Comorbid Common Biological Substrates in the Rat Model of Amyloid Beta-Induced Toxicity*. Front Pharmacol. 2022 Jan 3;12:799561. doi: 10.3389/fphar.2021.799561. PMID: 35046821; PMCID: PMC8763383.

5) Saviano A., Raucci F., Tallarico M., De Caro C., Di Martino S., Nesci V., Roberti R., Iannone L. F., Colia A. L., Dimonte S., Furgiuele A., Marino F., Ferrari M., Cosentino M., Bove M., Tucci P., Micale V., Leo A., Franco V., Maione F., Russo E. on behalf of SIF Working Group Pharmacognosy, *Phytotherapy and nutraceuticals Cannabidiol and the central nervous system: translating into clinics* on Pharmadvances 2021 June Volume 3, issue 2, doi: 10.36118/pharmadvances.2021.04.

6) Bove M., Colia A. L., Dimonte S., Trabace L. *Increase in Vitamin D prescriptions in a southern Italy region over 2011-2015 period* on Pharmadvances 2021 June Vol. 3, issue 2, doi: 10.36118/pharmadvances.2021.02.

7) Bove M, Schiavone S, Tucci P, Sikora V, Dimonte S, Colia AL, Morgese MG, Trabace L. *Ketamine administration in early postnatal life as a tool for mimicking Autism Spectrum Disorders core symptoms*. Prog Neuropsychopharmacol Biol Psychiatry. 2022 Jul 13;117:110560. doi: 10.1016/j.pnpbp.2022.110560. Epub 2022 Apr 20. PMID: 35460811.

8) Tucci P, Bove M, Sikora V, Dimonte S, Morgese MG, Schiavone S, Di Cesare Mannelli L, Ghelardini C, Trabace L. *Glucoraphanin Triggers Rapid Antidepressant Responses in a Rat Model of Beta Amyloid-Induced Depressive-like Behaviour.* Pharmaceuticals (Basel). 2022 Aug 26;15(9):1054. doi: 10.3390/ph15091054. PMID: 36145275; PMCID: PMC9500808.

9) Dimonte S, Sikora V, Bove M, Morgese MG, Tucci P, Schiavone S, Trabace L. *Social isolation from early life induces anxiety-like behaviors in adult rats: Relation to neuroendocrine and neurochemical dysfunctions*. Biomed Pharmacother. 2022 Dec 31;158:114181. doi: 10.1016/j.biopha.2022.114181. Epub ahead of print. PMID: 36592494.

10) Bove M., Schiavone S., Tucci P., Agosti L.P., Dimonte S., Palmieri M.A., Sikora V., Matteo M., Trabace L., Morgese M.G. "*Lifelong exposure to n-3 PUFA deficiency leads to anxiety-like profile in male and female adolescent rats: impact on spleen-brain axis*" submitted to British Journal of Pharmacology (ID: 2023-BJP-0421-RP) on April 27, 2023, under review.

11) Bove M., Morgese M.G., Dimonte S., Sikora V., Agosti L.P., Palmieri M.A., Tucci P., Schiavone S., Trabace L. "*Increased stress vulnerability in the offspring of socially isolated rats: behavioural, neurochemical and redox dysfunctions*" submitted to Neuropsychopharmacology (ID: NPP-23-0473) on May 12, 2023, under review.

Conference proceedings

1) Tucci P., Dimonte S., Morgese M.G., Lama A., Pirozzi C., Schiavone S., Bove M., Trabace L., *Impact of chronic psychosocial stress on insulin pathway and hepatic redox state: an in vivo study* on Pharmadvances, 2021 March Vol. 3, doi:10.36118.

2) M. Bove, V. Sikora, S. Dimonte, S. Schiavone, L. Trabace, *Redox dysregulation and drug-induced psychosis: unravelling new pathways and pharmacological targets* on Pharmadvances, 2021 March Vol. 3, doi:10.36118.

3) Dimonte S., Schiavone S., Tucci P., Trabace L., *Exposure to chronic social stress leads to anxiety- and depressive-like behavioural and neurochemical alterations in rats* on Pharmadvances, 2023 January Vol. 5, doi:10.36118.

4) Morgese M.G., Bove M., Dimonte S., Trabace L., *Lifelong exposure to n-3 PUFA deficiency leads to anxiety-like effects in adolescent rats: impact of sex on immune modulation* on Pharmadvances, 2023 January Vol. 5, doi:10.36118.

Oral communication

Dimonte S., Schiavone S., Tucci P., Trabace L., *Exposure to chronic social stress leads to anxiety- and depressive-like behavioural and neurochemical alterations in rats*, at 41° Italian Society of Pharmacology (SIF) Congress (November 16-19, 2022), Rome, Italy.

Posters

1) Tucci P., Dimonte S., Morgese M.G., Lama A., Pirozzi C., Schiavone S., Bove M., Trabace L., *Impact of chronic psychosocial stress on insulin pathway and*

hepatic redox state: an in vivo study, in the context of 40° Virtual SIF Congress (March 9-13, 2021).

2) Dimonte S., Schiavone S., Meli R., Trabace L., *Chronic psychosocial stress and insulin pathway in the liver of social isolated rats*, in the context of 8° European Virtual Congress of Pharmacology (EPHAR 2021) (December 6-8, 2021).

Co-authors

1) Schiavone S, Morgese MG, Dimonte S, Tucci P, Trabace L., *Impact of NADPH oxidase inhibition on ketamine-induced dysfunctions: preclinical findings*, 39° SIF Congress (November 20-23, 2019), Florence, Italy.

2) Bove M, Schiavone S, Tucci P, Dimonte S, Colia AL, Morgese MG, Trabace L., *Redox dysregulation and drug-induced psychosis: unravelling new pathways and pharmacological targets*, 40° virtual SIF Congress (March 9-13, 2021).

3) Bove M., Dimonte S., Colia A.L., Trabace L., *Postnatal ketamine administration as a novel tool to mimic Autism Spectrum Disorders symptoms in mice*, XIX Virtual Italian Society of Neuroscience (SINS) Congress (September 09-11, 2021).

4) Bove M, Morgese MG, Dimonte S, Tucci P, Sikora V, Schiavone S, Trabace L, *Ketamine administration in early postnatal life: a useful mice model to*

resemble Autism Spectrum Disorders core symptoms, 8° Virtual EPHAR Congress (December 6-8, 2021).

5) Bove M, Sikora V, Dimonte S, Schiavone S, Trabace L, N-*acetylcysteine decreases stereotyped repertoire, anxiety-like behaviour and neuroinflammation in a mouse model of Autism Spectrum Disorders-like dysfunctions*, 8° Mediterannean Neuroscience Society Conference (May 29th - June 2nd, 2022) Dubrovnik, Croatia.

Conferences as participant

1) XXII SIF Seminar on Pharmacology (November 19-20, 2019), Florence, Italy.

2) 39° SIF Congress (November 20-23, 2019), Florence, Italy.

3) Webinar: “The Charter & amp; Code in the New ERA” (April 15th, 2021).

4) XXIII Virtual SIF Seminar on Pharmacology (March 8th, 2021).

5) Minisimposio online su sperimentazione animale in biomedicina (October 29th, 2021).

6) XXIV SIF Seminar on Pharmacology (November 15-16, 2022), Rome, Italy.

7) Interdepartmental Scientific Seminars organized by University of Foggia (Italy) from April, 2022.

Awards

Oral communication: Dimonte S., Schiavone S., Tucci P., Trabace L. *Exposure to chronic social stress leads to anxiety- and depressive-like behavioural and neurochemical alterations in rats,* awarded as best oral communication in the context of 41° SIF Congress (November 16-19, 2022), Rome, Italy.

Attended courses during the PhD program

1) Linguistica (from March to May, 2020).

2) Gestione della ricerca, della conoscenza dei sistemi di ricerca e dei sistemi di finanziamento (from July to October, 2020).

3) Valorizzazione dei risultati della ricerca e della proprietà intellettuale (from April to June, 2021).

4) Informatica (from February to May, 2021).

5) Tossicologia ed integratori alimentari (from March to June, 2021).

6) Online Course: "Models of the Blood-Brain Barrier: Scientific tools to target the brain" organized by the Faculty of Pharmacy, University of Lisbona (March 18-20, 2021).

7) Training course "Biologia e gestione degli animali da laboratorio" organized by the Italian Ministry of Health (from July 11th to November 30th, 2022).

Ringraziamenti

Siamo giunti ormai al termine di questo arduo percorso, sembra ieri quando ho bussato alla porta e ho chiesto di fare un tirocinio volontario, sembra ieri quando ho iniziato con tanta ansia e tante incognite, sembra ieri quando ho accettato di intraprendere il corso di Dottorato.

Il mio primo ringraziamento va alla Professoressa Luigia Trabace che ho conosciuto per prima e con cui è nata fin da subito un'empatia che non so spiegare. I suoi occhi, i suoi gesti brevi e fermi mi hanno colpito fin da subito, la sua intraprendenza e determinazione mi hanno incoraggiato a iniziare e portare a termine questo percorso. Come capitano mi ha accolta sulla sua nave, perché lei non si preclude nulla, mi ha osservato lungo tutto il percorso e insieme agli altri componenti dell'equipaggio mi ha formato e dato fiducia. Con Lei ho imparato che tutti siamo utili e nessuno è indispensabile! E' proprio così! Dobbiamo avere l'umiltà e la consapevolezza che noi esseri umani non siamo completi e onnipotenti ma abbiamo bisogno di altri talenti per raggiungere il migliore dei risultati che si potrebbero ottenere. Cara Prof.ssa, questo è solo uno dei tanti consigli che mi ha dato, cercherò di tenerli a mente nel mio futuro! In questo percorso sono stata affidata ad una madrina e cioè la Prof.ssa Schiavone, per me Stefania. Lei è stata fondamentale nella realizzazione di questa tesi e va un ringraziamento speciale. Anche con Stefania sono entrata fin da subito in empatia e le vorrei dire GRAZIE per tante cose, servirebbe un libro per elencare il tutto. In questo percorso oserei dire che è stata la mia GUIDA, mi ha fatto subito

intendere cosa significa vivere nel mondo accademico, come ci si deve comportare. Non scorderò mai la sua cruda sincerità nel dirmi le cose, un modo che pochi hanno. La sua umiltà e la sua forza mi hanno fatto capire che bisogna andare avanti, che l'obiettivo bisogna raggiungerlo a tutti i costi e che in certe situazioni che capitano non sempre c'è un perché, è cosi e basta. Dopo magari lo saprai. A te Stefania dico Grazie perché hai avuto tanta pazienza con me, mi hai insegnato ad approfondire ogni cosa, a soffermarmi sui dettagli, a prestare attenzione, a *"controllare le cose 300000 volte"*, perché nella ricerca, e non solo, sono i dettagli che fanno la differenza e potrebbero purtroppo sfuggire. Non scorderò mai l'esperienza della SIF 2022 dove ho conquistato un premio nazionale. In quella circostanza avevo mille paure ma tu sei riuscita a tirare fuori il meglio di me. Come buon allenatore mi hai preparata e mi hai seguita con tanta dedizione. Tu mi hai accompagnata e mi hai fatto capire che tutto è possibile, basta volerlo. Cara Stefania, ogni tanto credi di non farcela, di non riuscirci, ma ti posso assicurare che se fosse così non starei a scrivere determinate cose. Non ho avuto solo una madrina ma anche un padrino, e cioè il Prof. Tucci, per me Paolo, il quale mi ha anche aiutato in questo lavoro di tesi. Da Paolo ho imparato che le cose possono realizzarsi anche senza ansia, che bisogna affrontare le situazioni con tranquillità e perché no!?!? Anche con un pizzico di ironia. Il suo "Stai tranquilla!" o "Non ti preoccupare" per me diventavano in certe occasioni una specie di carica per superare situazioni che in quei secondi risultavano non risolvibili. Caro Paolo, GRAZIE per la pazienza e per il supporto che in

determinate circostanze non è mancato. Un altro membro dell'equipaggio, a cui sono stata affidata, è la Prof.ssa Morgese, per me Maria Grazia. Il nostro rapporto è nato poco alla volta, siamo due caratteri diversi che piano piano si sono incontrati e hanno trovato un equilibrio. Ho conquistato la sua fiducia giorno dopo giorno. Cara Maria Grazia, a te dico GRAZIE perché anche tu mi hai formata, mi hai insegnato a chiedermi il perché di ogni cosa e che in ogni problema c'è una soluzione. Grazie per la tua vicinanza in determinati momenti e per il racconto delle tue esperienze. Tu dimostri come il sacrificio e la pazienza rappresentano gli ingredienti essenziali per riuscire in questo campo e non solo. Con te ho imparato che il mio carattere estroverso non sempre può venire fuori, bisogna gestirlo e solo quando occorre lo si deve usare, questa per me è una grande maturità raggiunta. Un'altra persona fondamentale nella mia formazione e a cui dico GRAZIE è la Dott.ssa Bove, per me Maria. Lei è un vulcano. All'inizio è stata dura perché avevamo metodi di lavoro e modi di fare diversi, poi abbiamo deciso di scoprirci a vicenda e di unire le nostre energie per completarci. Cara Maria, con te ho messo in pratica il lavoro di squadra, ho apprezzato tanto il tuo aiuto e ho imparato che non bisogna fissarsi sulle cose, che bisogna andare avanti. La passione per la ricerca ti ha condotto qui e ti posso garantire che è coinvolgente. Non ti dimenticherò mai per la tua continua disponibilità verso tutti. Ecco un altro ingrediente essenziale per la buona riuscita delle cose: la disponibilità verso l'altro. Col tempo il nostro reciproco sostegno non si è fermato solo alla professione ma lo è anche diventato da un punto di vista umano che spero continui

anche fuori di qui, grazie per le tante volte che ci siamo ascoltate e confrontate. Il mio percorso si è arricchito ancora di più quando sono andata a Malta. Come già mi aveva preannunciato la Prof.ssa Trabace, questa esperienza mi è servita tantissimo ed è arrivata in un momento particolare della mia vita. A Malta ho avuto modo di lavorare con il Prof. Mario Valentino, una persona di grande spessore, non solo dal punto di vista professionale ma anche umano. La sua umiltà è stata impressionante, lui stesso mi ha seguito durante la mia esperienza in laboratorio e in quei tre mesi mi sono sentita parte della sua squadra. Il suo approccio alla ricerca era sempre quello di voler scoprire e rimanere stupefatto alla scoperta, proprio come un esploratore. Il suo amore per il mare riassume tutto ciò. Caro Mario, GRAZIE perché con te ho scoperto i valori più nobili della vita e come crediamo di sapere tutto ma in realtà c'è sempre qualcosa da scoprire (*In Malta I had the opportunity to work with Prof. Mario Valentino, a person of great depth, not only from a professional point of view but also from a human point of view. His humility was impressive, he himself followed me during my laboratory experience and in those three months I felt part of his team. His approach to research was always to want to discover and be amazed at the discovery, just like an explorer. His love of the sea sums it all up. Dear Mario, THANK YOU because with you I discovered the noblest values of life and how we think we know everything but in reality there is always something to discover*). L'esperienza di questi 3 anni si è arricchita ancora di più quando è arrivato Vlad, il mio compagno di banco. GRAZIE a Vlad ho imparato che bisogna sempre puntare alla perfezione

delle cose, che non bisogna mai accontentarsi perché si può fare meglio. Con lui ho sperimentato l'aiutarsi a vicenda, non solo in laboratorio ma anche fuori come alla SIF 2022; inoltre non posso non ringraziarlo per il suo sostegno e aiuto nella stesura di questa tesi. Grazie per le risate che rallegravano la giornata e per il tuo come stai? Per il tuo interesse se avevo un sorriso oppure no e per la tua stima (*The experience of these 3 years was enriched even more when Vlad, my classmate, arrived. THANKS to Vlad because I learned that one must always aim for perfection in things, that one must never be satisfied because one can do better. With him I experienced helping each other, not only in the laboratory but also outside, like at SIF 2022; moreover, I thank him for his support and help in writing this thesis. Thanks for the laughs that brightened up the day and your "how are you?" For your interest whether I had a smile or not and for your estimation*). Nel nostro equipaggio non posso non menzionare "le girls": Ade, Martina e Lisa. Sono tre caratteri diversi che nel mio ultimo periodo in laboratorio mi hanno rallegrato. Mi hanno sopportata quando ero nervosa (Perdonatemi!!) e hanno gioito con me quando ho terminato. GRAZIE mille a tutte voi perché spiegandovi le cose mi sono riscoperta anch'io, la ricerca è bella anche quando si trasmette il sapere. Il mio desiderio è continuare comunque a sostenervi, anche a distanza. Ade, non avere paura, non precluderti niente perché questo percorso può sembrarti faticoso, tortuoso ma alla fine ciò che potrai ottenere è meraviglioso. Martina, cerca di andare oltre la tua timidezza, non permetterle di fermarti perché le emozioni iniziali sono sempre le più belle. Lisa, non avere paura, non

preoccuparti che il tuo impegno non venga visto perché niente sfugge e alla fine te ne accorgerai, vai avanti e segui la tua passione. Care ragazze, scopritevi giorno dopo giorno, unite i vostri talenti e innamoratevi della ricerca.

GRAZIE ancora a tutti voi del Laboratorio di Farmacologia di UniFg per ciò che mi avete dato, ognuno di voi con le sue caratteristiche mi mancherà e non voglio assolutamente perdervi, vi ho vissuto e mi avete vissuta in pieno. Farete sempre parte del mio bagaglio e spero di esservi anch'io entrata nel cuore. Se ora penso a come ho iniziato, a quello che sono oggi, mi rendo conto che mi sono veramente arricchita, che quei sacrifici hanno portato dei frutti. Inoltre, quella forza che tutti voi avete visto, oltre che da me stessa, proveniva dagli affetti sicuri della mia vita: la mia Famiglia e Antonio. Cari mamma e papà, ogni giorno vi dico GRAZIE per come mi avete formato. Mi avete dato gli strumenti per affrontare questo percorso: l'autostima, la forza e il senso di sacrificio. Insieme ai miei fratelli siete la mia bussola nella vita e il mio rifugio sicuro. Un GRAZIE speciale ad Antonio, il mio futuro marito, che si rivela ogni giorno il mio compagno di vita. Tanti sono stati i cambiamenti e le decisioni importanti prese durante il mio Dottorato, stiamo seminando con tanto coraggio e sono sicura che le soddisfazioni arriveranno, niente è perso e tutto serve. Caro Antonio, hai avuto tanta pazienza e non mi hai per niente limitato, anzi sei stato la mia parte razionale che in certi momenti particolari veniva a mancare. Per questo ti confermi una delle cose più belle che la VITA mi ha regalato. A tifare per me c'è anche la mia famiglia acquisita: i Caputo. Mi avete sempre sostenuto e i vostri occhi pieni di orgoglio nei miei

confronti sono stati per me in ogni istante un incoraggiamento a non mollare e ad andare avanti. Non posso non ringraziare i miei Amici, in Italia e a Malta. Mi avete dato carica ed energia.

Ebbene, si sono conclusi questi tre anni e come ho detto sinora ognuno di voi mi ha donato qualcosa, mi ha arricchito, ho sofferto tanto ma se mi guardo indietro sono orgogliosa di me stessa, di come ho concluso, di come sono stata battagliera, di come mi sono formata professionalmente e umanamente; se mi guardo avanti sono pronta ad affrontare nuove sfide e avventure perché adesso ho qualche strumento in più rispetto a tre anni fa.

Ad maiora semper…