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Behavioral, imaging, and neurochemical correlates of the neonatal clomipramine model of depression in the rat

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Sommario

Abstract

Depression is a widespread psychiatric disorder that represents a major cause of disability worldwide. Besides the significant mortality rate due to suicide, depression has been associated with an increased prevalence of coronary artery disease and type-2 diabetes. In spite of the accumulated knowledge on the heterogeneous collection of symptoms that characterize the disease, much has yet to be discovered about its pathophysiology. Risk factors include stressful life events, endocrine abnormalities, and genetic predispositions interacting with the environment. The development of animal models of depression have made it possible to study the behavioral and neurobiological correlates of the disease, although only a subset of the symptoms that characterize the human syndrome can be reproduced and studied in the animal. Clomipramine, a well-known tricyclic antidepressant, acts in an apparently paradoxical fashion when repeatedly administered to neonate rats. The treatment induces in the adult a series of behavioral and neurobiological effects similar to a depressive syndrome. The mechanisms underlying the phenomenon are still unknown, although hypotheses have been formulated on the potential depressogenic effects of clomipramine-dependent inhibition of REM sleep and alterations of the monoaminergic circuitry in a developing brain.

In the present dissertation, I report the results of an in-depth investigation of the behavioral and neurobiological correlates of the neonatal clomipramine administration model of depression, not yet described or only partially analyzed in the literature. For each of the experiments, groups of Sprague-Dawley rats were subjected from the 5th to the 21st post-natal day to a protocol of clomipramine administration (20 mg/kg i.p., twice a day). The animals were compared with groups of rats treated with saline injections according to an identical schedule. Both groups of animals were tested for locomotor activity and despair behavior with the forced swim test, for anhedonic behavior with the sucrose preference test and for exploratory activity and anxiety trait with the elevated plus maze test. In all behavioral tests performed, clomipramine-treated animals showed abnormalities, although sometimes subtle, that can be attributed to depression-like symptoms, compared to saline-treated animals. Morphometric studies conducted by means of magnetic resonance imaging, performed to detect possible volumetric abnormalities in brain regions usually associated with depression, showed a significant decrease in brain volume in treated animals, together with a significant enlargement of the lateral ventricles, but no significant changes in the hippocampus. Investigations of the neurobiological correlates of the clomipramine treatment yielded results that include: 1) Brain-derived neurotrophic factor levels were significantly decreased in the hippocampus, but not in the cortex, of treated animals; 2) serotonin transporter expression, measured as optical density of the immunohistochemical labeling of the marker in brain sections, was decreased in the hippocampus and cingulate cortex of treated animals compared to the controls; 3) expression of the glial fibrillary acidic protein, a marker of astrocytes, was by neonatal treatment affected in relation with age and sex. On the other hand, hippocampal neurogenesis rate and hippocampal granule neuronal morphology were not significantly affected by treatment. The data suggest sex-dependent differences in the effects of clomipramine, with female rats being more responsive than males to the neonatal treatment. Furthermore, the behavioral symptoms as well as the neurobiological consequences of the treatment appeared to reduce with aging, to the point of almost complete recovery at an advanced age

The neonatal clomipramine administration model was further characterized by testing the reaction of animals to an acute stressful event, in this case restraint stress realized by immobilizing animals with a wire mesh, on the rationale that such events can trigger or precipitate depression in humans and that a similar phenomenon may be observed in the animal model. For this study, a group of untreated rats, i.e. animals that were left undisturbed in their cages throughout their development, were added to the experimental design. An interaction between early life treatment and stress in adulthood was found in several experiments. Importantly, untreated animals showed substantial differences compared to the other two groups, suggesting that a significant fraction of the observed effects rather than being directly attributable to the pharmacological properties of clomipramine, may in fact depend on the procedure as a whole, including handling, injection discomfort, and separation from the mother. Overall, the studies reported here provide important elements for the evaluation of the efficacy and congruency of the early clomipramine treatment model of depression.

1 Introduction

1.1 Human depression

Depression is a widespread psychiatric disorder characterized by a combination of symptoms that interfere with a person's ability to work, sleep, study, eat, and enjoy once-pleasurable activities. In its more severe forms, depression is a debilitating and potentially devastating disease, whose neurobiological bases are still poorly understood.

1.1.1 Epidemiology and symptoms of human depression

Depression affects over 120 million people worldwide, and is projected to become the second most prevalent cause of illness-induced disability by 2020 according to the World Health Organization (Murray and Lopez, 1997). In addition to the higher mortality associated with suicide, depression complicates the prognosis of a wide variety of other chronic conditions, and depressed patients are more likely to develop coronary artery disease and type 2 diabetes. Despite its prevalence and high impact, understanding the etiology and pathophysiology of depression has been an elusive enterprise for the scores of dedicated researchers, both in basic science and in clinical research.

The reasons for the apparent paradox are, first and foremost, the intrinsic difficulty in observing subtle pathological alterations in the brain that justify the syndrome, and the fact that most depression cases occur idiopathically, in the absence of easily identifiable causal and/or precipitating factors.

The most common time of onset of the first episode of depression is between the ages of 30 and 40, while a second peak is between 50 and 60.

Depression can be diagnosed on the basis of a large variety of symptoms which, according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR, 2000), include:

- emotional symptoms: depressed mood, with feeling of sadness or emptiness, reduced interest and pleasure-seeking in activities usually enjoyed, suicidal thoughts or intentions;
- neurovegetative symptoms: sleep impairments (not enough or too much sleep), loss of appetite and weight;
- physical symptoms: loss of energy or a significant reduction in energy level;
- cognitive symptoms: difficulties in concentrating, holding a conversation, paying attention, or making decisions that used to be made easily.

The complex picture of symptoms, the course and severity of the illness, and its response to antidepressant treatment are all highly variable, suggesting that the term depression may in fact encompass distinct etiological and pathophysiological entities. To date, aside from the clinical assessment, no reliable diagnostic tests for depression are available, which makes it difficult to differentiate mild depression from "normal" reactions to hardships (Berton and Nestler, 2006).

1.1.2 Endogenous vs. reactive depression

An "old-fashioned" classification of depression distinguishes reactive depression from endogenous depression. According to this hypothesis, reactive depression is a direct consequence of a specific, highly stressful event or sequence of events, whereas endogenous depression, presenting no apparent external causative agent, is the result of a biochemical or genetic abnormality. This classification has been largely abandoned, also because it has proven of little use in predicting disease course, suicidal risk, or response to therapy (Pies, 2009).

Understanding the nature and causes of depression has long been the focus of intense research, but many aspects of the disease are still a matter of heated discussion. It is not always clear which neurobiological correlates are causes and which are effects of depression, although some theories have been developed to propose a background for the onset of depression, including psychological, psycho-social, hereditary, evolutionary, and biological arguments. Herein only the biological factors will be treated, with the intent that it will be possible to make a comparison between human and animal depressive syndrome.

1.1.3 Monoamine hypothesis and antidepressant therapy

The monoamine hypothesis of depression proposes that a depletion in the levels of serotonin, norepinephrine, and dopamine in the central nervous system (Fig.1.1) con-

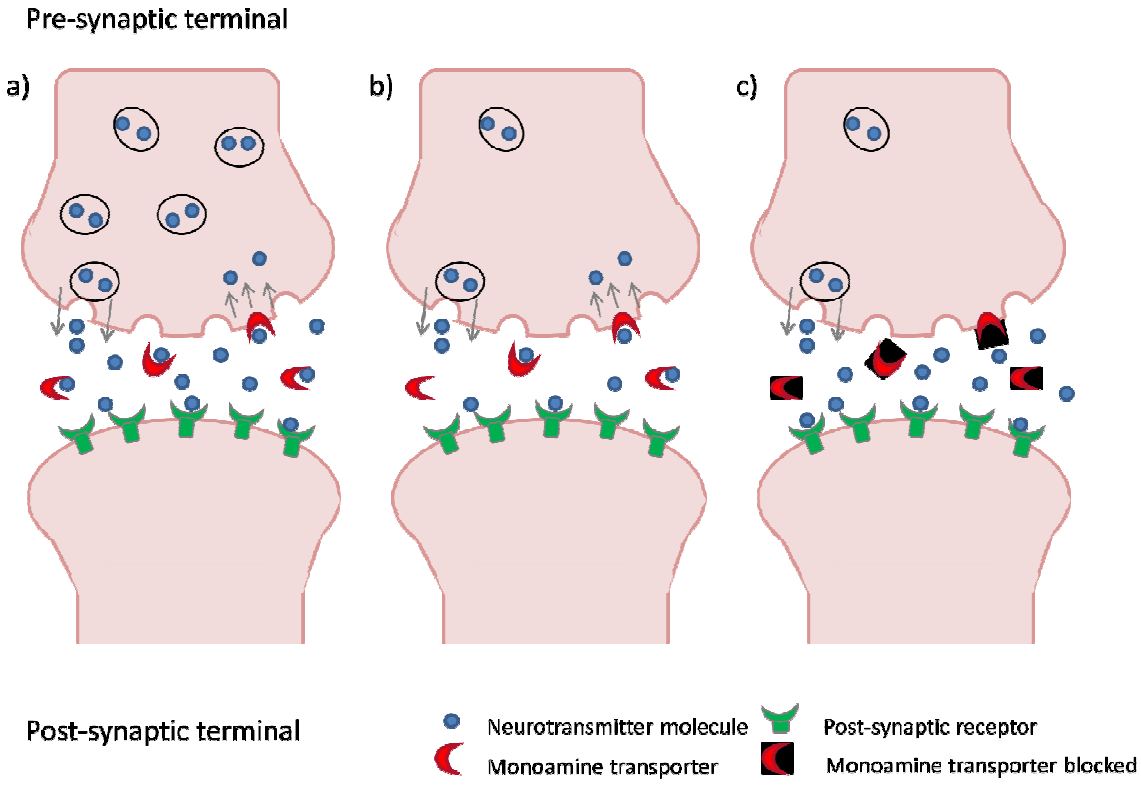

Fig. 1.1 Monoamine hypothesis of depression: a) neurotransmission in a healthy condition: monoamines are released in the synaptic cleft and bind to the postsynaptic receptors. In part, monoamines are recycled through the re-uptake mechanism; b) neurotransmission in depression: lower levels of neurotransmitters are responsible for depressive disorders; c) neurotransmission restored by antidepressant treatment: the inhibition of re-uptake increases the levels of monoamines at the synaptic cleft, allowing

stitute the basis of the pathophysiology of depression (Delgado, 2000). This hypothesis originated from early clinical observations: 1) reserpine, a drug used in the control of high blood pressure that depletes central monoamine stores, was found to induce in a subset of patients depressive effects, among which motor retardation and sedation (Delgado, 2000; Krishnan and Nestler, 2008); 2) imipramine and iproniazid, two structurally unrelated compounds that were not developed as antidepressants, but as an antihistamine and an antitubercolotic respectively, showed antidepressive effects by elevating mood of patients (Castren, 2005).

For many years, the neurobiological bases of depression have been linked to the mechanisms of action of antidepressants. By elevating the levels of neurotransmitters, alleviation of the symptoms of depression is obtained, although the experimental depletion of monoamines in healthy individuals does not alter mood (Krishnan and Nestler, 2008). This observation suggests that the cause of depression is far from being a simple deficiency of central monoamines, but rather the result of the contribution of many factors.

For many years, the pharmaceutical industry focused the development of antidepressant agents, as shown in Table 1.1, on the rationale of the monoamine hypothesis, i.e. that, increasing the levels of the monoamines serotonin, dopamine, and noradrenaline at the synaptic cleft, either by inhibiting the neuronal reuptake mechanism, such as the selective serotonin reuptake inhibitors (SSRI) do, or by inhibiting degradation, like monoamine oxidase inhibitors, can help to restore the normal neurotransmission thus inducing an amelioration of the symptoms of depression. Both classes of drugs act by immediately increasing the levels of monoamines, but their beneficial effects on patients' mood are manifested only after a few weeks of treatment (Krishnan and Nestler, 2008).

Recent drug discovery strategies aim at the development of novel antidepressant agents whose target is other than the recovery of neurotransmission. One important purpose of these new classes of drugs is to obviate the delay of the onset of therapeutic effects observed with classical amine-based therapies. Actions of the novel drugs (see Table 1.2) are directed to disparate targets that range from some classes of receptors (κ opioid, CB_1 cannabinoid, melatonin, NPY, etc.) to regulatory pathways of inflammation and blood coagulation, to gene transcription. All the novel antidepressant therapies are still at a pre-clinical stage of research (Berton and Nestler, 2006).

Class of antidepressant agents	Mode of action	Examples
Tricyclics	Inhibition of mixed noradrenaline and serotonin reuptake	Imipramine, desipramine
Selective serotonin reuptake inhibitors (SSRIs)	Inhibition of serotonin-selective reuptake	Fluoxetine, citalopram
Noradrenaline reuptake inhibitors (NRIs)	Inhibition of noradrenaline-selective reuptake	Atomoxetine, reboxotine
Serotonin and noradrenaline reuptake inhibitors (SNRIs)	Inhibition of mixed noradrenaline and serotonin reuptake	Venlafaxine, duloxetine
Monoamine oxidase inhibitors	Inhibition of monoamine oxidase A (MAO_A). Inhibition of MAOB does not have antidepressant effects	Tranylcipromine, phenelzine
Lithium	Although its many effects, it is still unknown the antidepressant mechanism of lithium	
Atypical antidepressants	Unknown	Bupropion, mirtazapine, tiamepti-ne

Table 1.1 - Currently available antidepressant treatments (Berton and Nestler, 2006).

Antidepressant agents	Target	Actions
κ opioid receptor antagonists	Opioid peptide dynorphin	Systemic or site specific (nucleus accumbens) administration blocks dynorphin action, responsible for depression-like behaviors in rodents. κ antagonists have been found to reduce the immobility in the rodent model of forced swim test (Mague *et al*., 2003).
CB_1 cannabinoid receptor agonists or antagonists	CB_1 receptor and its endogenous ligands	CB_1 receptors are highly expressed in brain regions such as amygdala and the disruption of their activity elicits anxiety-like behaviors in rodents. This effect suggests that anxiolytic tone is inherently mediated by endogenous cannabinoids (Kathuria *et al.*, 2003).
Proinflammatory pathways regulators	Cytokines	Proinflammatory molecules (like cytokines and interferon-γ) are responsible for sickness behavior, a sort of depressive syndrome characterized by a decrease in a number of activities, like feeding, exploration, and sexual activity in animals; humans medicated with interferon-α showed depressive symptoms that were successfully treated with antidepressants (Dunn *et al*., 2005).
Melatonin receptor agonists (ex. Agomelatine)	Melatonin receptor	Melatonin has anti-depressive like effects in animal models, maybe interfering with hypothalamic-pituitary-adrenal axis; in a transgenic mouse model of depression, with decreased glucocorticoid receptor in the brain, agomelatine treatment reduced the immobility time in the forced swim test (Barden *et al*., 2005).
Galanin ligands	Serotonergic and noradrenergic neurons	Galanin inhibits the firing and release of noradrenergic and serotonergic systems besides anatomically coexisting with these two major monoamines in several brain regions. Galanin is implicated in cognition, nociception, feeding and sexual behavior and it is supposed to play an important role in stress (Holmes *et al*., 2005b).
Neuropeptide Y (NPY) agonists	NPY receptor	Anxiolitic effects of NPY are suggested by, for example, the evidence that there is an up-regulation of NPY mRNA levels in the amygdala after chronic stress, and that transgenic rats with overexpression of NPY are behaviorally less sensitive to stress (Charney, 2004).
Histone Deacetylase (HDACs) inhibitors	Gene transcription	There are observations on drugs inhibiting gene transcription with anti-depressant effects (ex. imipramine). Brain regions involved in these actions are not known with certainty yet (Berton and Nestler, 2006).
Agents interfering with tissue plasminogen activator (tPA) action	tPA	tPA is known to promote stress-induced synaptic plasticity and anxiety-like behavior. Corticotropin-releasing factor can upregulate tPA activity in the amygdala consequently influencing neuronal activation and behavioral response (Matys *et al*., 2004).

Table 1.2 - Novel antidepressant therapies in preclinical phase of drug discovery research.

Non-pharmacological forms of antidepressant therapy range from psychotherapy, recommended for mild depression, to electroconvulsive therapy, a shock treatment that, inducing changes in brain chemistry, has been demonstrated to be highly effective in reversing symptoms of depressive syndromes resistant to pharmacological treatment (Berton and Nestler, 2006). Another non-pharmacological therapy is represented by deep brain stimulation, which, stimulating a region of the cingulate cortex , has been shown to improve depressive symptoms (Berton and Nestler, 2006).

1.1.4 Dysregulation of the Hypothalamic-Pituitary-Adrenal Axis and stress

The so-called hypothalamic-pituitary-adrenal (HPA) axis is an anatomical complex constituted by the hypothalamus, the hypophysis, and the adrenals glands that through both neuronal and hormonal interactions and feedback loops controls and regulates physiological processes such as digestion, temperature, immune response, sexuality, energy usage, and mood. The HPA axis plays a crucial role in how the organism reacts to stressful events, injury, and trauma.

The regulation of HPA axis is illustrated in Fig. 1.2. The corticotropin-releasing factor (CRF) is released by parvocellular neurons of the paraventricular nucleus of the hypothalamus under an excitatory influence from the amygdala. Through the hypophyseal portal system, CRF reaches the anterior pituitary and stimulates it to release the adrenocorticotropin hormone (ACTH). ACTH, via systemic blood circulation, reaches the adrenal cortex inducing the release of glucocorticoids. By means of negative feedback, glucocorticoids inhibit CRF and ACTH synthesis and release, and, consequently, their own synthesis (Nestler *et al*., 2002; Barden, 2004).

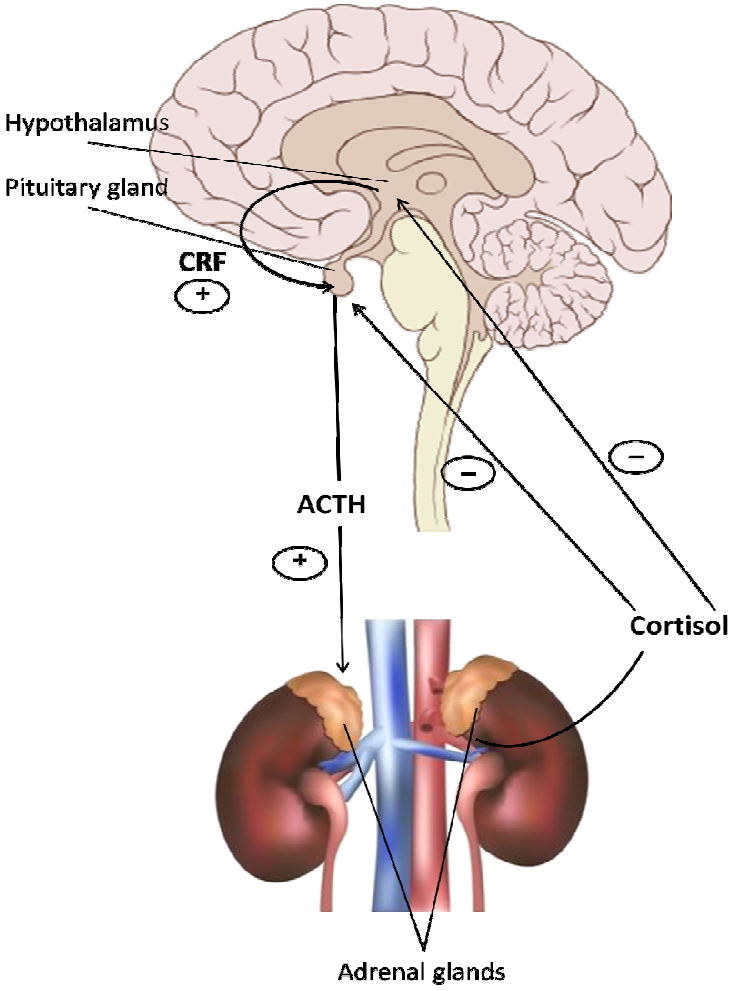

Fig. 1.2. Regulation of HPA axis, decribed in the text. Adapted from Nestler *et al*., 2002.

Small but significant increases in serum concentrations of glucocorticoids, in particular cortisol, in depressive patients suggest a role of a dysfunctional HPA axis in the

pathophysiology of depression (Krishnan and Nestler, 2008). Indeed, some depressed patients present excessive activation of the HPA axis, an abnormality that can be reversed to correct functionality by antidepressant treatment (Nestler *et al*., 2002; Barden, 2004). In this respect, a possible mechanism by which antidepressants, such as the tricyclic agents amytriptiline (Reul *et al.*, 1993) and imipramine (Kitayama *et al.*, 1988) and the monoamino oxidase A inhibitor moclobenide (Reul *et al*., 1994), could act is the increase, through stimulation at the genomic level, in the concentrations of cellular corticosteroid receptors to make the HPA system more susceptible to feedback inhibition by cortisol (Barden, 2004).

HPA axis is strongly activated by stress (Aguilera, 1994; Aguilera and Rabadan-Diehl, 2000), which is widely acknowledged to be a precipitating factor of depressive syndromes especially in genetically predisposed individuals. Although stress per se has not been demonstrated to cause depression, the early phases of depression are associated with enhanced responsivity to stress. Early life experiences are considered a relevant modulator of the development of individual vulnerability to stressful events and, consequently, to depression (Kubera *et al*., 2011).

Stress decreases BDNF expression (Duman and Monteggia, 2006), increases pro-inflammatory cytokines concentrations (O'Connor *et al.*, 2003), decreases neurogenesis and induces dendritic atrophy through BDNF- and/or HPA-axis-dependent mechanisms (Duman and Monteggia, 2006), affects hippocampal function and structure (Lucassen *et al.*, 2010). All these actions contribute to the concept that stress should be regarded as a major risk factor for depression. The importance attributed to stress in establishing depression is reflected in the large use of stress-induced animal models of depression, where animals that are subjected to a single or to repeated stressful events at any age show behavioral and neurochemical markers of depression (Kubera *et al*., 2011).

1.1.5 Genes and environment

Across the life span, stressful life events can influence the onset and course of depression, as said in section 1.1.4, but not all people succumb to depressogenic effect of stress (Caspi *et al.*, 2003). The diathesis-stress model of depression attempts to explain individual's behavior as a result of genetic vulnerability together with individual's sensitivity to stressful events (Caspi *et al*., 2003).Behavioral genetic research, supporting this prediction, aims at the documentation of a relationship between genes and depression.

The disorder is highly inheritable, even if no genetic abnormalities have been linked with certainty yet (Nestler *et al*., 2002). It should be noted that the identification of genetic alterations that may be responsible for the disorder has proven difficult not just in the case of depression, but more in general for psychiatric disorders, due to the complexity of each one. It is likely that the respective genetic bases lay in alterations of groups of genes, rather than of a single gene (Nestler *et al*., 2002).

One of the most discussed studies in this research field is the one performed by Caspi and collaborators that was aimed at: a) demonstrating of a correlation between the functional polymorphism in the promoter region of the serotonin transporter gene (SLC6A4) and vulnerability to develop a depressive syndrome and b) investigating of the impact of gene variation on life stress influence on depression (Caspi *et al.*, 2003). The longitudinal study was not able to demonstrate a direct association between the serotonin transporter gene and depression, even if the interaction gene*environment extends

to the natural development of depression in a representative sample of subjects followed (Caspi *et al.*, 2003).

1.1.6 The inflammatory hypothesis of depression

Immunological mechanisms have been included among the neurobiological correlates that characterize depression. During inflammation, cells of the immune system produce pro-inflammatory cytokines such as interleukine-1α and β (IL-1α and IL-1β), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) which elicits various endocrine, autonomic, and behavioral alterations that together coordinate the "nonspecific symptoms of sickness" (Dantzer *et al.*, 2008; Lucassen *et al.*, 2010). According to recent hypotheses, pro-inflammatory cytokines may not only induce sickness behavior, but also a depressive syndrome in physically ill patients who have never suffered previous depressive episodes or mental disorders (Dantzer *et al.*, 2008).

These patients appear feverish, nauseated, lacking appetite, and not interested in any social activity; they get tired easily and have a fragmented sleep; moreover, they show a depressed and irritable mood, and a certain degree of cognitive impairment, such as lack of attention and memory (Dantzer *et al*., 2008). These symptoms largely overlap the set of typical symptoms of depression.

In rodents, central or systemic administration of IL-1β or TNF-α induces sickness symptoms, like decreased motor activity, sociality, liquid and food intake, and altered sleep pattern, cognition, and pain-sensitivity (Dantzer *et al*., 2008).

In humans, depressive symptoms develop in a third of patients treated with the recombinant human cytokines interleukine-2 (IL-2) and interferon- α (IFN-α), as anticancer therapies (Dantzer *et al*., 2008). Furthermore, increased levels of chemokines and adhesion molecules, of cytokines and acute-phase proteins have been observed in depressed patients (Raison *et al*., 2006).

Peripherally secreted cytokines access the brain through three different routes: 1) leaky regions of theblood brain barrier, 2) binding to specific transporters expressed in the brain endothelium, and 3) activation of vagal afferent fibers that transmit the signal to specific brain nuclei which than serves as a relay station to other brain nuclei including the paraventricular nuclei in the hypothalamus. Once in the brain, a central cytokine network, made of neurons and glia, expresses cytokine receptors and amplify the signal, inducing behavioral and hormonal effects. These changes are reflected in the alteration of the metabolism of serotonin, noradrenaline, and dopamine in brain areas belonging to the limbic system, and in the stimulation of the HPA axis in the secretion of hormones (Raison *et al*., 2006).

The driving factor for inflammation in depressed patients could be psychological stress: stress is a common risk factor for depression and it is known that in laboratory animals a large variety of stressors, such as inescapable tailshock, footshock, social isolation, immobilization, restraint, and open-field exposure, increase expression of pro-inflammatory cytokines, with IL-1ß being the most explored cytokine in regard, in brain regions involved in the emotionality and in the periphery (O'Connor *et al*., 2003).

Paroxetine has been found to improve IFN-α-induced depression (Musselman *et al*., 2001) and it has been proven that antidepressants inhibit the production and/or release of pro-inflammatory cytokines and induce the production of anti-inflammatory cytokines. These observations suggest that antidepressants, reducing inflammatory process, induce an amelioration of the symptoms of depression, even if not all symptoms seem to be responsive, such as fatigue or psychomotor slowing (Raison *et al*., 2006).

Some studies, however, have failed to find a correlation between inflammation and depressive severity or have found disparate and occasionally opposite correlations for different pro-inflammatory cytokines (Raison *et al.*, 2006). These observations suggest that it is not possible to assert with certainty that inflammation has a role in the pathophysiology of depression (Raison *et al*., 2006).

1.2 Neurobiological correlates of depression

1.2.1 Adult hippocampal neurogenesis and neuroplasticity

Neurogenesis is the process of generating new cells in the brain from neural progenitor stem cells. Early neurodevelopmental studies, including Ramon y Cajal's classical observations, asserted that the formation and differentiation of neurons in the mammalian brain is restricted to the prenatal and early postnatal age (Ming and Song, 2005). The notion of a hard-wired brain in the adulthood was so engrained that the later discovery of adult neurogenesis came as a surprise (Ming and Song, 2005). It is currently well established that the phenomenon occurs in at least two brain areas, the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus, whereas outside these two regions neurogenesis seems to be limited or non-existing (Ming and Song, 2005; Ehninger and Kempermann, 2008). The fact that adult neurogenesis occurs in all mammalians species studied to date, including humans (Eriksson *et al*., 1998), suggests an important role for the phenomenon (Fuchs and Gould, 2000), although to this day its full significance is not well understood.

In principle, of course, the generation of new functional neurons in the adult brain should be regarded as the ultimate expression of structural plasticity. On the other hand, given the numerical and topographical limitations of neurogenesis, the relationship between the phenomenon and the general domain of neuronal plasticity is complex and hard to pinpoint. In rodents, it seems that the formation and survival of new neurons is correlated with learning and short-term memory (Gould *et al*., 1999a; Gould *et al*., 1999b; Kempermann, 2002; Leuner *et al*., 2004). Hippocampal cell proliferation in rodents has been reported to be positively influenced by many factors, such as voluntary exercise (Farmer *et al.*, 2004), sleep deprivation (Grassi Zucconi *et al*., 2006), and environmental enrichment (Kempermann *et al*., 1997). Furthermore, neurogenesis in the rodent hippocampus has been shown to increase following antidepressant treatment (Malberg *et al*., 2000; Dranovsky and Hen, 2006; Warner-Schmidt and Duman, 2006; Paizanis *et al*., 2007; Krishnan and Nestler, 2008). These observations may represent a link between hippocampal neurogenesis and depression. Antidepressants' regulation of neurogenesis seems to range from cell proliferation to differentiation and survival (Malberg *et al*., 2000). Interestingly, all tests performed with different classes of antidepressant drugs resulted in an increased proliferation and survival of new neurons in the dentate gyrus of rodent hippocampus (Paizanis *et al*., 2007; Pittenger and Duman, 2008). On the other hand, actively blocking neurogenesis does not elicit a depression-like effect, as shown in the open-field, the light-dark choice, and the elevated plus maze tests, although the block affects hippocampus-dependent forms of fear-conditioning, long-term spatial and working memory (Sahay and Hen, 2007).

These results may support the idea that the increase in hippocampal neurogenesis is a sort of driving force to enhance the antidepressant response but, at the same time, there are no observations that confirm that impairments in the rates of neurogenesis are involved in the core features of depression (Krishnan and Nestler, 2010). Even in

the presence of convincing data correlating depression-like symptoms in the rodent with a dysfunctional hippocampal neurogenesis, much has yet to be explored in the human hippocampus and the limited number of publications on this issue offer contrasting evidence. Indeed, postmortem analyses of hippocampal tissue from depressed patients have not revealed a decreased neurogenesis, but rather an increase in neuronal and glial density in conjunction with decreased neuronal cell body size (Stockmeier *et al*., 2004). On the other hand, it has been shown that antidepressant treatment induces an increase in neural progenitor cells both in non-human primates (Perera *et al*., 2007) and in humans (Boldrini *et al*., 2009).

Elevated levels of glucocorticoids, produced by HPA axis when acitivated by stressful events, represent a negative factor for hippocampal neurogenesis rate and neuroplasticity. Glucorticoids have been shown to reduce the birth of new neurons in the hippocampal granule cell layer (Fuchs and Gould, 2000) and to induce atrophy of CA3 pyramidal neurons, with a reduction of apical dendritic branch points and of the length of apical dendrites, and a loss of dendritic spines specialized in the reception of excitatory glutamatergic synaptic inputs (Sapolsky, 2000). Transient glucorticoid overexposure induces an alteration of neuronal morphologic features, thus it can be deleterious to explicit hippocampal memory pathways (Sapolsky, 2000). Impaired hippocampal function consequent to such damages may contribute to some of the observed cognitive abnormalities of depression. Re-growth of dendritic processes could occur if glucocorticoid exposure stop (Sapolsky, 2000).

Ont the other hand, antidepressant therapy has been shown to positively influence hippocampal cell proliferation and plasticity. Fluoxetine, a well-known SSRI antidepressant agent, modulates synapse density in CA1 and CA3 after 5 days of treatment, but CA3 with a higher peak only after 14 days of treatment; this asynchrony in the synaptoplasticity effects may be due to the delayed integration of new granule cells into the hippocampal circuitry. It is known that neurogenesis is enhanced within two weeks of fluoxetine treatment and that newborn hippocampal cells directly innervate and activate only CA3 pyramidal neurons through mossy fibers (Fig. 1.3), so this could be the reason of the later onset of new synapses in CA3 (Hajszan *et al*., 2005). It is presumable that hippocampal synaptic remodelling is, if not the first, an early stage of restoration of normal functioning of hippocampus (Hajszan *et al*., 2005).

In a study in the olfactory-bulbectomy model of depression in rodents, chronic administration of amytriptiline, a tricyclic antidepressant, was found to block the de-

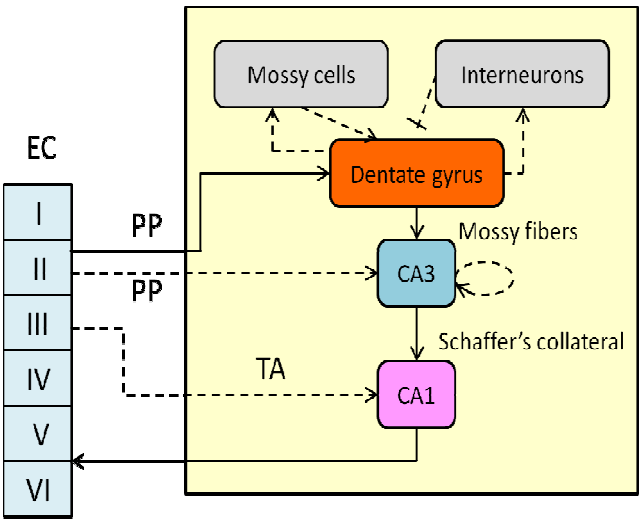

Fig. 1.3 Rodent hippocampal circuitry: diagram of the neural network. CA3 receives projections from layer II neurons of entorhinal cortex (EC) through the direct connection of the perforant pathway (PP) and through an indirect connection, in which the dentate gyrus is a middle passage. CA3 pyramidal neurons convey the information to CA1 pyramidal neurons through Schaffer collaterals and CA1 pyramidal neurons send back-projections to deep-layer neurons of the EC. CA1 receives direct projections from EC through the temporoammonic pathway too. Granule cells of the dentate gyrus also project from interneurons of the hilus which send back inhibitory or excitatory stimuli (adapted

crease in spine density in dentate gyrus, CA1 and CA3 cell layers (Pittenger and Duman, 2008). More studies are needed to test the action of different classes of antidepressant drugs since it has been observed that reduction in dendritic arborization is blocked/reversed by chronic administration of tianepine, an atypical antidepressant, but not by fluoxetine, in a chronic stress model of depression (Pittenger and Duman, 2008).

1.2.2 Brain-Derived Nerve Factor

Neurotrophic factors, besides the regulating action on neuronal growth and differentiation, are associated with plasticity and survival of adult nerons and glia.

At the end of the 1990s, the hypothesis of a role of neurotrophic factors in the pathophysiology of depression raised the interest of many scientists. According to the hypothesis, an altered expression of neurotrophin levels may contribute to the development of depression by inducing neuronal atrophy and cell loss in key limbic brain regions involved in depression, such as the hippocampus (Nestler *et al.*, 2002; Duman and Monteggia, 2006).

Attention has been focused for many years on the brain-derived nerve factor (BDNF), possibly the most prevalent neurotrophic factor in adult brain. The great majority of blood BDNF is localized in platelets, from which it is released following platelet activation. In depressed patients, blood levels of BDNF are not different from those in healthy controls, but it is possible that in depressive syndromes the ability of platelets to release BDNF is altered (Castren and Rantamaki, 2010). It is unclear whether serum BDNF levels reflects brain BDNF levels, since BDNF does not cross the blood-brain barrier (BBB); plasma BDNF could influence brain regions in which BBB is leaky, such as parts of the hypothalamus (Castren and Rantamaki, 2010). A large amount of literature has shown that stress can cause damage and atrophy of neurons in several brain structures and a decrease in BDNF expression, maybe via a stress-induced glucocorticoid mechanism (Smith *et al*., 1995), but chronic antidepressant treatment has been shown to restore BDNF levels in these regions (Nibuya *et al*., 1995). Nowadays, BDNF signalling is believed to play an important role in the mechanism of action of antidepressants in the improvement of depressive symptoms, although the role of BDNF itself in the onset of depression is still unclear (Castren and Rantamaki, 2010).

Signs of hypomania following intratechal BDNF administration were observed in patients with amyotrophic lateral sclerosis, thus supporting the hypothesis of a role for BDNF in regulating mood (Castren and Rantamaki, 2010). In animal models of depression, in contrast to the observed anti-depressant-like effects when injected into hippocampus (Shirayama *et al*., 2002) and midbrain (Siuciak *et al*., 1997), BDNF increased pro-depressant effects when administered into the ventral tegmental area and nucleus accumbens (Eisch *et al*., 2003). In a rat model of chronic mild stress, BDNF protein expression was found decreased in the hippocampal dentate gyrus, but not in the CA region (Gronli *et al.*, 2006). In a study on a chronic unpredictable stress model of depression, however, the opposite effect was found (Larsen *et al*., 2010).

It is worth mentioning a study performed in transgenic mice with a single-nucleotide polymorphism in the gene encoding BDNF, whose exposure to a stressful environment induced an increase in anxiety-like behaviors that were not normalized by fluoxetine treatment (Chen *et al*., 2006). These results demonstrate that BDNF may represent an example of the gene-environment interaction responsible of the onset of mood disorders, possibly of a depressive syndrome, as previously discussed in section 1.1.5,

1.2.3 Neuroanatomy of depression

The limbic system, constituted by a group of brain structures surrounding the brainstem (the cingulate gyrus, hippocampus, the hypothalamus, amygdala, antherior thalamic nuclei, the prefrontal and the enthorinal cortex) was identified as playing a role in the experience of emotion and in the storing of memory (Palazidou, 2012). The emergence of neuroimaging techniques , like magnetic resonance imaging (MRI), provided evidence of the malfunction of the brain structures and the connections that form the limbic region in depressed patients, suggesting a central role of this system in the pathophysiology of depression (Palazidou, 2012). The prefrontal cortex (PFC), the amygdala and the hippocampus are the brain structures most studied in relation to depression (Palazidou, 2012).

The hippocampus is the most widely studied region in relation to depression because it is fundamental for learning and memory functions, it is rich in corticosteroid receptors providing regulatory feedback to the HPA axis, and it is one of the two regions in which neurogenesis occurs in adulthood (see section 1.2.1).

In the human depressed patients, MRI studiesof the hippocampus, provide information about the correlation among neuronal atrophy, volumetric changes and depression *in vivo* while in postmortem studies it is possible to investigate the cellular aspects responsible of volume changes. A high number of post-mortem studies showed morphological and morphometric changes of the hippocampus of depressed patients, including volume loss (Bremner *et al*., 2000; Sheline, 2000; MacQueen *et al*., 2003; Neumeister *et al*., 2005), gray matter alterations and neuropil reductions (Bremner *et al*., 2000; Sheline, 2000). Therefore, it is not clear why the hippocampus is decreased in volume since neuron density is not reduced (Stockmeier *et al*., 2004). A few studies, reporting no differences between patients and healthy subjects (Ashtari *et al*., 1999; Vakili *et al*., 2000) maybe did not consider effects of sex, age, anatomical definition, type of depressive syndrome, or antidepressant treatment response (Schmidt and Duman, 2007; Kempton *et al*., 2011).

Other reductions in the volume of brain regions have been found in the frontal cortex, caudate nucleus, putamen, pituitary gland and core nuclei of the amygdala (Sheline, 2000). Besides the results above described, a very recent meta-analysis study reported in depressed patients compared to controls about a significant increase in volume of adrenal gland, basal ganglia, and thalamus and a significant enlargement of lateral ventricles (Kempton *et al*., 2011).

1.3 Animal models of depression

Models of depression have been developed to reproduce the symptoms of depression in laboratory animals in order to study behavioral patterns and biological mechanisms. It is difficult to realize an animal model that perfectly represents human depression: animals lack consciousness of self, self-reflection and consideration of others as well as traits of depressed mood, low self-esteem, and suicidality. On the other hand, other hallmarks of depression can be reproduced and investigated in animals, including behavioral, physiological, hormonal, morphological, and neurochemical modifications (Berton and Nestler, 2006; Nestler and Hyman, 2010).

In rodents there is a set of symptoms of depression, common to human depression, that can be reproduced and investigated. They include:

- changes in appetite and/or weight gain, that can be easily measured in animals;
- alterations in sleep pattern: disturbances in circadian rhythm, especially insomnia or excessive sleeping, can be measured using electroencephalography;
- anhedonia: a marked loss of interest in pleasurable and rewarding actions can be measured with intracranial self-stimulation, preference for a palatable reward (for example, sucrose) and social withdrawal;
- anxiety-like behavior: this symptom has a high prevalence in depression; in animals it can be investigated with behavioral paradigms, such as the elevated plus maze test and the open field test, or with neurochemical analyses, like hormonal dosage of cortisol;
- behavioral despair: the inhability to cope with a helplessness and inescapable situation is a common feature measured in animals with the forced swim test and the tail suspension test;
- fatigue or loss of energy: reduced activity in home cage or other stimulating environments with tests that measure locomotor activity;
- difficulty in performing even easy tasks: this can be reflected in the scarce hygiene of animals, like dirty and ruffled coat;

As said above, some symptoms, like recurrent thoughts of death/suicide and feelings of guilt cannot be modeled in animals but other investigations can be performed on neuroanatomical features, like hippocampal neuronal arborization and death, or neurochemical analysis of depression-targeted substances, like neurotrophic factors (Cryan and Holmes, 2005).

1.3.1 Validation of an animal model of depression

The minimal requirements for animal models of depression are *face*, *construct* and *pharmacological validity*.

Face validity is the degree of homology of the model with human depression. The symptomatology in rodents ranges from social isolation, lack of appetite with weight loss, anhaedonic behavior, circadian alterations, and HPA axis abnormalities. It is possible that these symptoms are not present at the same time and persist only for a certain period of animal's life (Willner and Mitchell, 2002; Krishnan and Nestler, 2010).

Construct validity consists in the causality of the syndrome: the etiology should be the more possible resembling the one that triggers human depression. This is a challenging requirement: today we still do not know the definitive causes of depression, even if we have learned a lot about factors characterizing it (Willner and Mitchell, 2002; Krishnan and Nestler, 2008, 2010).

Pharmacological validity, known also as *predictive validity*, is met when depressive-like behaviors of the animal model are reversed by the same treatment modality that is effective in humans. Anyway, this is not a mandatory requirement, since some animal models are reproduced in order to study the antidepressants' mechanism itself, that is still largely unknown (Krishnan and Nestler, 2010).

Another potential requirement that could be taken in account is *pathological validity*: animals should show the same physiological, cellular, and molecular changes observed in post-mortem studies on brains of depressed patients (Krishnan and Nestler, 2010).

Besides these demanding conditions, of course it is desirable that the behavioral changes can be monitored objectively and that the animal model is reproducible between investigators.

1.3.2 Animal models of depression

As mentioned above, many of the symptoms of depression cannot be easily investigated in laboratory animals and the lack of known genes involved in vulnerability to depression are major impediments in depression research (Nestler *et al*., 2002).

In Table 1.3, the most widely used animal models of depression are presented. A substantial fraction of all available animal models of depression rely on exposure to various types of stressors and response to antidepressant therapy, but some of these, such as the forced swim test (Kitayama *et al.*) and the tail suspension test, are no more considered as real models of depression, but have been found to be useful paradigms in the pharmaceutical industry to assess the efficacy of new medications in the screening stage of drug development (Nestler and Hyman, 2010). The learned helplessness model can be included in this type of tests because they all have a common paradigm: to subject animals to an acute, short-duration (only a few minutes) stress to make them to respond in an active or passive way and maybe provoking a despair behavior measurable as time spent in immobility during the stressful event. An acute antidepressant treatment in animals that underwent these short stressful events is really effective; but criticisms against the validity of these animal models were raised, since it is known that in human depression antidepressants are administered chronically for weeks or months to obtain an effect within a few weeks, but single doses are able to improve symptoms in animals in a few hours or days (Nestler and Hyman, 2010). Even if the learned helplessness model simulates only an increased risk for precipitation of depression (Willner and Mitchell, 2002), it is still one of the most studied models of depression, especially for the demonstrated involvement of a genetic predisposition to develop signs of helplessness symptoms (Cryan and Mombereau, 2004).

Chronic stressors used on normal adult animals induce symptoms of anhedonia, that can be measured as a reduced consumption of a sucrose solution, which chronic, but not acute, treatment with antidepressants is able to reverse. Chronic stressors used to induce depression in laboratory animals include chronic mild or unpredictable stress and psychosocial stress. Chronic mild or unpredictable stress is represented by a set of different stressful events to which the animals are subjected: cage tilting, inversion of light/dark cycle, immersion in cold water, food or liquid deprivation, and paired housing are some of the most common stressors used (Nestler *et al*., 2002).

Chronic social defeat is a paradigm of psychosocial stress, in which rodents are subjected to repeated bouts of social subordination. The intensity of this kind of stress is more severe than that seen in most humans and it induces not only anhedonia, but social withdrawal too (Nestler and Hyman, 2010). Prolonged exposure of adult rodents to social isolation induces anhedonia as well, and chronic antidepressant treatment is effective in reversing this symptom(Nestler and Hyman, 2010). It has been shown that early adverse life events can trigger an abnormal development: traumas in childhood may result in increased sensitivity to stress exposure in adulthood and increased vulnerability to stress-induced disorders, depression included (Heim and Nemeroff, 2001).

MODEL	METHOD OF INDUCTION	BEHAVIORAL CORRELATES OF DEPRESSION
STRESS IN ADULT-HOOD		
Learned helplessness	Animals are exposed to uncontrollable and inescapable shocks and consequently fail to escape from a situation in which escape is possible (Vollmayr and Henn, 2001).	Short lasting deficits in escape, cognitive and rewarded behaviors, such as sucrose preference
Chronic mild/unpredictable stress	Animals are exposed to a series of mild stressors (isolation or crowded housing, food or water deprivation, inversion of light/dark cycle, cage tilting, wet bedding, low room temperature, noises, etc.) presented in a continuous unpredictable fashion every day for at least two weeks (Nestler and Hyman, 2010).	Long lasting changes of behavioral parameters: decreased sucrose preference and intracranial self-stimulation, poor fur condition
Psychosocial stress	An example is the resident-intruder paradigm, in which an adult male (intruder) is introduced into the home cage of an unfamiliar aggressive conspecific (resident) (Rygula *et al*., 2005); similarly, in another paradigm, an aggressive male can be introduced into a stable social group. A further paradigm is the creation of social groups and then mixing them (Blanchard *et al*., 2001).	Anhedonia, decreased locomotor activity and social withdrawal
DEVELOPMENTAL		
Early-life or prenatal stress	Maternal separation for 1-24h per day during the first two postnatal weeks (Sterley *et al.,* 2011). Prenatal stress is performed by exposing dams, in the early stages of pregnancy, to repeated footshock or in the late stages to mild stress of handling (Ward *et al*., 2000).	Increased anxiety- and depression-related behaviors and abnormal locomotor activity
Clomipramine and other antidepressant agents	Chronic drug administration in the postnatal period for two or three weeks induces a depressive syndrome in adulthood (Willner and Mitchell, 2002).	Decreased sexual activity, aggressive behavior and rewarded behaviors; increased immobility in the FST
LESIONS		
Olfactory bulbectomy	Bilateral removal of the olfactory bulbs induces changes in synaptic strength and loss of spine density in limbic circuitry (Song and Leonard, 2005)	Hyperactivity in open field, impaired spatial learning in water maze
PHARMACOLOGICAL		
Reserpine	Reserpine induces a depletion of central stores of neurotransmitters (Delgado, 2000)	Motor retardation and sedation
Psychostimulant withdrawal	Escalating doses of cocaine or amphetamine administration for several days (Barr *et al*., 2002)	Increased immobility in the FST and tail suspension test; reward deficits
IMMUNE STIMULATION		
Proinflammatory cytokines	Central or systemic administration of IL-1β or TNF-α induces sickness behavior, a syndrome similar to depression (Dantzer *et al*., 2008).	Decreased motor activity, sociality, liquid and food intake, and altered sleep pattern, cognition, and pain-sensitivity
GENETIC		
Selective breeding	Specific criteria concerning performance in a behavioral test, such as TST, are used for selection and consequent breeding of animals (El Yacoubi *et al*., 2003).	Great variability within the models
Genetically engineered mice	Genes for serotonine and norepinephrine receptors and transporters, dopamine ß-hydroxylase, monoamino oxidase A, BDNF and CREB constitute the candidate genes for the genetic mutant mice (Cryan *et al*., 2002; Urani *et al*., 2005).	Behavioral syndromes are dipendent from the gene selected

Table 1.3. Summary of common rodent models of depression

Early-life handling and maternal separation, have been proven to be valid models

of depression: the stressful event to which pups are subjected has life-long behavioral and neuroendocrine rebounds, that can be reversed by antidepressant treatment (Nestler and Hyman, 2010). Moreover, mouse models of postnatal stress have been produced to study the interaction between a specific gene and the environment (Holmes *et al*., 2005a).

Chronic administration of antidepressant drugs like clomipramine, zimeldine, and citalopram in the early postnatal period have been shown to induce in the adult rats a spectrum of symptoms resembling those of depression: decreased aggressive behavior and sexual activity in males, anhedonia, REM sleep abnormalities, and motor agitation in some tests and motor retardation in other tests. These long-lasting symptoms were shown to be reversed by antidepressant treatment or REM sleep deprivation (RSD) (Vogel *et al*., 1990a).

A lesion model is a non-stress based animal model that relies on the assumption that deficits in regulation of neuronal circuitry can cause depression. The olfactory bulbectomy in rodents induces a disruption of the limbic-hypothalamic axis, thus the behavioral abnormalities observed are not only consequence of loss of smell, but largely of compensatory mechanisms of neuronal reorganization, included changes in synaptic strength and loss of spine density in limbic regions, like hippocampus and amygdala (Song and Leonard, 2005). The hyperactivity observed in the open field paradigm is reversed by antidepressant treatment (Cryan *et al*., 1999).

Reserpine treatment induces decreased locomotor activity and reduced body temperature by non-selectively depleting monoaminergic stores in the central nervous system. Iproniazid, a drug developed against tubercolosis and now used as antidepressant, can reverse the effects of reserpine on hypomotility in rats. It is still not clear how reserpine lowers mood and which monoamine is predominantly made unavailable by its action. The validity of the reserpine model is still under investigation (O'Neil and Moore, 2003).

Psychostimulant withdrawal induces in humans typical symptoms of a depressive syndrome; in rodents, amphetamine or cocaine withdrawal may help to comprehend the neurobiological mechanisms that underlie the development of depression (Barr *et al*., 2002). The symptoms are transient but are highly analogous to some aspects of human depression.

Another model of depressive-like behavior is represented by the administration of TNF-α in rodents. Animals show a spectrum of symptoms such as anorexia, decreased social behavior and locomotor activity, as well as activation of the hypothalamic-pituitary-adrenal axis. All these symptoms characterize the sickness behavior, that resemble a depressive syndrome (Kaster *et al*., 2012). It has been proved that antidepressants inhibit the production and/or release of pro-inflammatory cytokines and induce the production of anti-inflammatory cytokines. These observations suggest that antidepressants, reducing inflammatory process, induce an amelioration of the symptoms of depression, even if not all symptoms seem to be responsive, such as fatigue or psychomotor retardation (Raison *et al*., 2006).

Genetic approaches offer the possibility to identify the genes that are determinant to develop a depressive syndrome and to reproduce in animals the human conditions. Several approaches were exploited to find target genes involved in the onset of depression and the result is that depression is a syndrome characterized by multiple genetic mutations (Bucan and Abel, 2002). Genetically engineered mice have been successfully developed to investigate depression; nowadays, mouse mutants target monoaminergic receptors and transporters and enzymes necessary for monoaminergic

synthesis and degradation (Urani *et al*., 2005). The limitations of this kind of models is that the effects of only one gene at a time can be studied, thus animals produced do not represent entirely a realistic model of depression.

Also selective breeding of mice and rats is a successful strategy, taking advantage of differences in the individual responsiveness in paradigm of depression- or antidepressant-like behavior such as immobility levels in the FST, or susceptibility to learned helplessness (El Yacoubi *et al*., 2003).

1.3.3 Sex differences in animal models of depression

Epidemiologic research has repeatedly reported about the difference between men and women in the rates and courses of depression; in particular it has been found that in females morbidity is twice as common as in males, and females had the first episode at a younger age compared to males (Marcus *et al*., 2005).

The serotonergic system was found to be implied in a sexual dimorphism: in depressed women, cerebrospinal fluid levels of 5-hydroxy-indoleacetic acid was found to be increased, and, in general, whole brain serotonin synthesis and 5-HT_2 receptor binding attitude were significantly decreased compared to men (Dalla *et al*., 2010). For what concerns animals, normal female rats showed increased serotonergic activity, synthesis and levels of metabolites in a study of sex differences in rat brain serotonin (Carlsson and Carlsson, 1988).

There is also recent evidence about sex differences in antidepressant response: women appear to respond in a different way according to the class of antidepressant agents, maybe due to a hormonal "background". Indeed it has been shown that menopause influences the response to therapy (Dalla *et al*., 2010).

The investigation of sex differences in the neurobiology of depression and antidepressant response is important to improve diagnosis of depression and may provide gender-based antidepressant therapies which take account of sex differences in pharmacokinetics and pharmacodynamics (Dalla *et al*., 2010).

Most animal models of depression were produced using male rodents and only later they were applied in females, but with certain difficulties due to not only basal sex differences but also to differences that emerged after certain manipulations. The exploration of these differences could be useful to understand the heterogeneous development of depression in men and women and the sex influence in response to antidepressant treatment.

It is largely acknowledged that female rats do not express learned helplessness behavior as males do and even a complex study did not succeed in demonstrating that sexual hormones may be imputable for the lack or decreased expression of the learned helplessness behavior in females (Dalla *et al*., 2008).

In response to a set of chronic mild stressful events, female and male rats showed different performances in behavioral assessments: 1) both males and females showed a decrease in sucrose preference, but males at a major degree; anyway, sucrose intake has been shown to be higher in unstressed females compared to males, so these basal evidence could suggest that this paradigm is not an appropriate parameter; 2) females spent less time in explorative activity in an open field compared to males; 3) in response to an additional novel stressor, the FST, females showed a higher despair behavior (Dalla *et al*., 2005). Anyway, in FST per se, not only as an additional stressor in a stress-induced model of depression, females showed a higher immobility compared to males

(Dalla *et al*., 2011). Furthermore, only females showed increased corticosteroid levels and serotonergic activity (Dalla *et al*., 2005).

In the model of lipopolysaccharide–induced sickness many differences between males and females were found: a higher rewarded behavior with the sucrose preference in females, a reduced food intake only in males, a decreased locomotor activity mainly in males, an HPA axis activation only in females, dimorphic hypothalamic serotonergic response, an hippocampal activation of serotonergic activity only in females, and an increased serotonergic activity in prefrontal cortex and striatum only in females (Pitychoutis *et al*., 2009).

Collectively, sex differences are influenced in both the behavioral and neurobiological aspects. In human society it must be taken in account also that psychosocial factors may mediate or influence the individual predisposition to the risk of depression onset and the response to antidepressant treatments.

Despite the fact that none of the presently available animal models is able to shape all aspects of depression, there are useful paradigms that help in the investigation and improvement of antidepressant drugs and in the validation of neurobiological theories about depression.

We chose to investigate correlates of depression in the neonatal clomipramine model because: 1) it can be produced without specialized equipment; 2) it does not require traumatizing animals; 3) the effect of treatment lasts for months, allowing to conduct a large variety of tests; 4) it is a valid model to study altered brain functions and physiology; 5) there is a large amount of literature demonstrating its validity as model of depression, as described in the following section.

1.4 The neonatal clomipramine model in the rat

Clomipramine is the 3-chlorinated derivative of imipramine (Fig. 1.4) and both drugs belong to the family of the tricyclic antidepressants.

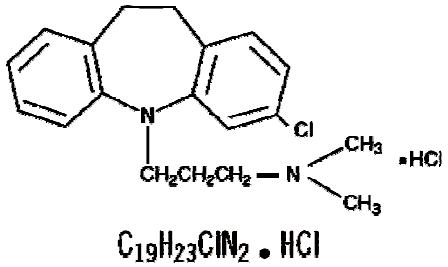

Fig. 1.4 - Chemical structure of clomipramine hydrochloride.

Clomipramine (Anafranil®) principally acts as a reuptake inhibitor of serotonin, dopamine, and noradrenaline in the synaptic space and it is also a powerful REM-sleep suppressant. Because of its mixed action that implies numerous side effects, it does not represent the preferred agent prescribed for an antidepressant therapy. Anyway, it is frequently used for the treatment of obsessive compulsive disorder (OCD), panic disorder, chronic pain and narcolepsy too.

A large amount of data from literature support the hypothesis, made for the first time by Vogel and co-workers, of a paradoxical depressant action of clomipramine in rodents neonatally treated (Vogel *et al*., 1990a). In spite of the thorough investigation of this animal model, it is still not clear if the endogenous depression, affecting adult animals that were exposed to clomipramine in early life (CLI animals), is caused by the suppression of REM sleep or by the interference on aminergic neurotransmission of an immature central nervous system (CNS).

Vogel's work was inspired by Mirmiran and colleagues, who evaluated the impact of neonatal REM sleep on adult behavioral development. REM sleep deprivation (RSD), accomplished by clomipramine administration during the neonatal period, caused in male rats, as adults, sexual and locomotor impairments, and, in advanced age, a permanent increase of REM sleep and a higher number of REM sleep onset periods (Mirmiran *et al.*, 1981; Vogel *et al*., 1990a). Mirmiran and co-workers interpreted the results obtained as a relevant indication about the importance in the early life of a normal REM sleep pattern for the development of normal adult behavior and sleep. Vogel, on the other side, interpreted Mirmiran's findings as evidence of a complex syndrome that resembles human endogenous depression. It is known that depressed patients show an alteration of sexual and locomotor activity, and an impaired REM sleep pattern, thus suggesting that if adult rats, neonatally exposed to clomipramine, show the same symptoms, they could be considered valid models of human endogenous depression (Vogel *et al*., 1990a).

Each animal model of depression needs to be tested for its validity, and in particular, according to Vogel's proposal to validate the neonatal clomipramine model, animals should show: 1) several behavioral abnormalities typical of human endogenous depression such as decreased aggressiveness, sexual activity, and pleasure-seeking activity, as well as motor agitation or retardation; 2) an alteration of the normal REM sleep pattern, since depressed patients usually exhibit decreased REM latency, increased REM sleep onset periods, increased REM phasic events, and, after a period of RSD, an abnormal REM sleep rebound; 3) an amelioration of the behavioral abnormalities induced by treatment with antidepressant agents like in human endogenous depression (Vogel *et al*., 1990a).

Vogel and collaborators carried out a study to validate the animal model. Behavioral abnormalities observed in CLI rats, compared to animals treated with a saline solution (Sal animals) as a control group, were presented as follows:

1) in a test for aggressive behavior, pairs of rats, each pair consisting of one CLI animal and one SAL animal, fought in response to randomly delivered intermittent foot shock: CLI rats consistently lost in shock-induced aggression, showing significantly fewer offensive fighting responses, and significantly more defensive fighting responses (Vogel *et al*., 1988);

2) in a set of nonsexual pleasure-seeking assessments, CLI rats showed, when 7 months old, diminished response to hypothalamic intracranial self-stimulation at both single and multiple stimulus intensities, a lower interest in the exploration of a novel object in an open field, but no differences compared to Sal rats in the sucrose and saccharine consumption, even if the tests were monthly repeated from age 3 months to age 10-11 months (Vogel *et al*., 1990a);

3) in sexual testing of males, three strains of 3-month old rats were compared: even if Sprague-Dawley and Wistar rats exhibited a basal lower sexual activity than Long-Evans rats, CLI rats from each strain showed a lower sexual activity in terms of number of mounts, intromissions, and ejaculations and of mount latency and post-

ejaculatory pauses, but only in Long-Evans rats the decrease was significant (Vogel *et al*., 1990a);

4) in two different locomotor activity tests, CLI animals showed contrasting behavior: hyperactivity appeared to be age- and procedure-dependent (Hartley et al., 1990). Both tests were repeated monthly from age 2 months to age 8 months. In the first test, animals were put for two minutes on each of five consecutive days in a circular open field whose floor was marked in pie slice-shaped sectors. An observer recorded the movements of animals by signing the sectors through which animals passed. The test was considered stressful because the short assessment did not allow habituation to the environment. CLI animals were significantly more active than Sal at ages 4 and 6 months, and, as first noted by Mirmiran (Mirmiran *et al*., 1983), the activity was confined in the periphery of the open field. In the second test, a computerized apparatus (Digiscan, Omnitech Electronics®) measured spontaneous motor activity (total activity and ambulation) in a plexiglas chamber for 16 minutes on each of five consecutive days. This assessment was always performed one week following the open field test. Only 3-month old CLI rats were significantly more active than Sal rats; any other significant differences were not found (Hartley *et al*., 1990).

5) In a EEG study, the sleep pattern of 6- and 11-month old rats was observed: 6-month old CLI rats, but not 11-month old rats, showed in basal conditions a higher REM sleep percent, a significantly shorter REM latency, and significantly more REM sleep onset periods compared to Sal rats; the abnormalities were mainly in the light period. On days 2 and 3 after a 72-h RSD, carried out with the small platform over water method, both 6- and, even if in a much lesser degree, 11-month old rats showed a higher REM sleep rebound than SAL rats. REM sleep stimulation by RSD was more persistent in CLI than in Sal rats (Vogel *et al*., 1990b).

6) In several contests, antidepressant treatments improved the symptoms above described of CLI rats. RSD, proven by many scientists to ameliorate symptoms of human endogenous depression (Adrien, 2002), and imipramine were used as antidepressant treatments, even though they clearly act in a different manner. In a test for aggressive behavior, the same mentioned in i), CLI rats, treated with both or with either imipramine or RSD by small platform over water method, showed an increased shock-induced fighting; imipramine significantly reduced hyperactivity of CLI rats in the open field test; and both imipramine and RSD improved the sexual performance of CLI rats compared to Sal rats (Vogel *et al*., 1990a).

Vogel and collaborators interpreted their results as clear signs that clomipramine is able to induce in rodents an endogenous depression when administered in the early life. Diminished aggressiveness, pleasure-seeking behavior, and sexual activity, as well as motor agitation, and REM sleep disturbances are all symptoms of human endogenous depression. Vogel brought to light all these aspects in CLI rats and made several interesting considerations. First, hyperactivity shown in the open field test can be hypothesized as an overreaction of rats to a stressful situation and the permanence of rats in the periphery was oriented toward escaping the apparatus, not exploring it. Hyperactivity and hyper-responsivity to stress are, again, typical features of depressed patients (Hartley *et al*., 1990). Second, the diminished entity of REM sleep disturbances in basal conditions of CLI aged rats suggest that clomipramine plays a self-limited impairment with a spontaneous remission after several months (Vogel *et al*., 1990b). Third, data suggest that endogenous depression of CLI rats has a gradual onset, maybe due in part by a delayed, life-event independent, long-term increase of REM sleep, and animals show a gradual recovery with remission of initial symptoms. These findings confirm that CLI rats are af-

fected by an endogenous depression more than a reactive depression, is the second one, on the other hand, being typical of stress-induced animal models (Vogel *et al*., 1990b).

Furthermore, Vogel made some hypotheses on the activity of chronically administered antidepressant agents: is brain able to compensate for acute effects of drugs? Maybe, unlike the adult brain, the immaturity of a neonate brain does not allow a compensation for the effects of treatment with antidepressant drugs (Vogel *et al*., 1990a). Early life treatment can produce permanent or temporary effects on the functions of a brain that is still in a very critically formative stage: brain growth in rodents begins antenatally, reaches a peak in the development at post-natal day (PND) 5-10 and continues until the 3rd-4th post-natal week; synaptogenesis begins before birth, undergoes its most extensive development between PND5 and PND20 and goes on until PND40; development of neurotransmitter systems occurs up to post-natal 5-6 weeks (Maciag *et al*., 2006). So it can be hypothesiszed that neonatal treatment with clomipramine "shakes" a developing nervous system that is not able to compensate the insult. As a consequence of this, antidepressant drugs could cause a permanent upregulation of post-synaptic receptors, or an impairment of the normal sleep pattern, or a decrease in aminergic neurotransmission: any of these effects is a sufficient condition to produce depression. These considerations explain the paradox that administration of antidepressant drugs in adults improve depression, while in neonates may produce depression (Vogel *et al*., 1990a).

Velazquez-Moctezuma completely disagree with Vogel's opinion that early administration of clomipramine can cause behavioral abnormalities due to RSD or to the augmented availability of amines at the synaptic level: neither rats neonatally treated with scopolamine, a cholinergic antagonist with a potent suppressive mechanism of REM sleep, nor rats treated with idazoxan, a α2-receptor antagonist that can increase adrenergic activity, displayed an increase in sexual activity or in time spent in immobility in the Porsolt's FST (Velazquez-Moctezuma *et al*., 1993).

Many scientists interested in the paradigm of neonatal clomipramine administration tried to find the explanation for behavioral abnormalities observed in CLI rats by measuring the levels of aminergic neurotransmitters. Decreased serotonin levels were found in some brain areas of CLI rats, such as frontal cortex, hypothalamus, hippocampus, septum, striatum, and brain stem; further, noradrenaline was found significantly decreased in frontal cortex, hippocampus, septum, striatum, brain stem and hypothalamus and dopamine was significantly decreased in the hippocampus, frontal cortex, hypothalamus, striatum and brainstem (Vijayakumar and Meti, 1999; Bhagya *et al*., 2011). But a fortnight period of chronic escitalopram treatment between the 2nd and 3rd postnatal month as antidepressant therapy was able to restore the levels of serotonin, noradrenaline and dopamine levels in the hippocampus, frontal cortex, hypothalamus, striatum, and brainstem (Bhagya *et al*., 2011).

Andersen and coworkers found in CLI rats different aminergic laterality in some brain areas: early clomipramine treatment appears to influence the limbic regions of nucleus accumbens, amygdala, prefrontal cortex and striatum, but not hippocampus, in a different degree by shifting laterality of serotonin and dopamine. These effects may play a role in the emergence of depression. Furthermore, the perturbation of dopaminergic and serotonergic systems with clomipramine treatment may lead to inhibition of a normal development of brain, since it is known that neurotransmitters act as brain trophic factors (Andersen *et al*., 2002). Not only serotonin levels were influenced by neonatal clomipramine treatment, but also serotonin circuitry and neuronal excitability. In CLI rats a diminished immunoreactivity, persistent into adulthood, to serotonin transporter (SERT) was observed in fiber networks of cortical areas (Maciag *et al*., 2006). Further-

more, in CLI rats treated in adulthood with either zimelidine, an SSRI antidepressant, or with repeated RSD, serotonergic neurons of nucleus raphe dorsalis showed a lack of response to the inhibitory effect of citalopram. Actually, serotonergic neurons of CLI rats appeared to be already hyporeactive to citalopram under basal conditions, i.e. without zimelidine treatment or RSD; in Sal rats, on the other hand, the firing was reduced as a consequence of citalopram administration (Maudhuit *et al*., 1996). This result raises questions about the relationship between serotonergic system's functionality and abnormalities in behavior.

Clomipramine treatment in the neonatal age was found to affect the levels of other important substances. Orexin, for example, is a hypothalamic peptide somehow linked to serotonin deficiency in depressives and involved in the wake-promoting process. Its levels are significantly decreased in young CLI rats (35 days old), but the pattern is inverted in the adults (3 or 4 months old). A possible hypothesis to explain this phenomenon is that orexin changes are associated with sleep disturbance typical of depression: increased levels found in adult CLI rats are compatible with many features of sleep associated with depression, since orexin promotes wakefulness and suppresses sleep (Feng *et al*., 2008).

Also BDNF levels were significantly decreased in the hippocampus of CLI animals (Cassano *et al*., 2006). BDNF is the most diffused trophic factor in the brain, where it participates to very important processes such as neuronal growth, long-term potentiation and learning. As described in par. 1.5 altered BDNF levels have been associated in humans with the major depressive syndrome.

An altered sensitivity of central cholinergic system is another one of the possible neurochemical correlates of behavioral abnormalities in CLI rats: early-life clomipramine administration enhances the hypothermic response to oxotremorine, an agonist of the muscarinic acetylcholine receptor, effect that can be reversed by RSD performed with the small platform over water method (Prathiba *et al*., 2000); furthermore, the soluble form of acetylcholinesterase enzyme was found significantly higher in CLI compared to Sal rats in the hippocampus, but lower in the frontal cortex . Since the cholinergic stimulation of brain has been reported to increase REM sleep, the augmentation of REM sleep in CLI rats may also be due to increased cholinergic activity (Mavanji and Datta, 2002).

Taken together, all these results about the altered levels of cholinergic-monoaminergic neurotransmitters, of BDNF, and orexin in different brain regions, suggest that depression-like symptoms of this animal model may be due to a general imbalance of numerous neurochemical correlates.

A large amount of literature reports about an intricate inter-relationship CRF, HPA axis, glucocorticoids, monoamines and mood disorders. It has been hypothesized that in depressed patients an altered regulation of the ACTH and, subsequently, an enhancement of cortisol levels, may be due to an impaired rhythmicity of the HPA axis (Bonilla-Jaime *et al*., 2010). CLI rats exhibited significantly higher corticosterone levels than control group at time-points 0h and 6h after the onset of light phase, but no differences were detected in the dark phase, thus resulting in a substantial change of the circadian activity of corticosterone. On the other hand, the levels of corticosterone were restored by a 4-day RSD, performed with the small platform over water method, to values comparable to those found in Sal rats (Prathiba *et al*., 1998) and reduced also by fluoxetine treatment (Bonilla-Jaime *et al*., 2010).

Body weight gain was found to be reduced in CLI rats at different ages: during the treatment period (Andersen *et al*., 2002; de Souza *et al*., 2004), at the end of it (Andersen

et al., 2002), and, in the adulthood, at age 3 months (Vijayakumar and Meti, 1999; Cassano *et al*., 2006). In parallel, also food intake at age 3 months was decreased (Vijayakumar and Meti, 1999). In depressive patients diminished body weight and food intake are common symptoms, thus these data are a further confirmation of the validity of the animal model of neonatal clomipramine administration.

Evaluations of behavioral abnormalities were conducted by many authors, sometimes with results mismatching from those described by Vogel and collaborators. Most of the experimentation performed by Vogel used the Sprague-Dawley rat strain, but some comparisons were carried out by Vogel himself and by other scientists also in Wistar and Long-Evans rats, but without any relevant result that could lead to think about a strain-correlated different responsivity to neonatal clomipramine administration.

In the FST, many authors confirmed the observation of an increase in immobility of CLI rats (Velazquez-Moctezuma *et al.*, 1993; Bhagya et al., 2008; Feng *et al*., 2008), but Yoo and coworkers found no differences among CLI and Sal rats, and imipramine treatment or physical training of CLI rats did not produce any change too (Yoo *et al*., 2000). CLI animals showed no decrease in immobility, but a significant increase in climbing was reported by Cassano (Cassano *et al*., 2006).

Sexual activity was confirmed to be decreased by many authors (Velazquez-Moctezuma *et al*., 1993; Feng *et al*., 2001; Maciag *et al*., 2006). Locomotor activity was reported to be increased in male CLI rats compared to Sal rats (Andersen *et al.*, 2002; Maciag *et al*., 2006) and also compared to female CLI rats (Andersen *et al*., 2002), confirming hyperactivity as a typical trait of CLI rats.

The results obtained in the sucrose preference test (SPT) are useful to recognize the anhedonic behavior, typical of human endogenous depression. In this animal model the SPT performed by other groups had different results from Vogel's ones: while Vogel found no differences between CLI and Sal animals in sucrose consumption, other groups observed that CLI rats consumed lower levels of sucrose than Sal rats (Cassano *et al*., 2006; Bhagya *et al*., 2008; Bhagya *et al*., 2011), but chronic escitalopram treatment of CLI rats restored sucrose preference (Bhagya *et al*., 2011)..

Further behavioral tests were conducted to investigate the following issues:

1. anxiety trait: in the elevated plus maze (EPM) test contradictory results were obtained. Andersen observed that CLI rats, at PDN70, spent a significantly lesser time in the open arms compared to Sal rats (Andersen *et al*., 2002), while Cassano found the opposite result at PND90 (Cassano *et al*., 2006). In a more recent work, Andersen confirmed the high anxiety trait of CLI rats in the EPM test, with the observation that CLI rats spent more time than Sal rats in the closed arms of the instrument, and, in another test, the marble burying test, CLI rats buried more novel objects than Sal rats (Andersen *et al*., 2010);

2. cognitive impairment: in the Morris water maze (Cassano *et al*., 2006) and in the radial arm maze test (Bhagya *et al*., 2008; Bhagya *et al*., 2011), CLI rats showed a spatial memory impairment, and chronic escitalopram treatment significantly restored it to scores typical of Sal rats (Bhagya *et al*., 2008; Bhagya *et al*., 2011).

3. somatic growth, maturation and development of sensory-motor reflex: the tests, performed in neonatally citalopram treated rats, showed that development of these parameters was delayed (Deiro *et al*., 2004). De Souza and coworkers found that CLI rats showed an alteration of some physical developmental parameters such as reduced body growth, reduced growth of the antero-posterior and latero-

lateral axis of the body, reduced tail and head sizes were recorded at specific PNDs during the treatment period. Clomipramine treatment did not alter the maturation of any of the already assessed physical characteristics such as ear unfolding, auditory conduit opening, eruption of the lower incisors, and eyes opening. Furthermore, clomipramine treatment did not significantly alter the temporal pattern of onset of the tested reflexes (righting, vibrissa placing, cliff avoidance, negative geotaxis, auditory startle, and free-fall righting) (de Souza *et al*., 2004).

Recently, Andersen and coworkers proposed that the neonatal clomipramine administration is able to produce a model of OCD, a condition mediated by specific cortico-striatal- thalamic-cortical circuits and characterized by anxiety, perseveration and , behavioral inflexibility, and working memory impairments (Stein *et al*., 2000; Andersen *et al*., 2010). According to Andersen, CLI rats present behavioral characteristics consistent with an OCD-like profile in humans: the anxiety-like behavior was assessed with the EPM test and the murble burying task (already mentioned), while the perseverative-like behavior was observed with the simple spontaneous alternation task; an impairment in reversing direction and in a working memory task suggested a certain degree of learning impairment (Andersen *et al*., 2010).

Interestingly, Andersen and collaborators argued that the neonatal clomipramine paradigm reproduced in their labs was an animal model of depression in a previous work (Andersen *et al*., 2002), reporting much evidence of it. We noticed that the period and duration of treatment differ, even if slightly, from the one reported in the description of the OCD model. It is known that the post-natal period is a very important phase and that each of the first post-natal weeks is characterized by the formation of specific structures and development of specific functions in the brain. Thus, we wonder if the slight difference in the treatment period could be crucial for the determination of either a syndrome or the other. It is hard to believe it, since many authors, who defined CLI rats affected by an endogenous depression, treated animals in different periods (but always in the first post-natal 4 weeks) with different durations, administration routes (subcutaneous or intra-peritoneal) and doses of clomipramine (from 15 to 30 mg/kg), and the results were comparable from an author to another.

2 Study 1 – Neurogenesis in clomipramine-treated rats

2.1 Introduction

As already described in section 1.2.1, hippocampal neurogenesis has been shown to be affected in animal models of depression, but data from neurogenesis studies have never been reported by any author who investigated this animal model of depression.

Neurogenesis rate was studied with the employment of a double immunohistochemistry and the markers chosen were 5'-bromo-2'-deoxyuridine (BrdU) and doublecortin. BrdU is one of the most used markers of newly generated cells and it is an analogue of thymidine which is incorporated in the DNA of proliferating cells during the S-phase of the cell cycle. BrdU staining conveys the total cell proliferation rate, but the identification of newly born neurons in the dentate gyrus of the hippocampus requires a specific additional marker, that does not label non-neuronal cells and is able to label all types of neurons. Double immunostaining with BrdU and DCX was necessary to demonstrate that DCX-labeled (DCX+) cells were clearly the newly generated neurons since DCX is a cytoplasmic microtubule-associated protein involved in two important phases of neuronal development, migration and differentiation (Rao and Shetty, 2004). A very detailed description of a range of morphologies of DCX+ cells was provided by Plumpe (Plumpe *et al*., 2006), who divided cells expressing DCX in 6 categories, reflecting a continuum, according to the presence and the shape of apical dendrites. DCX is transiently expressed during a period lasting approximately 3-4 weeks, and it is not present in mature neurons. The period of expression extends from a proliferating progenitor cell stage, to a postmitotic phase in which cells present long dendrites (Plumpe *et al*., 2006).

Following a specific protocol of BrdU administration and performing a double immunostaining with BrdU and DCX, it is possible to study the influence of a long-term or short-term stimuli on the birth and maturation of new neurons.

As mentioned in section 1.2.1, several experimental conditions have been observed to affect proliferation and survival of newly generated neurons in the rodent hippocampus, either positively, such as enriched environment, physical exercise, and learning, or negatively, like stress and glucocorticoids. These observations suggest a role of neurogenesis in the adaptation to new environmental and physiological conditions and in the regulation of cognitive functions and behavior, also in mood disorders such as depression (Meerlo *et al*., 2009). SD has been long studied as a factor influencing neurogenesis: it has been shown that short (<1 day) SD does not strongly affect neurogenesis, on the contrary it can even lead to an increase of it (Grassi Zucconi *et al.*, 2006), but it significantly suppresses cell proliferation if it is prolonged (Meerlo *et al*., 2009). The repeatedly reported clinical data about the antidepressant effect, even if transient, of one-night SD on patients (Adrien, 2002) induced us to investigate on a possible role of SD on mood and hypothesize a correlation with changes in neurogenesis rate. Enforcing wakefulness in laboratory animals can be carried out with several methods, such as gentle handling, forced locomotion in a slowly rotating drum, forced locomotion on a treadmill, or the small platform over water method (Meerlo *et al*., 2009). Each method has its own imperfections unrelated to sleep loss, but they all can be avoided with some devices (Meerlo *et al.*, 2009). The gentle handling method was chosen among the several methods in order to induce a total SD with a procedure aimed at possibly excluding confound-

ing variables known to affect neurogenesis, such as stress, physical exercise and enriched environment (Grassi Zucconi *et al*., 2006).

Behavioral assessment with the FST was part of a set of observations already described in literature: this test was necessary to assess that the procedure of drug administration reproduced a behavioral model of the neonatal clomipramine treatment comparable with data reported in literature. FST was first introduced by Porsolt (Porsolt *et al*., 1977) as a tool to measure the antidepressant efficacy of drugs in reducing the despair behavior of rodents when put in a helplessness situation, such as a cylinder full of water from which animals cannot escape. Animals basically struggle during the first seconds of the test, but then they give up and slow down or stop their movings in water doing nothing but floating. It has been repeatedly observed that antidepressant agents are able to reduce time spent in immobility of rodents in the FST (Cryan *et al*., 2005).

The first experiment performed was designed to investigate:

- despair behavior in a helplessness paradigm;
- hippocampal neurogenesis in basal conditions and effects of a short-term SD on it;
- possible morphological changes in dendritic arborization of hippocampal granule cells due to early-life clomipramine treatment.

2.2 Materials and methods

2.2.1 Animals

Pregnant Sprague-Dawley rats (Harlan), were put on a 12:12h light/dark cycle (lights on at 8:00 am) and maintained at room temperature (20±2°C) with food and water *ad libitum*. At birth, pup rats were left with their own dams and never separated from them a part for 5 minutes twice a day, each day, until PND4, during which they were habituated to be handled by experimenters.

2.2.2 Treatment

From post-natal day (PND) 5 until PND21 animals were intra-peritoneally (i.p.) injected, twice daily, with either a 20mg/kg solution of clomipramine (Sigma-Aldrich) or an equivalent volume of 0.9% sodium chloride solution (Fresenius Kabi). During the period of treatment, animals were weighed daily, in order to check the body growth rate. After weaning, animals were divided by their own dams and housed singularly. After a period of habituation, rats underwent behavioral assessment.

2.2.3 Forced swim test

At 2 months of age, depressed behavior was tested with the forced swim test. The procedure adopted differs from the classical Porsolt test (Porsolt *et al*., 1977) for the use of a large cylindrical pool (Ø 132 cm) instead of the standard, 30 cm-wide, cylinder. The subjects' motor activity was video-recorded and analyzed with the help of the Ethovision® software, whose output was the calculation of the amount of time spent in swimming, struggling, as an attempt to escape from the cylinder, and distance moved

(m). The assessment for each animal was performed over two days and consisted of a 15-minute training on day 1 and a 5-minute test on day 2.

2.2.4 Sleep deprivation

At 4 months of age, animals entered the final stage of the protocol.

Animals were divided in two groups: a group of animals underwent 10 hours of sleep deprivation by gentle handling, starting at the beginning of their circadian resting phase (8:00 am, lights on), while another group remained undisturbed. During the gentle handling procedure, animals were kept awake by tapping on or gently shaking the cage, touching their tails or whiskers, gently pushing them, etc. (Grassi Zucconi *et al*., 2006).

2.2.5 BrdU injections

All animals, both sleep deprived and non-sleep deprived, received two injections of BrdU (100 mg/kg i.p.; Sigma-Aldrich), four and two hours before the end of the sleep deprivation period. At the end of sleep deprivation (6:00 pm) all animals underwent the perfusion protocol.

2.2.6 Perfusion and brain processing

Animals were deeply anesthetized with pentobarbital (80 mg/kg i.p.), transcardially perfused with a cold NaCl 0.9% solution (Fresenius Kabi) followed by ice-cold 4% paraformaldehyde (Sigma-Aldrich) in PBS. Brains were removed, post-fixed overnight in the same paraformaldehyde solution, and cryoprotected in a 30% sucrose solution until they sank. Brains were subsequently frozen at the microtome and cut in 40 µm-thick coronal sections, which were preserved in PBS 0.1M with 0.1% NaN_3 until histological processing.

2.2.7 BrdU-DCX immunohistochemistry

Sections from 24 animals, 12 males and 12 females, each sex group comprehending 6 sleep-deprived and 6 control animals, were first processed for BrdU immunohistochemistry and then stained for the DCX protein. All washes were performed in 0.01M PBS containing 0.1% of Triton X-100.

BrdU immunohistochemistry. After incubation in 0.75% H_2O_2 in 0.1M PBS for 20 min for peroxidase inactivation and DNA denaturation with an incubation in 2N HCl in 0.1M PBS for 30min at 37˚C followed by 10 minutes in borate buffer (pH 8.5), sections were blocked in 3% BSA + 0.3% Triton X-100 in PBS for 90min. Then sections were incubated overnight with a monoclonal mouse anti-BrdU (NCL-BrdU, Novocastra, 103014; 1:500) diluted in PBS containing 1% BSA+ 0.3% Triton X-100 at 4˚C. Incubation in secondary antibody (biotinylated goat anti-mouse IgGs; 1:200) diluted in PBS containing 1%BSA+0.3% Triton X-100 lasted 2h at room temperature. Then the staining was developed with the ABC kit (incubation for 1h at RT) followed by an incubation in 0.1M PBS containing 0.05% NiCl + 0.025% DAB +0.003% H_2O_2 for 5 min.

DCX immunohistochemistry. After incubation in 0.75% H_2O_2 in 0.1M PBS for 20 min for peroxidase inactivation, sections were blocked in 5% BSA + 0.3% Triton X-100 in PBS for 90min. Then sections were incubated overnight with a polyclonal goat anti-

DCX (Santa Cruz; 1:500) diluted in PBS containing 1% BSA+ 0.2% Triton X-100 overnight at room temperature; incubation in secondary antibody (biotinylated horse anti-goat IgGs; 1:200) diluted in PBS containing 1% BSA+ 0.2% Triton X-100 was performed for 2h at room temperature. Then the staining was developed with the ABC kit (incubation for 1h at RT) followed by an incubation in 0.1M PBS containing 0.05% DAB + 0.03% H_2O_2 for 5 min.

2.2.8 Cell counts

Stained sections were examined with a 40x objective under a light microscope. Counts of labeled cells were performed in the subgranular zone of the entire extent of dentate gyrus by visual inspection in 4 evenly spaced sections for each animal, approximately beginning at Bregma -2.45 and ending at Bregma -5.65 of the hippocampus, according to the stereotaxic coordinates reported by Paxinos and Watson (Paxinos and Watson, 2005). The total number of BrdU-labeled (BrdU+), BrdU/DCX-labeled (BrdU/DCX+) and DCX-labeled (DCX+) cells was acquired by the same operator under sex- and treatment-blind conditions. Furthermore, newborn cells, defined by Plumpe as granule cells with very short or no processes (Plumpe *et al.*, 2006), were identified and counted in order to evaluate SD effect on proliferation rate.

2.2.9 Cell morphology

Morphological analyses were conducted on a sample of DCX-labeled cells (72 neurons, 3 per animal) to investigate dendritic arborization rate, cell body size (perimeter and area), number of dendritic nodes, and total dendritic length. Well-differentiated DCX-labeled neurons chosen for the analysis exhibited dendrites with the following features: (i) they were vertically orientated and extended into the molecular layer of the dentate gyrus; (ii) not cut off in the immediate proximity to the soma; and (iii) with minimal overlap with the dendrites of close cells to avoid ambiguous tracing.

The analysis was carried out with a 100x objective, using a semiautomatic neuron tracing system (Neurolucida 8.0, Microbrightfield®), linked to a Nikon motorized microscope. Cells tracings were acquired with Neurolucida's Autoneuron module and the three-dimensional analysis of reconstructed neurons was performed by using the Neuroexplorer software (Microbrightfield®) and choosing the Branched structure analysis to determine all the parameters listed above.

2.3 Results

2.3.1 Body weight

Animals' body weights measured in the morning of each day of the treatment period were chosen for the analysis. The graph in Fig. 2.1 clearly shows how clomipramine treatment influenced the rate of body growth: in CLI animals body weight was significantly reduced compared to SAL animals (Repeated measures analysis, PND as a repeated factor, $F_{(1,53)} = 29.670$, $p < 0.001$).

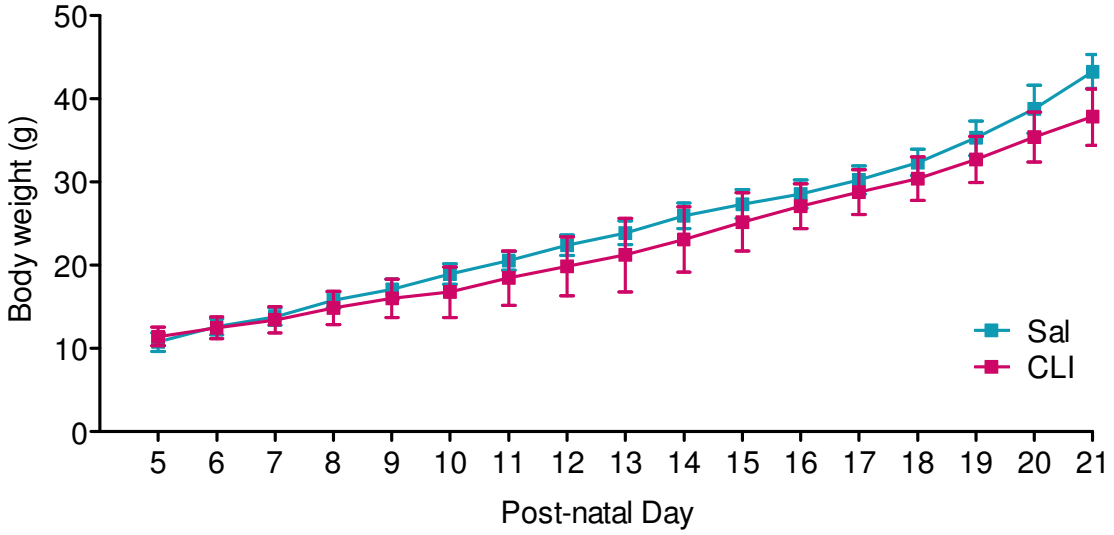

Fig. 2.1. Body growth rate of CLI and SAL rats during the 17-day period of treatment.

2.3.2 Behavioral assessment with the forced swim test

In Fig. 2.2, graph a) represents time spent in immobility during the 5-minute test, while graph b) shows distance moved by animals during the test. None of the parameters analyzed in the performance of the FST showed significant differences due to either treatment or sex.

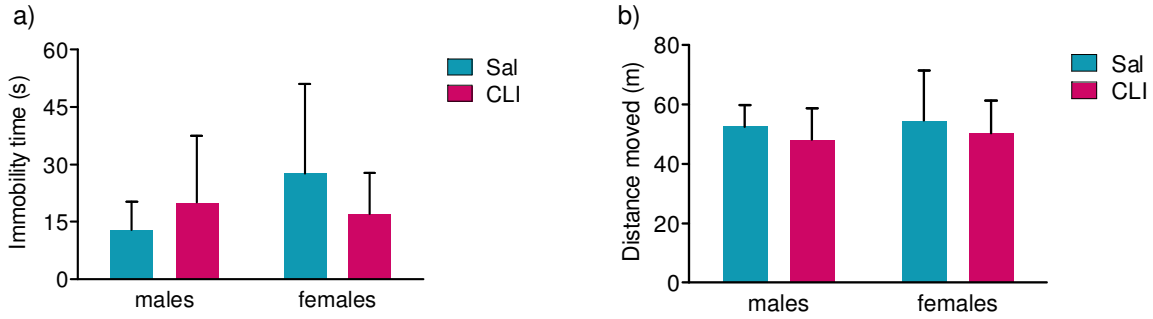

Fig. 2.2. a) time spent in immobility and b) distance moved by animals during the FST

2.3.3 BrdU+ and DCX+ cell counts

Graphs reported in Fig. 2.3 show total BrdU+ (a) and total DCX+ (b) cells counted in the dentate gyrus of 4 evenly spaced sections of hippocampus. Given the sample size and the within group variance, we cannot draw conclusions on between-group differences . An observation, that can be made by looking at the graphs in Fig. 2.3, is that females show a higher number of both BrdU+ and DCX+ cells, in spite of the variability of cell counts.

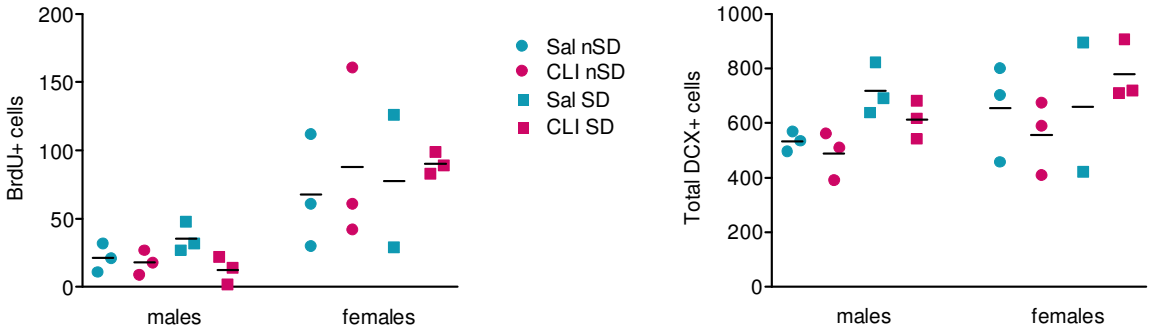

Fig. 2.3 - a) Total BrdU+ cell counts: all cells that appeared immunostained with BrdU, double labeled cells included, were considered in the counting; b) total DCX+ cell counts: the cells that appeared double-labeled represent new neurons. In both analyses, females showed a higher number of cells.

2.3.4 Cell morphology

Representative reconstructions performed with the Neurolucida software are reported in Fig. 2.4, together with microphotographs of the plotted cells.

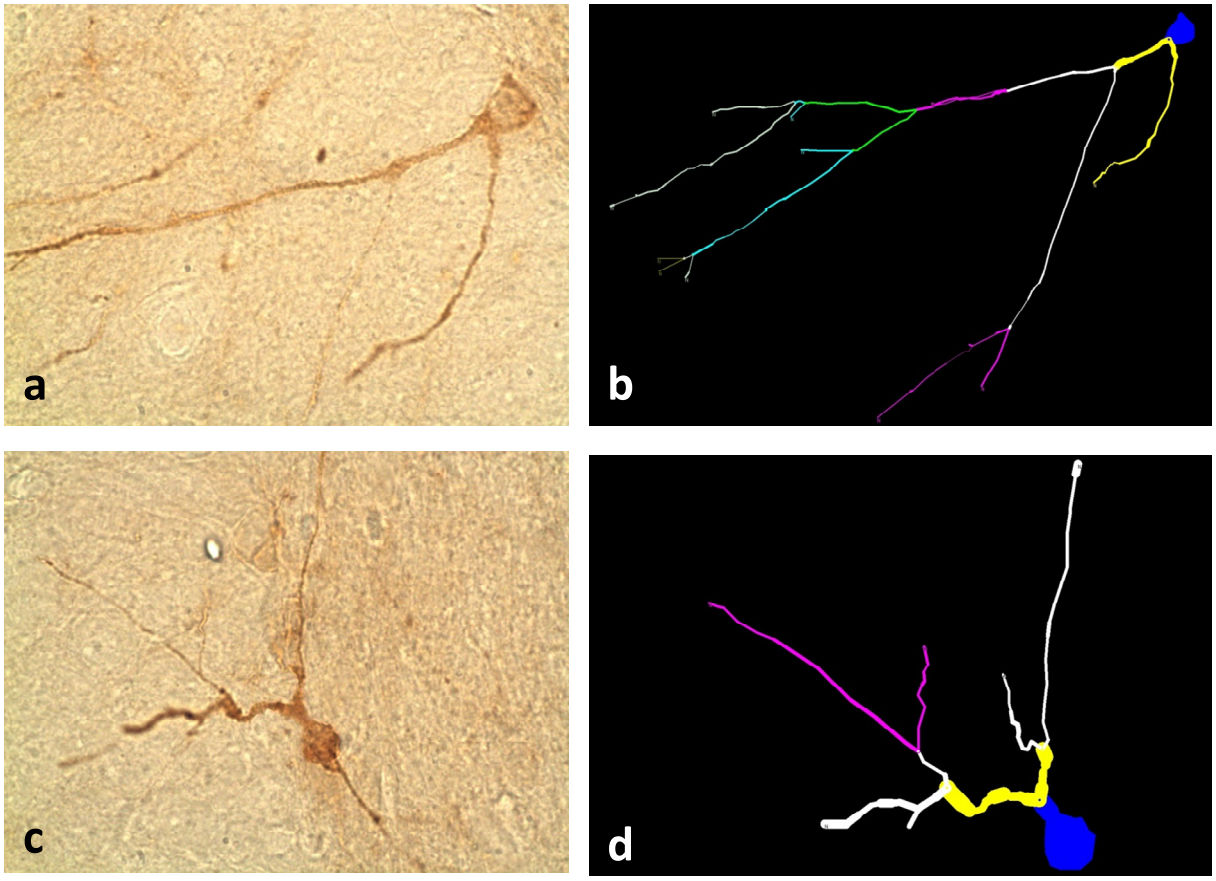

Fig. 2.4 - Representative pictures from neuron reconstruction with Neurolucida: a) and c) are microphotographs of DCX+ neurons from, respectively, a Sal and a CLI animal, and b) and d) are their reconstructions

The graph in Fig. 2.5 shows, for each animal, the total dendrite length of each hippocampal granule cell reconstructed with the Neurolucida software. Cells showed a great variability in the total dendrite length not only within the same experimental group, but also within the same animal. The low number of cells analyzed showed a high variability in the features considered, and, for this reason, a statistical evaluation of sex and treatment effects was not performed. A larger population of reconstructed cells is needed to make a reliable statistical analysis to estimate sex and treatment effects.

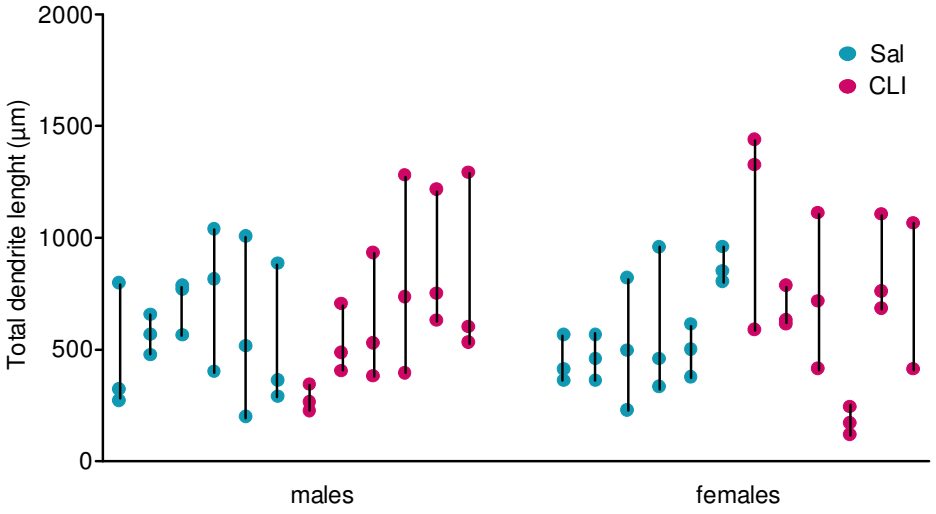

Fig. 2.5 - Total dendrite length of cells analyzed with the Neurolucida software.

We could appreciate qualitative differences in the dendritic arborization between CLI and SAL animals: newly generated neurons in the SAL group (Fig. 2.6A) appear to have longer processes that extend vertically and transverse both hippocampal granule cell layer and the inner region of the molecular layer, while neurons of the CLI group (**Errore. L'origine riferimento non è stata trovata.**B) were more irregular and appeared dystrophic, with convoluted helical-shaped processes.

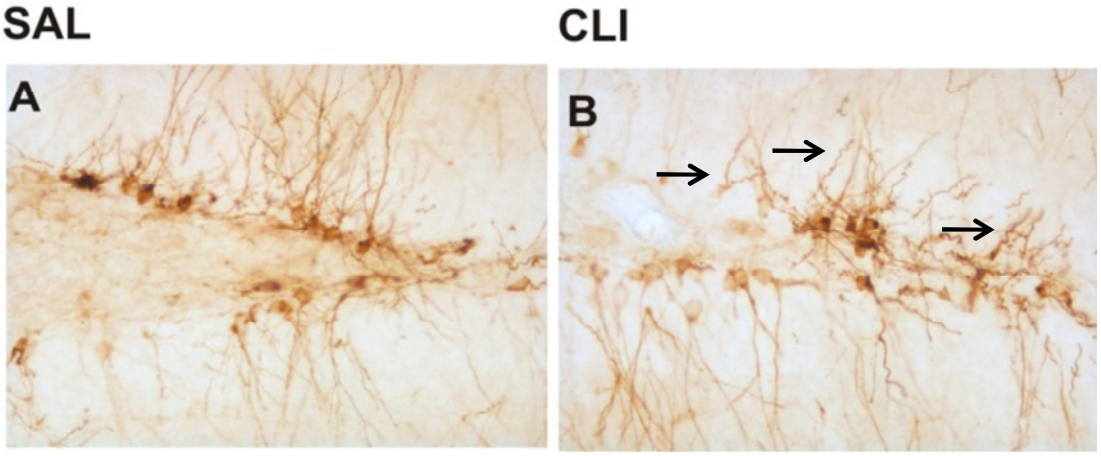

Fig. 2.6 - Microphotographs of the BrdU/DCX immunostaining from representative A) Sal and B) CLI animals respectively: dendrites of the CLI animal appear shorter and less arborized than those of SAL animal.

2.4 Conclusions

These preliminary data from the investigation of the relationships between depression, sleep deprivation, and neurogenesis offered interesting clues to the future directions of the study, but also point out clear limitations that need to be addressed.

Preliminary behavioral results showed the need to collect more data, given the variability found in the studied groups. In this respect, the employment of different tasks, such as sucrose preference test, activity tests, and food intake monitoring, became necessary to better characterize symptoms of depression in our model.

Sleep deprivation effect on hippocampal cell proliferation was not consistently demonstrated, but with regard to neurogenesis and morphological studies, the population of animals considered in each group was very low and the variability was too high to get statistical significance.

3 Study 2 - Behavioral, imaging, and neurochemical characterization of the neonatal clomipramine model in the young and aging rat

3.1 Introduction

Results from Experiment 1 gave useful indications on the need of a deeper behavioral characterization. In Experiment 2 behavioral investigation included the classical Porsolt's FST for the measure of despair behavior (Porsolt *et al*., 1977) and by a further test to investigate anhedonic behavior, the sucrose preference test (SPT). SPT is a reward-base test for depression which measures a rat's appetite for a highly palatable sucrose solution. A decrease of sucrose consumption in a limited access paradigm has been long used to determine hedonic deficits. Sucrose consumption can be measured as absolute intake or percentage of consumed sucrose solution over the total amount of liquid drunk in the 2h-test following a stressful period of food and liquid deprivation. Some authors consider SPT not to be a convincing measure of anhedonia because sucrose intake can be affected by many factors not related to the hedonic state of the animals, such as prolonged effects of stress procedures, food and water deprivation, and reduced metabolic need of sucrose intake due to loss of body weight (Strekalova *et al*., 2004). Although all these negative opinions, nowadays SPT is largely accepted as a tool to investigate the anhedonic behavior in animal models of depression.

In order to investigate if volume changes of several brain regions observed in depressed patients were evident also in animals, a brain imaging analysis was performed. A MRI study showed in olfactory bulbectomized rats, compared to sham controls, significant volumetric changes such as decreased volumes of frontal cortex and amygdala and an enlargement of lateral ventricles, but no changes in the volume of hippocampus (Wrynn *et al*., 2000), that is one of the brain regions found to be reduced in humans (Bremner *et al*., 2000; Sheline, 2000; MacQueen *et al*., 2003; Neumeister *et al.*, 2005). A further study performed on the chronic mild stress rat model of depression with tridimensional MRI technique confirmed the absence of volumetric differences in the measure of hippocampal extent among stressed animals and controls (Delgado y Palacios *et al*., 2011). On the other hand, rats chronically restrained for 6h/day for 21 consecutive days showed a reduction in hippocampal volume compared to a measurement performed before the stress procedure (Lee *et al*., 2009). This result may suggest that the stress procedure adopted to induce depression could be determinant in provoking brain volume changes. None have described about brain regions measurements in our model, so we performed the analysis on several regions involved in the pathophysiology of depression.

The increasing evidence of the role for BDNF in the mechanism and treatment of depression has been reported by many authors in both clinical and preclinical studies, in particular in animal models of stress-induced depression. Determination of brain BDNF levels in the rat hippocampus has been already performed in this animal model of depression through Western blot analysis: CLI animals showed a reduction of BDNF compared to SaL animals (Cassano *et al*., 2006). The confirmation of this result with an investigation of BDNF levels in hippocampus and in cortex was performed with ELISA technique.

Glial cells are the most abundant cells within the central nervous system and are represented by three different types of cells: oligodendrocytes, microglia, and astrocytes. We focused our attention on astrocytes, the most abundant glial cells constituting about one third of the mass of the brain. They have the function to support neurons, metabolize neurotransmitters, maintain ion homeostasis in the extracellular tissue, regulate neuronal migration, secrete growth factors and participate in the immune and inflammatory responses, and in the constitution of the BBB (Fuchs *et al*., 2004); moreover, it has been hypothesized that astrocytes may regulate formation, maturation, and maintenance of synapses through a dynamic communication with neurons (Slezak and Pfrieger, 2003). As already said, significant decrements in the hippocampal volume of depressed patients was repeatedly reported (Stockmeier *et al*., 2004) and histopathological correlates of this evidence included loss of glial cells, reduction in synapses, increase in neuronal density, and reduction in neuronal size (Drevets *et al*., 2008). Furthermore, post-mortem studies on brains of depressed patients demonstrated altered expression of glial fibrillary associated protein in prefrontal cortex (GFAP) (Si *et al*., 2004). In laboratory animals, GFAP mRNA expression was decreased in the prefrontal cortex of chronic unpredictable stress model of depression, and reversed or prevented by chronic riluzole treatment (Banasr *et al*., 2010), and decreases in GFAP immunostaining and number of GFAP-labeled cells in the hippocampus were reported after chronic psychosocial stress (Czeh *et al*., 2006). In Wistar-Kyoto rats, a genetic model of anxiety and depression, Gosselin and coworkers found, compared to control Sprague-Dawley rats, a lower GFAP immunoreactivity in the infralimbic, prelimbic, cingulate cortex, basolateral amygdala and hippocampal CA3 and dentate gyrus regions (Gosselin *et al*., 2009). These results suggest a glial pathology correlated with depression (Banasr *et al*., 2010) but they do not give any information about the alteration of glial function or about a possible correlation with behavioral impairments.

In many animal models of depression, sex differences were reported (Dalla *et al*., 2010), but there is no literature about this issue for the neonatal clomipramine model. Another important factor that we examined was aging: Vogel reported about a remission of some of the effects induced by neonatal clomipramine administration late in life (Vogel *et al*., 1990a). Herein, behavioral and neurochemical effects of age were investigated in three different age groups of rats.

Experiment 2 was aimed at a deeper characterization of the neonatal clomipramine model of depression in a larger cohort of animals with the purposes of:

- investigating abnormalities associated with depression:
    - weight changes
    - anhedonia
    - behavioral despair
    - brain volume changes
    - BDNF levels
- studying neural plasticity:
    - hippocampal neurogenesis
    - astrocytic density
    - neuron morphology
- investigating any effects of sex and age.

3.2 Materials and methods

3.2.1 Animals

All animals were treated with the same procedures adopted in Experiment 1, described in section 2.2.1.

3.2.2 Treatment

The period, the route of administration and the doses were the same as those described in section 2.2.2.

3.2.3 Experimental procedures and groups of animals

The diagram below (Fig. 3.1) shows the experimental procedures to which 3 groups of animals were subjected. The three groups of animals differed for the age to which they were tested in behavioral paradigms and successively sacrificed to allow histological studies on brain tissue.

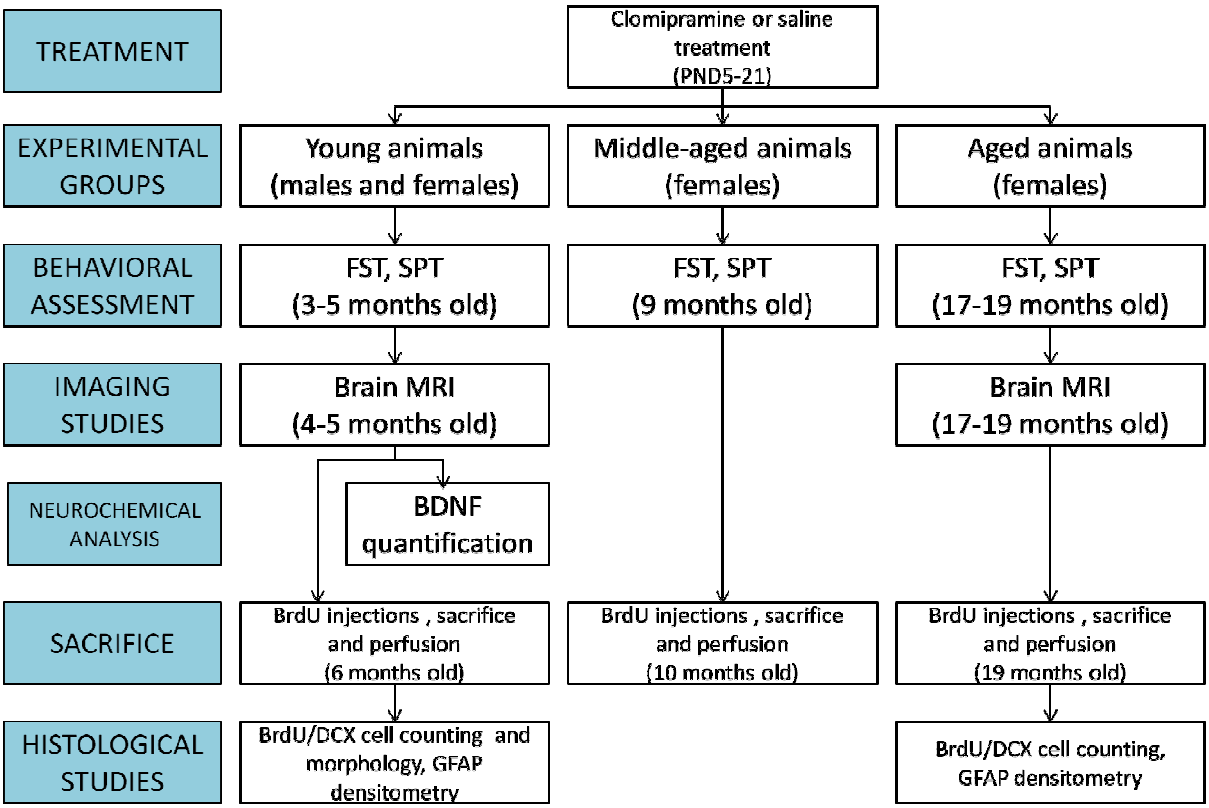

Fig. 3.1 - Time-line of the experimental procedures of the three age groups of animals.

3.2.4 Behavioral assessment

3.2.4.1 Forced swim test

According to the largely validated protocol established for the first time by Porsolt (Porsolt *et al*., 1977), the animals were placed for 5 minutes in an acrylic glass cylinder (h 50cm x Ø 30cm) filled up to 30 cm with lukewarm water, with no possibility to escape. A 15-minute training session was performed 24 hours before the test session. Both training and test sessions were performed during the light phase and recorded by a video camera; video footage was analyzed by a rater unaware of the treatment condition in order to determine time spent in immobility, swimming, climbing, and diving. A rat was considered immobile if the only movements that it made were aimed at keeping the head above the water with the barycenter of animal body essentially in the same position; climbing was judged as an active attempt of stepping over the walls of the cylinder, with forceful thrashing movements and rat forelimbs against the walls; active swimming was judged when animals moved all 4 paws causing a continuous movement of the body barycentre; diving was performed by rats swimming below the water towards the bottom of the cylinder.

All animals from the three age groups were tested in order to make an age comparison of the performances.

3.2.4.2 Sucrose preference test (SPT

The sucrose preference test (SPT) is largely adopted to measure anhedonia, i.e. the incapacity to experience pleasure in situations that prelude to pleasure, like sleeping, eating, social relations, and sexual activity. Anhedonia is a key symptom of human depression.

In this paradigm, rats' responsiveness to reward is tested by offering them a solution of 1% sucrose and tap water. The protocol we used was the same as described by Bhagya and coworkers (Bhagya *et al*., 2008), and it consisted of three steps:

- a 48-hour training session during which rats had free access to food and to both plain water and a sucrose solution; every 24 h, the position of the 2 bottles was swapped to prevent position preference and bottles were weighed in order to quantify liquid consumption;
- an 18-hour period, at the end of the training session, during which rats were deprived of food and liquid;
- a 2-hour test session, after the period of deprivation, during which rats were allowed free access to both water and sucrose solution, but not food. Again, liquid consumption was determined.

All animals belonging to three age groups were tested and total liquid intake and percentage of sucrose consumed over total liquid intake were analyzed in both habituation and test phases.

3.2.5 Brain MRI volumetry

Volumetric analysis was performed using non-invasive magnetic resonance imaging (MRI), a technique that allows measurements in vivo. Table 3.1 shows the list of the 38 animals selected for the analysis.

Group	Sex	Treatment group	Number of animals
Young	Female	CLI	5
		Sal	5
	Male	CLI	8
		Sal	8
Aged	Female	CLI	7
		Sal	5

Table 3.1 - List of the animals used for the MRI analysis of volumetry of total brain and several brain regions. Middle-aged animals were excluded from the analysis.

Animals were anesthetized by mechanical ventilation of a mixture of oxygen containing 2% of isofluorane and placed in a custom-made head holder with an integrated surface head coil. Body temperature was monitored throughout the scan. Scans were performed on a 4.7T Bruker Biospec tomograph (Bruker, Germany) equipped with a 33cm bore magnet (Oxford ltd, UK). A tripilot image was used for reproducible anatomical orientation of 18 coronal rapid-acquisition relaxation enhancement (RARE) images (TR=4000 ms, TE = 19.4 ms, TEeff 43.8 ms, RARE factor four, six averages, spatial resolution 0.068 x 0.068 x 0.75 mm^3, 0.75mm slice thickness with 0.1 mm gap, field-of-view 3.5 x 3.5 cm2, matrix 512 x 384) which were positioned perpendicular to a line connecting the superior end of the olfactory bulb with the superior end of the cerebellum according to Wolf (Wolf *et al.*, 2002). Using an in-house developed, Matlab based, image analysis program, the regions of interest (Wiegers *et al.*) were manually outlined on the images by a rater who is familiar with basic rat neuroanatomy and using a standard rat brain atlas (Paxinos and Watson, 1998) for reference. The extent of the hippocampus was traced on five consecutive slices in the individual animals. The most anterior hippocampal slice included corresponded to a level approximately -2.16 mm posterior to bregma, the most posterior hippocampal slice included corresponded to -6.00 mm posterior to bregma. The delineation of the mPFC started at the first appearance of the forceps minor of the corpus callosum (approximately 4.20 mm from Bregma) and ended at approximately 0.96 mm from Bregma, including prelimbic cortex, infralimbic cortex areas and anterior cingulated cortex areas 1 and 2. In more details, the medial prefrontal cortex was measured in 5 consecutive slices. Lateral and third ventricles were delineated in 11 consecutive slices. The brain volume was outlined on 14 consecutive slices, starting at 4.68 mm from the Bregma rostrally and ending at -6.36 mm from the Bregma caudally. Volumes were calculated by multiplying the number of pixels by the pixel size (0,0036992 mm3/pixel) accounting for the interslice gap and were given in mm3.

3.2.6 BDNF analysis

A group of 10 males, 5 CLI and 5 Sal rats, were selected from the young group of animals. They were deeply anesthetized with pentobarbital, their brains were removed and hippocampus ant total cortex were rapidly taken out and at once stored at -80°C until the time of analysis.

Tissues were homogenized by using a dounce homogeniser in 10 volumes (weight/volume) of extraction buffer (0.01 M Tris–HCl buffer, pH 7.4, containing 1 mM EDTA, and a protease inhibitor cocktail (Sigma Aldrich), left at 4°C for 10 minutes and centrifuged at 4 °C for 10 min at 14,000 rpm. Supernatants were recovered, aliquoted and stored at -80°C. Protein contents of lysate were determined with Bio-Rad Protein

Assay (Bio-Rad Laboratories, Milan, Italy) using bovine serum albumin as a standard (Sigma Aldrich).BDNF levels were measured by anti-human BDNF sandwich-ELISA according to the manufacturer's specifications (R&D Systems), being the mature BDNF protein 100% conserved among humans and rats (Maisonpierre *et al*., 1991). Briefly, half-area microtiter plates (Costar) were coated with capture anti-BDNF antibody by an overnight incubation at RT. After blocking of non-specific binding with Reagent Diluent (PBS containing 1% BSA) for 1h at RT, plates were incubated with the samples diluted in Reagent Diluent (dilution 1:8 of samples from hippocampus, 1:2 from cortex, according to Elfving (Elfving *et al*., 2010)) or with standard curve ranging from 47 to 1500 pg/ml for 2 h at RT with shaking. Then, the antigen was incubated with the biotinylated anti-BDNF detection antibody for 2 h at RT with shaking and with steptavidin-HRP for 20 min at RT. The addition of 3,3',5,5'-tetramethylbenzidine started the colour reaction. The reaction was stopped 20 min later with 1M HCL solution, and the absorbance was immediately measured at 450nm (EL 800 Universal Microplate reader, Bio-Tek instruments, Inc). BDNF concentrations were determined from the regression line for the BDNF standard and expressed as ng BDNF/mg total proteins. All assays were performed in duplicate which were averaged for statistical comparisons.

3.2.7 BrdU injections

All animals, received two i.p. injections of BrdU (100 mg/kg, Sigma-Aldrich), four and two hours before sacrifice.

3.2.8 Perfusion and brain processing

All animals were subjected to the same procedures described in section 2.2.6.

3.2.9 BrdU-DCX immunohistochemistry and cell counting

Brain sections from the young and aged group were processed for the double immunohistochemistry with BrdU and DCX markers as described in section 2.2.7.

3.2.10 Cell counting

Cell counts were performed as described in section 2.2.8.

3.2.11 Cell morphology

As described previously (section 2.2.9), morphological analyses were conducted to investigate dendritic arborization rate, cell body size (perimeter and area), number of dendritic nodes, and total dendritic length of DCX+ granule cells of the subgranular zone of the dentate gyrus of hippocampus. In this experiment, not only the Branches Structure analysis was performed, but also the concentric spheres analysis of Sholl, in order to measure branching pattern of the dendritic growth away from the soma (Arisi and Garcia-Cairasco, 2007). In the basic procedure, the number of intersections of dendritic processes with rings of increasing radii centred in the cell soma are counted. The Sholl profile of a cell is then obtained by plotting the number of intersections *versus* the radial distance from the cell soma. The morphological study was performed only on neurons of young animals, chosen with the same criteria previously reported.

3.2.12 Glial fibrillary acidic protein (GFAP) immunohistochemistry and densitometric analysis of astrocytic immunosignal

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein exclusively found in the cytoskeleton of astrocytes, and is therefore used as a specific marker of these cells. Brain sections from young and aged animals were processed for GFAP immunoreactivity (GFAP i-r) measured as optical density (OD).

GFAP immunostaining: after incubation in 1% H_2O_2 in 0.1M PBS for 20 min for peroxidase inactivation, sections were blocked in 5% BSA + 0.3% Triton X-100 in PBS for 90min. Then sections were incubated overnight with a polyclonal rabbit anti-GFAP (DAKO; 1:500) diluted in PBS containing 1% BSA+ 0.2% Triton X-100 overnight at 4°C; incubation in secondary antibody (biotinylated horse anti-rabbit IgGs; 1:200) diluted in PBS containing 1% BSA + 0.2% Triton X-100 was performed for 2h at room temperature. Then the staining was developed with the ABC kit (incubation for 1h at RT) followed by an incubation in 0.1M PBS containing 0.05% DAB + 0.03% H_2O_2 for 3-5 min. All the washes were done with 0.01M PBS containing 0.1% of Triton X-100.

Densitometry: photographs at high magnification were taken with a digital camera connected to a microscope (objective 40X) and background signal (non specific GFAP i-r signal) was calculated for each frame chosen from the areas of interest (Table 3.2) in coronal sections selected between rostro-caudal levels -2.88 mm to +2.2 mm (relative to Bregma, Paxinos and Watson, 1998). GFAP i-r values were normalized to OD values with the following formula:

$$1-(OD_x/OD_b)$$

where ODx is the OD of the brain area considered, and OD_b is the OD of the background measured in the same section of the brain area analyzed.

Brain area	frames per side per animal	Bregma
Cingulate cortex	4	0.00
Motor cortex	2	0.00
Parietal cortex	2	-3.48
Hippocampal hilus	4	-3.48
Hippocampal CA2	4	-3.48

Table 3.2 - Number of frames selected from the brain areas of interest according to Paxinos and Watson, 1998.

3.3 Results

3.3.1 Body weight monitoring

Each animal's body weight was monitored twice a day until the end of the treatment period.

In the chart below (Fig. 3.2) the body weight of a subgroup of all CLI and Sal animals is reported as a function of time: a treatment effect was observed, with CLI animals showing a significant decrease in body growth rate compared to Sal animals during the

period of treatment (Repeated measures ANOVA with $F_{(1,61)} = 38.91$, $p<0.0001$). No sex differences in body growth rate were observed during the period of treatment.

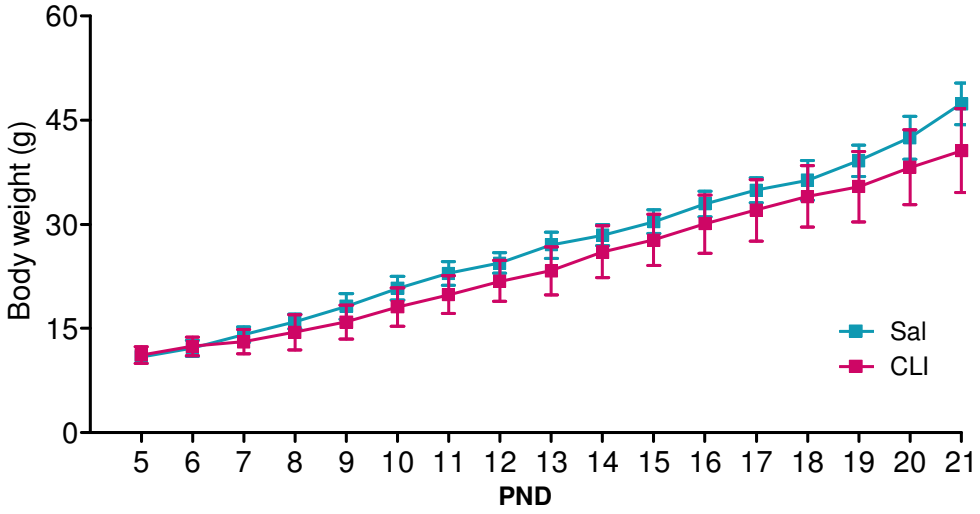

Fig. 3.2 - Body growth rate during the 17-day period of treatment.

3.3.2 Behavioral assessment

3.3.2.1 Forced swim test

3.3.2.1.1 Treatment and sex correlation in the young group

The chart below (Fig. 3.3) reports the amount of time spent in immobility by young animals for each minute of the 5-min test. The graph shows that females, and in particular the group of CLI females, spent more time in immobility compared to males already at the first minute of the test, while the amount of time spent in immobility is the same at the end of the test. A Repeated Measures ANOVA showed a significant effect of time ($F_{(4,136)} = 60.597$, $p<0.0001$) and of sex ($F_{(1,37)} = 10.579$, $p<0.05$), indicating that, during the 5-minute test, immobility time progressively increases for all the animals, and that females showed greater immobility than males. Furthermore, a significant interaction effect time*sex*treatment ($F_{(4,136)} = 2.808$, $p<0.05$) was shown, with CLI female rats spending more time in immobility than the other groups of animals.

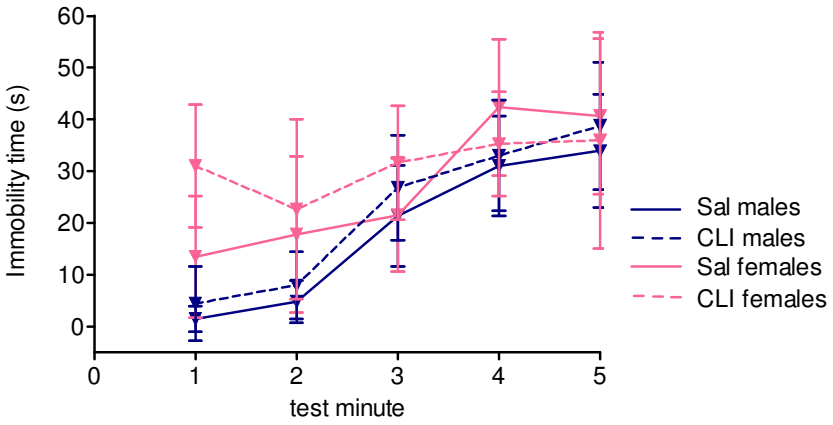

Fig. 3.3 - Immobility during FST in young animals. The chart reports time spent in immobility each minute of the 5-min test.

In the graph below (Fig. 3.4), time spent climbing by each group of rats is shown: only female rats seem to show differences in the performance, with CLI females spending the lowest amount of time in climbing activity compared to Sal females and also to males. Even if a certain variability within the groups of animals is evident, a significant treatment effect ($F_{(1,37)}$ =4.456, p<0.05), with CLI rats spending a lower amount of time climbing than Sal rats, and a trend towards a sex*treatment interaction effect ($F_{(1,37)}$ =2.94, p=0.096) were found with a Two-way ANOVA.

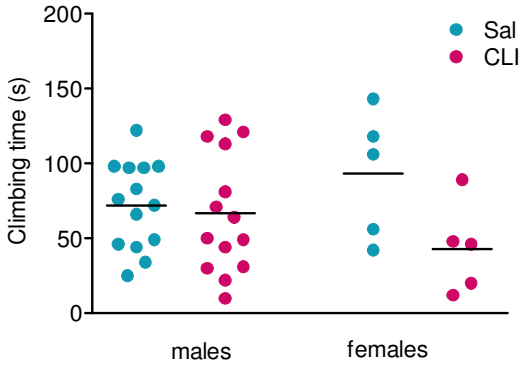

Fig. 3.4 - Climbing activity during FST in young animals.

3.3.2.1.2 Treatment and age correlation

FST performance was compared among all the age groups of animals, and is described in the following two charts, showing time spent in immobility (Fig. 3.5A) and climbing (Fig. 3.5B) by CLI and Sal rats. While young and middle-aged rats showed a similar attitude in immobility rate, aged rats significantly spent more time in immobility compared to the other two age groups (Two-way ANOVA, $F_{(2,32)}$ = 3.594, p<0.05). As shown in Fig. 3.4 and described in section 3.3.2.1.1, CLI young rats showed a significant effect of treatment in climbing activity but the same cannot be said for the middle-aged and aged groups, as confirmed by the trend towards a treatment*age interaction ($F_{(2,32)}$

= 3.040, p=0.64) effect found with Two-way ANOVA. Furthermore, only a trend towards an age effect was found ($F_{(2,32)}$ = 2.667, p=0.88).

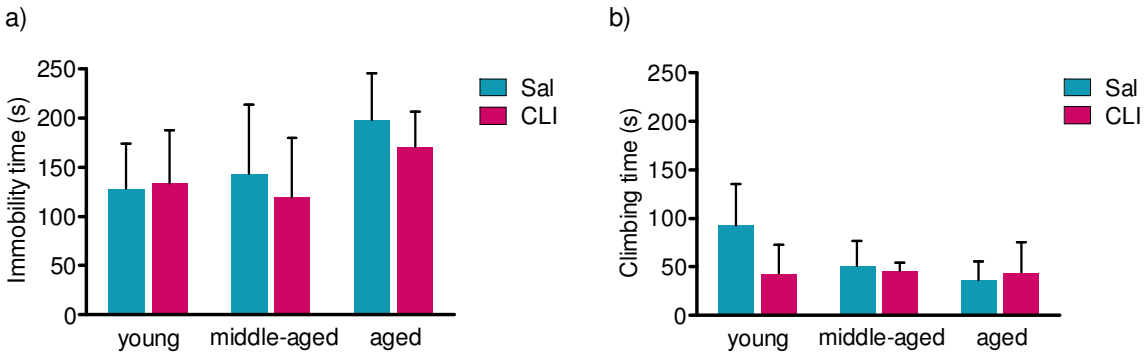

Fig. 3.5 - Comparison of a) immobility and b) climbing activity in the FST among the three age groups.

3.3.2.2 Sucrose preference test

3.3.2.2.1 Treatment and sex correlation in young animals

The chart below (Fig. 3.6) shows the total liquid intake over the 48-hr training phase, separately for water and sucrose consumption: all the animals preferred sucrose, with no differences in water intake. In particular, CLI male rats showed a significantly higher sucrose consumption compared to Sal male rats (p<0.05). Female animals did not show the same preference, as demonstrated by the significant main effect of sex ($F_{(1,37)}$ =3.92, p<0.05) and by the sex*treatment interaction effect ($F_{(1,37)}$ =10.622, p<0.01) on sucrose consumption.

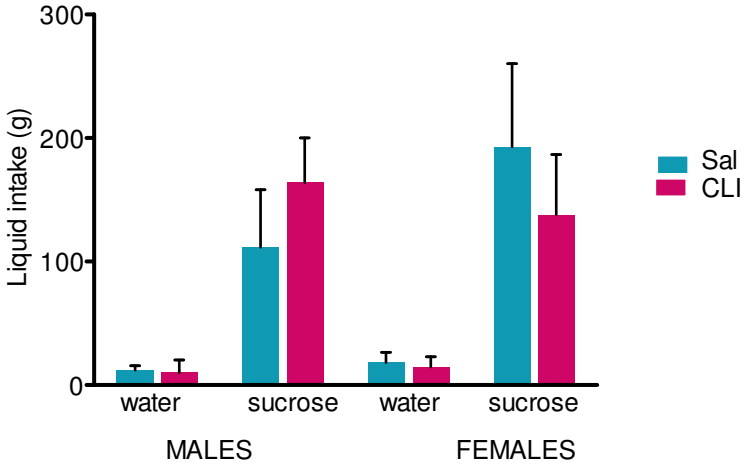

Fig. 3.6 - Total water and sucrose solution intake, after the 48h habituation phase of the SPT of the young group of animals.

The chart below reports the percentage of sucrose consumption, over the total liquid intake, measured during the 2h-test phase (Fig. 3.7). The higher sucrose consumption of CLI male rats compared to Sal male rats was evident, whereas females showed no

differences due to treatment. As in the habituation phase, a significant effect of sex (Two-way ANOVA, $F_{(1,37)}$ =4.89, p<0.05) and a sex*treatment interaction (Two-way ANOVA, $F_{(1,37)}$ =9.05, p<0.01) were found, suggesting that the hedonic behavior of animals did not consistently change after the 18h stressful event of food and water deprivation.

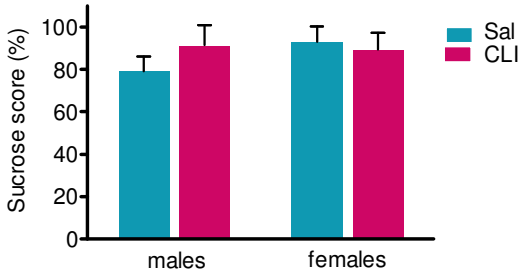

Fig. 3.7 - Sucrose preference score in the young group of animals in the 2h-test.

3.3.2.2.2 Treatment and age correlations

Graph reported in Fig. 3.8 shows the sucrose consumption over the total liquid intake in the three age groups during the 2h test: a treatment effect seems to be present in the middle-aged group, with CLI rats exhibiting a lower consumption of sucrose compared to Sal rats. Furthermore, aged rats, without any distinction between CLI and Sal animals, consumed less sucrose than the other two age groups. Multivariate ANOVA showed a significant age effect ($F_{(2,32)}$ = 9,795, p=0.001), but no treatment or treatment *age interaction effects in the 2h-test. Further analysis showed a significant effect of age in sucrose preference scores measured in both the first and the second day of habituation (data not reported) and a trend towards a treatment effect ($F_{(1,32)}$ =3.839, p=0.06) in the second day of the habituation phase (not shown). The Post-hoc analyses (Bonferroni) showed a significant difference between the aged and the young group in all the three phases of the behavioral test, and a significant difference between the aged and the middle-aged group in the habituation phase, but not in the 2h test.

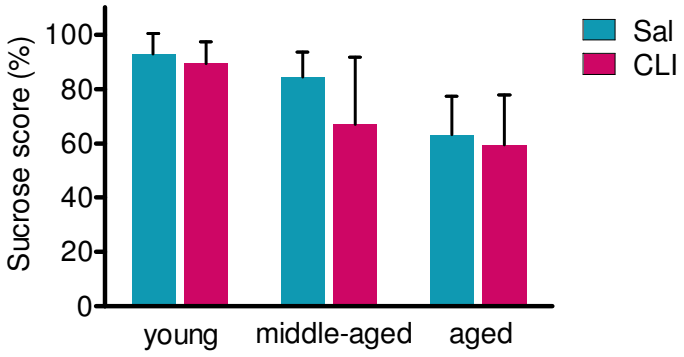

Fig. 3.8 - Sucrose preference score after the 2h test in the three age group.

3.3.3 Brain MRI volumetry

3.3.3.1 Young animals

The graphs in Fig. 3.9 show the volumes of a) total brain and b) hippocampus measured in the young group of animals measured in mm^3.

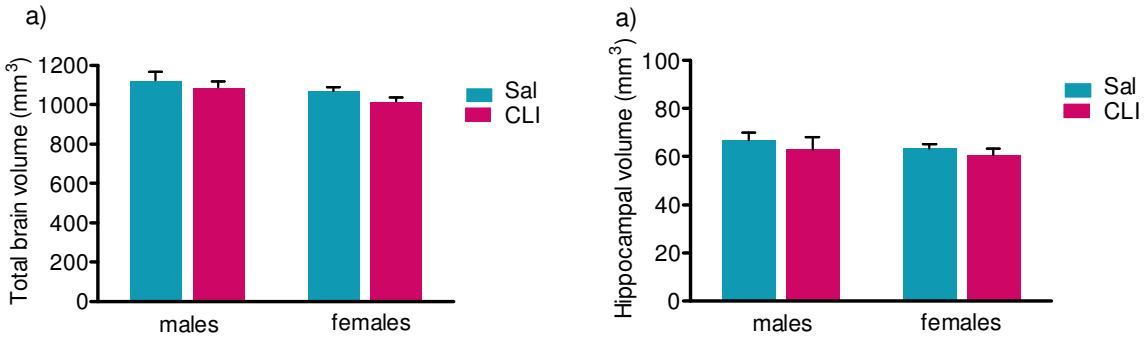

Fig. 3.9 - Volumetric analysis of a) total brain and b) hippocampus of young animals.

A Two-way ANOVA demonstrated a treatment effect in total brain volume ($F_{(1,25)}$ =11.84, p<0.05) with CLI rats showing a smaller brain compared to Sal rats. Also hippocampal volumetric analysis, performed without any manipulation/normalization of data, confirmed the presence of a treatment effect: in CLI rats we observed a smaller hippocampus compared to Sal rats ($F_{(1,25)}$ = 5.23, p<0.05). Interestingly, we found a significant effect of treatment on volume of lateral ventricles: CLI rats (Fig. 3.10A) exhibited enlarged ventricles compared to Sal rats (Fig. 3.10B) ($F_{(1,25)}$ =5.28, p<0.05). Treatment did not affect the volume of prefrontal cortex and did not affect in a different way the volume of brain regions of males and females.

We made a further investigation of the hippocampal reduction and of the ventricular enlargement observed by performing Univariate ANOVA for each of the two regions with total brain volume as covariate. Hippocampal volume of CLI animals showed no more significant reduction compared to Sal animals, while total ventricles were confirmed to be significantly enlarged in CLI animals compared to Sal animals ($F_{(1,25)}$ = 4.624, p<0.05). This important observation has been already reported in depressed (Kempton *et al.*, 2011) and schizophrenic patients (Kempton *et al.*, 2010; Meduri *et al.*, 2010).

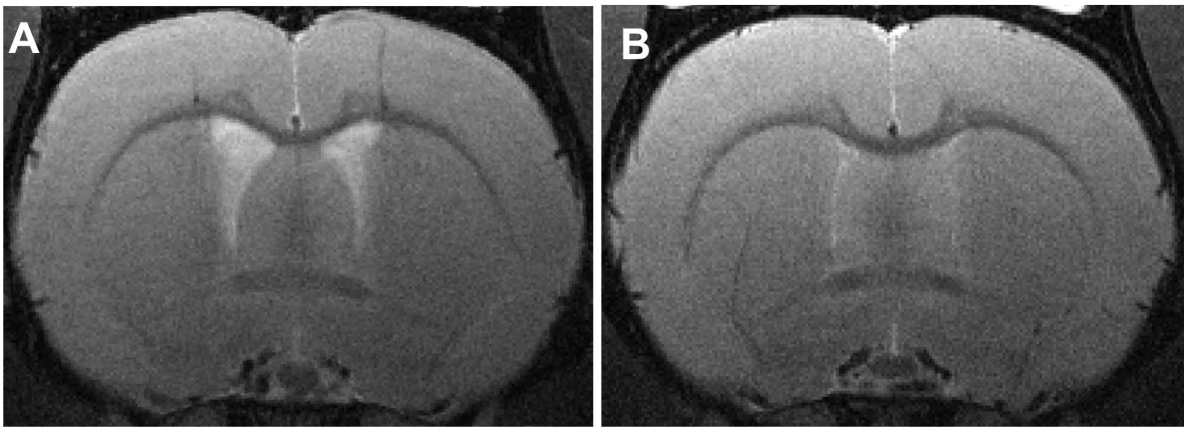

Fig. 3.10 - MRI total ventricular volumetry in the young group of animals: A, CLI rats; B, SAL rats.

3.3.3.2 Age comparisons

In a comparison of total brain volumes between young and aged animals, no age effects were found. Further, aged brains did not show any differences due to a treatment effect (not shown).

The graphs reported in Fig. 3.11 show the comparison made between young and aged rats of a) prefrontal cortex (PFC) and b) hippocampal volumes. For what concern PFC, the chart shows slight differences between CLI and Sal animals, and a little reduction in volume for aged rats compared to young. Results from a Two-way ANOVA showed trends towards age (Two-way ANOVA, $F_{(1,21)}$ =3.170, p=0.09) and treatment (Two-way ANOVA, $F_{(1,21)}$ =3.948, p=0.06) effects.

For what concerns the analysis of hippocampal volumes (Fig. 3.11B), aged rats showed a bigger hippocampus compared to young animals, but not relevant differences due to treatment. A significant effect of age (Two-way ANOVA, $F_{(1,21)}$ =6.813, p<0.05) was found without any manipulation of data, and this result was confirmed also in a Univariate ANOVA with total brain volume as covariate factor ($F_{(1,21)}$ =13.751, p<0.01).

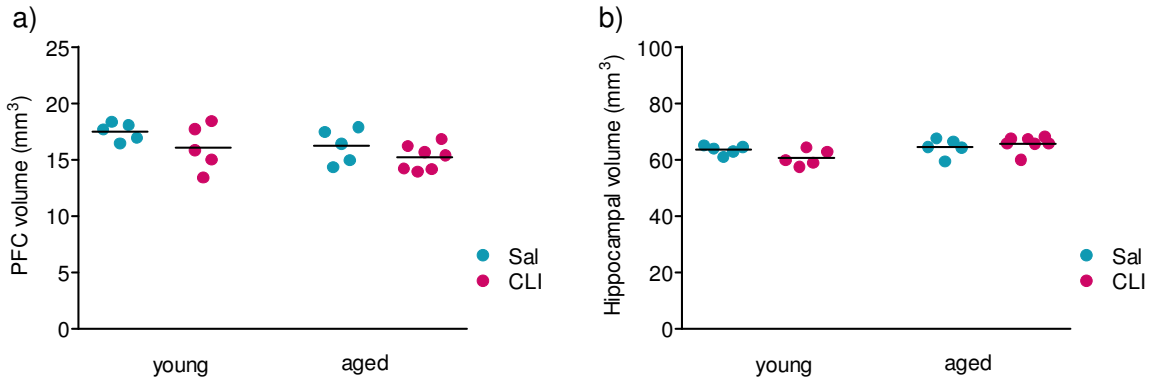

Fig. 3.11 - MRI volumetric analysis of a) prefrontal cortex, and b) hippocampus in aged and young rats.

3.3.4 BDNF analysis

The chart in Fig. 3.12 shows the quantity of BDNF, expressed as quantity of BDNF (ng) over the total amount of protein (µg), measured in hippocampus and total cortex. A significant decrease in BDNF levels was found in hippocampus of CLI rats (One-way ANOVA, $F_{(1,8)} = 14.394$, $p<0.01$) but no difference were found in cortical levels.

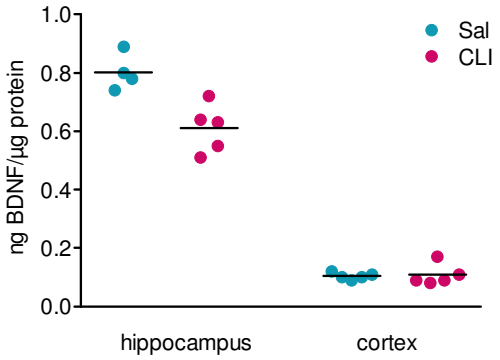

Fig. 3.12 - Levels of BDNF in hippocampus of Sal and CLI rats.

3.3.5 Cell counting

3.3.5.1 Young animals

The graphs below report the total number of DCX+ (Fig. 3.13A) and BrdU+ (Fig. 3.13B) cells. Females showed a lower number of both DCX+ and BrdU+ cells. A treatment effect can be noticed only in BrdU+ cell counts, with CLI animals showing a lower number of cells compared to Sal animals in both males and females. A Multivariate ANOVA showed a significant sex effect in both total DCX+ cell counts ($F_{(1,22)} =21.208$, $p<0.001$) and BrdU+ cell counts ($F_{(1,22)} =4.384$, $p=0.05$) and a treatment effect was observed in BrdU+ cell counts ($F_{(1,22)} =5.289$, $p<0.05$).

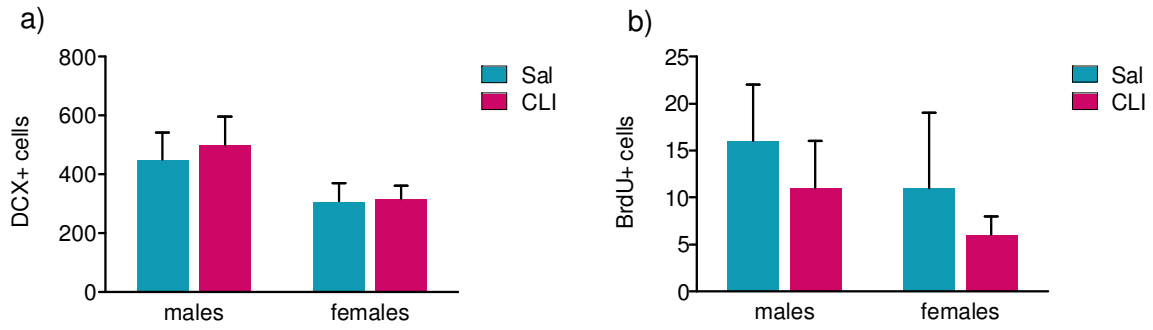

Fig. 3.13 - Effects of sex and treatment on total counts of a) DCX+ and b) BrdU+ cells.

3.3.5.2 Age comparisons

As shown in the graph below (Fig. 3.14A), the number of DCX+ cells was significantly lower in aged animals than in young animals. A two-way ANOVA showed an age effect ($F_{(1,19)}$ =221.604, p<0.001). Age effect was significant also for BrdU+ ($F_{(1,19)}$ =10.158, p<0.01) and for double-labeled (Fig. 3.14B) cell counts ($F_{(1,19)}$ =16.787, p=0.001). Representative microphotographs from BrdU/DCX immunohistochemistry in young and aged animals are shown in Fig. 3.15.

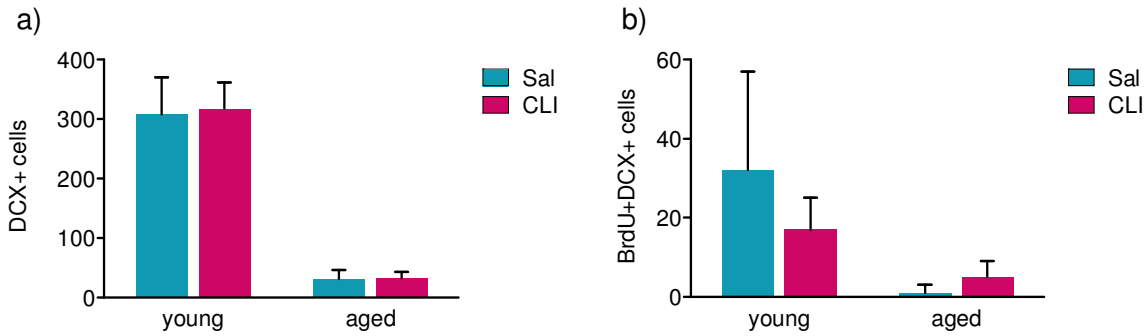

Fig. 3.14 - Age effect on a) DCX+ and b) double-labeled cells of the subgranular zone of the hippocampus.

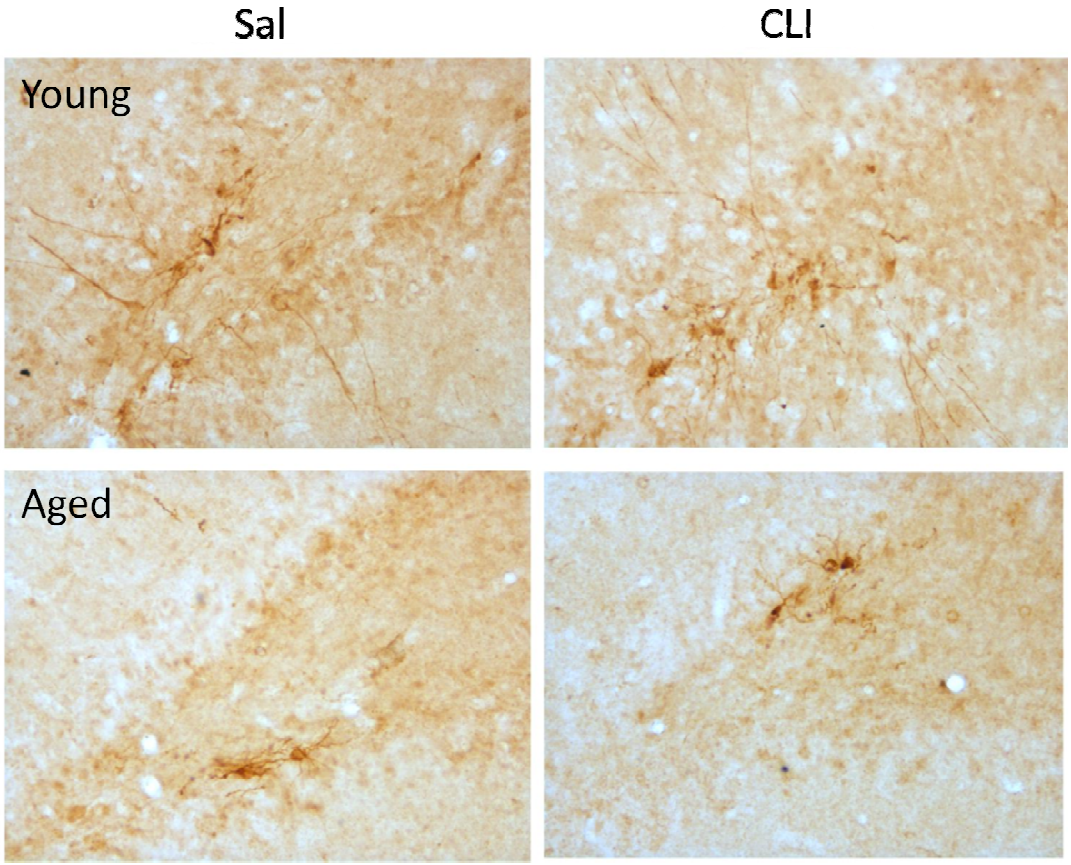

Fig. 3.15 - Representative microphotographs of DCX+ cells in young and aged animals.

3.3.6 Cell morphology

The statistical analysis of the parameters measured was performed as described by Rao (Rao *et al.*, 2005): the value of every parameter was first calculated separately for each animal and then means and standard deviations were determined for the total number of animals included per group.

DCX+ cells were analyzed for the total length of apical dendrites, total number of nodes and for the pattern of dendritic growth and of node distribution away from the soma. No differences in total number and pattern of distribution of dendritic nodes were found; branching pattern of the dendritic growth away from the soma revealed a sex difference in the first 10 µm of distance from soma, with granule cells of females showing a significantly higher branching than those of males (Multivariate ANOVA, $F_{(1,22)}$ =11.887, p<0.01)(Fig. 3.16, marked by red circle). Furthermore, a trend towards a treatment effect (Multivariate ANOVA, $F_{(1,22)}$ =2.748, p=0.093) was found at 30 µm of distance from soma, with CLI rats exhibiting a higher branching than Sal rats) (Fig. 3.16, marked by green circle). Dendritic arborization over a distance of 150 µm was significantly reduced or even not present in some of the cells analyzed.

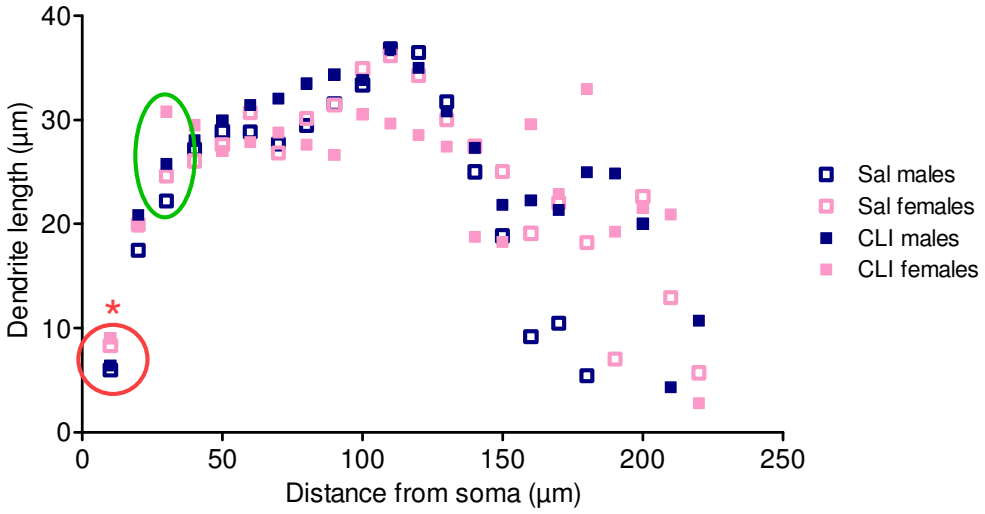

Fig. 3.16 - Morphological Sholl profile of young animals: effect of sex (red circle) and a trend towards a treatment effect (green circle) on the dendritic arborization pattern of DCX+ granule cells analyzed in the subgranular zone of the hippocampal dentate gyrus.

Chart reported in Fig. 3.17 shows a significant treatment*sex interaction effect in total dendrite length: CLI males showed a major total dendrite length compared to Sal males, while in females the trend is opposite (Multivariate ANOVA, $F_{(1,22)}$=5.07, p<0.05).

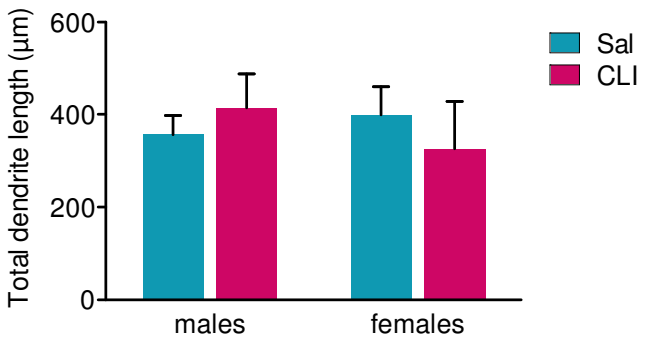

Fig. 3.17 - Total dendrite length measured in DCX+ granule cells of young animals.

A further morphological analysis was performed on the soma, evaluating characteristics such as perimeter, area (referred to XY projection of the 2D images reconstructed at the plane of the sharpest focus, i.e. the focus calculated at the midpoint of the Z-plane extreme focuses), feret maximum, that is the longest diameter of the soma, and feret minimum, the longest diameter perpendicular to the feret maximum. Females showed decreased values in all the components examined, as shown in the four graphs reported in Fig. 3.18. Furthermore, only females seemed to exhibit a certain degree of treatment effect, with CLI animals showing decreased perimeter, area, and Feret maximum and minimum. Statistical analysis (Multivariate ANOVA), whose results are entirely reported in Table 3.3, showed a sex effect in all parameters measured. A sex*treatment interaction effect and a trend towards a treatment interaction effect were found only in the measure of Feret maximum, with CLI females showing a decreased Feret maximum compared to Sal females, while no effect was observed in males. A trend towards a sex*treatment effect was found also in the measure of perimeter, again with the same observations made for Feret maximum.

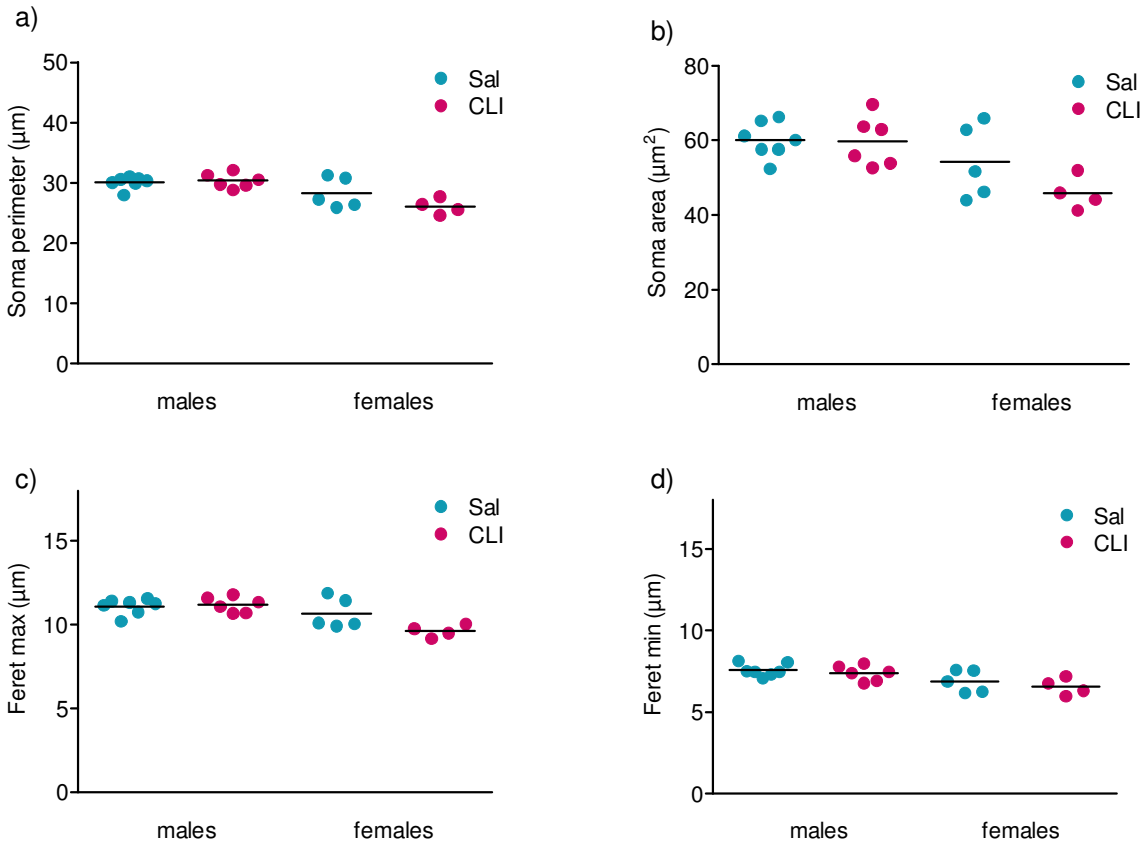

Fig. 3.18 – Somatic neuron components analyzed in selected DCX+ cells of young females and males with the Branched structure analysis: a) perimeter, b) area, c) Feret max, and d) Feret mimum.

Somatic parameter	Sex		Treatment		Sex*treatment	
	F	*p* value	F	*p* value	F	*p* value
Perimeter	19.567	<0.001*	2.121	0.162	3.408	0.081
Area	11.551	0.003*	2.162	0.159	1.914	0.183
Feret maximum	15.448	<0.001*	3.580	0.075	5.293	0.034*
Feret minimum	11.766	0.003*	1.301	0.269	0.104	0.751

Table 3.3 – Multivariate ANOVA results of the analysis of morphological somatic parameters of DCX+ cells in young animals. Animals: 13 males, 9 females; 12 Sal, 10 CLI. (*)= statistical significant results for α value =0.05.

3.3.6.1 Glial fibrillary acidic protein (GFAP) immunohistochemistry and densitometric analysis of astrocytic immunosignal

3.3.6.1.1 Young animals

All areas in which GFAP i-r was measured showed a significant sex difference with female rats exhibiting a higher astrocytic density compared to male rats. In Fig. 3.19, graphs relative to GFAP i-r measured in a) sensitive cortex and b) hippocampal hilus show that CLI females have a lower astrocytic density compared to Sal females, while in males no treatment effect is evident. Table 3.4 reports the results of the Multivariate ANOVA performed. As expected, in the region of motor cortex, no treatment effect was found. Furthermore, all brain regions, with the exception for motor cortex, showed a significant sex*treatment interaction effect, with CLI females showing a lower astrocytic density compared to Sal females, but no differences in males.

Brain region	Sex		Treatment		Sex*treatment	
	F	*p* value	F	*p* value	F	*p* value
Cingulate cortex	156.691	<0.001*	1.812	0.194	8.667	0.008*
Motor cortex	51.437	<0.001*	0.490	0.493	3.354	0.083
Parietal cortex	32.832	<0.001*	4.660	0.044*	6.391	0.020*
Hippocampal hilus	70.576	<0.001*	5.446	0.031*	15.885	0.001*
Hippocampal CA2	58.232	<0.001*	1.662	0.213	7.019	0.016*

Table 3.4 - Statistical results of Multivariate ANOVA of GFAP i-r in different brain regions. N=23, 11 CLI rats and 12 Sal rats; 14 males and 9 females.

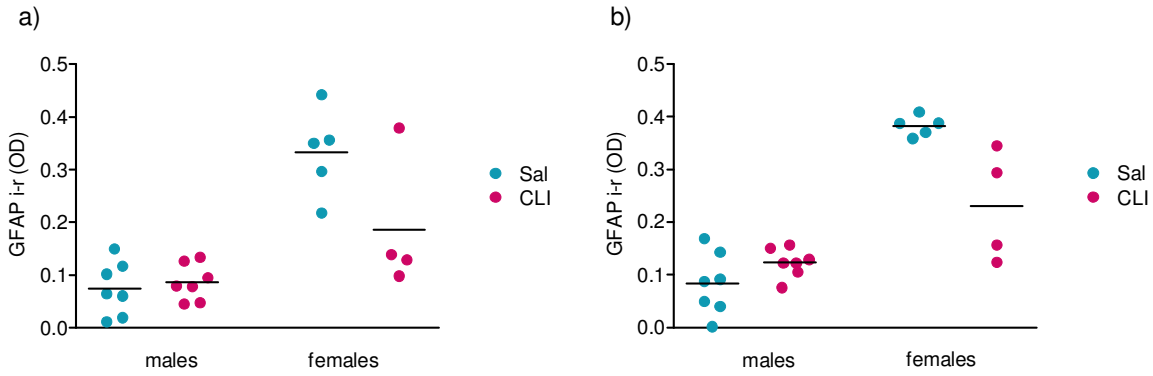

Fig. 3.19 - GFAP i-r expressed as optical density (OD) in the sensitive cortex (a) and hippocampal hilus (b) of young male and female rats. In these two regions there was evidence of sex, treatment and sex*treatment interaction effects.

3.3.6.1.2 Age comparisons

In Fig. 3.20, scatter plots relative to GFAP i-r in a) cingulate cortex and b) hippocampal CA2 show a different pattern of the effect of clomipramine treatment: CLI aged animals exhibited an augmented GFAP i-r compared to Sal aged rats, while in young animals the trend is opposite. In Table 3.5 all results from the Multivariate ANOVA performed are reported. In the comparison between young and aged female rats, a trend to an age effect was found only in motor cortex, with young rats showing a higher GFAP i-r than aged rats . No treatment effects were evident in any region. Effects of age* treatment interaction were found in all regions analyzed. In Fig. 3.21 representative microphotographs of the GFAP immunohistochemistry in both young and aged animals are shown.

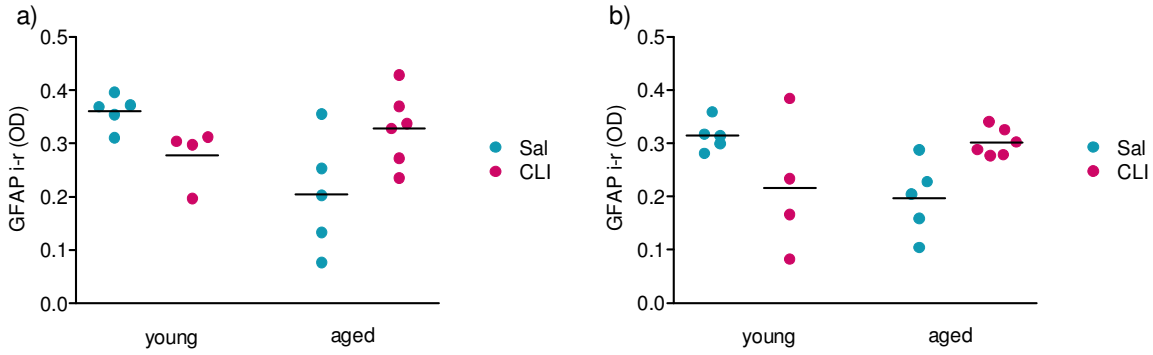

Fig. 3.20 - Age comparison in GFAP i-r in cingulate cortex (a) and hippocampal CA2 (b) of female rats. Neonatal clomipramine treatment shows an inversion of effect in GFAP i-r in the elderly compared to early age.

Brain region	Age		Treatment		Age*treatment	
	F	*p* value	F	*p* value	F	*p* value
Cingulate cortex	2.639	0.124	0.406	0.533	10.143	0.006*
Motor cortex	4.238	0.056	0.258	0.619	7.347	0.015*
Sensitive cortex	0.005	0.943	0.000	0.983	11.840	0.003*
Hippocampal hilus	0.064	0.804	0.1692	0.212	7.237	0.016*
Hippocampal CA2	0.266	0.613	0.015	0.904	10.776	0.005*

Table 3.5 - Statistical results of Multivariate Anova of GFAP i-r in different brain regions. N=20, 10 CLI rats and 10 Sal rats; 9 young and 11 aged.

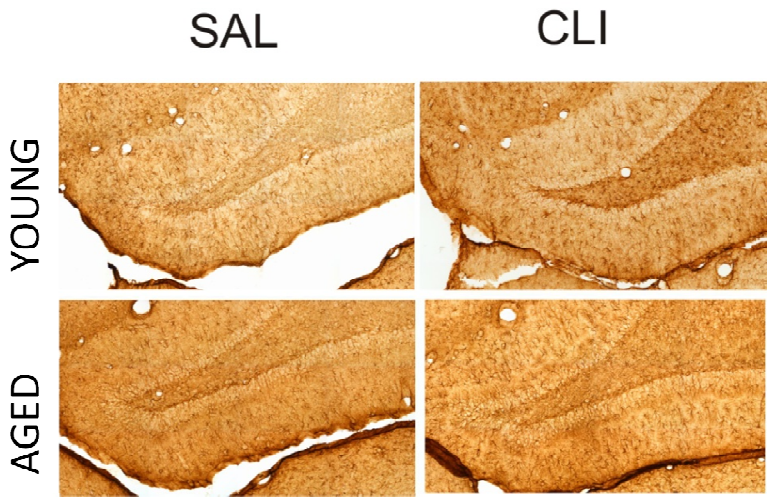

Fig. 3.21 – Representative microphotographs from GFAP immunohistochemistry in the hippocampus of young and aged animals.

3.4 Conclusions

The results obtained so far raise several considerations on the effects of neonatal clomipramine treatment in males and females and their persistence during adulthood and aging.

- In young animals, the most straightforward indication obtained is the decrease in total brain volume and an enlargement of total ventricular volume of CLI compared to Sal rats, as demonstrated by the MRI studies. These results are in line with clinical (Sheline, 2000; Kempton *et al.*, 2011) and preclinical studies on other animal models of depression who showed a reduction of brain volume and an enlargement of lateral ventricles (Wrynn *et al.*, 2000).
- BDNF decrease in CLI animals may be the responsible of the qualitative observations on morphological dystrophic features of neurons observed in previous experiment (section 2.3.4).

- Behavioral testing yielded different effects in females vs. males, suggesting a sex-dependent susceptibility to the treatment or possibly a different recovery time. The data reported described for the first time a gender-dependent effect in the neonatal clomipramine model of depression, in line with many studies that repeatedly reported sex differences in animal models of depression and antidepressant response (Dalla C. *et al*., 2009); sex differences during aging remain to be investigated.
- In aged animals, behavioral, neurogenic, and brain structural changes typical of aging, with a flattening of the effects of treatment suggest a degree of long-term recovery from the picture seen in young rats;
- The group of middle-aged animals may represent an intermediate phase between the early treatment effects and the subsequent recovery.

These findings confirmed in part data reported by Vogel (Vogel *et al*., 1990a): the effects of neonatal clomipramine treatmentare likely to appear in the adulthood and to persist for a certain period of time. To the best of our knowledge, the effects on animals before the three months of age have not yet been described.

4 Study 3 – Effects of acute stress in clomipramine treated rats

4.1 Introduction

The last experiment was aimed at the investigation, in the neonatal clomipramine model, of the possibility that a stress event, in the adult age, might exacerbate behavioral depressive symptoms and influence some neurobiological substrates. It has been repeatedly reported that patients suffering from depression show an increased reactivity to stress and contemporarily show a dysregulation of the HPA axis (Holsboer, 2000; Holsboer and Ising, 2010). Life experiences characterized by a stressful component elicit a complex variety of behavioral and physiological changes believed to contribute to ideal coping of the organism with the situation including the activation of HPA axis (Girotti *et al*., 2006). Considering that in human life stressful events are daily and, as said above, can trigger a latent depressive syndrome, also through the chronic elevation of glucorticoid levels due to HPA axis activation (Girotti *et al*., 2006), it is necessary to consider that maybe it is paid too much attention in protecting lab animals thus creating a not-realistic environment. The behavior of CLI animals could be masked by the protected environment in which they live. Could a controlled acute stress in the adulthood induce the coming out of behavioral depressive symptoms of early-life treated animals?

The study was divided into two phases:

4. behavioral and neurobiological characterization of neonatal clomipramine exposure in the animals;
5. investigation of behavioral and neurobiological correlates of the interaction between pharmacological treatment in early life and an acute stress event in the adult age.

Restraint stress is the type of stress chosen for our aims: it is a kind of mild stressoror, often used in protocols of chronic mild stress induced depression in rodents (Bessa *et al*., 2009; Reich *et al*., 2009), and it is considered to be primarily a psychological stress because it does not produce pain or direct physical insult (Girotti *et al*., 2006).

Among the procedures for behavioral assessment, here the elevated plus maze (EPM) test was adopted to study anxiety trait in our animals in basal conditions and after a stressful event. The maze is a cross-shaped apparatus elevated from the floor, with 2 open arms, two enclosed arms with high walls, and an open central zone. Rodents are put in the central zone with the head in the direction of one open arm and left free to move on the apparatus for 5 minutes. No training is performed, so the behavior of animals is spontaneous and unconditioned. The test is a paradigm on the relationship between novelty, fear of the open spaces and exploration (Rodgers and Dalvi, 1997). The measure of time spent and of number of entries in both open and closed arms are employed as measures of locomotor and exploratory activity. The more animals spend time in closed arms, the more they show an anxious behavior.

Another method to study exploratory behavior, locomotor activity and anxiety trait is the open field test, that herein is represented by a larger cage (for the description, see paragraph 4.2.3.2) enriched by several objects. Animals in a novel enriched environment show reduced aggression, anxiety, fear, stress and an increase in learning

abilities (Ilin and Richter-Levin, 2009) and are induced to react to novelty and to explore it.

In order to complete a series of neurochemical analyses performed in the previous experiments, the serotonin transporter (SERT) distribution was analyzed with the immunohistological technique in several brain regions of our animals. SERT is a membrane protein that transports the neurotransmitter serotonin from synaptic spaces to presynaptic membrane, in order to block serotonin's action and recycle it. Because of its function, SERT has become in the last years one of the favorite targets of the most used antidepressant agents, the SSRIs.

SERT was found also in cells outside the central nervous system, including blood platelets, lymphocytes, and enterochromaffin cells of the intestinal epithelium. A decrease in peripheral blood platelet SERT binding is a biomarker of depression (Rivera-Baltanas *et al*., 2012). Further observations about a decrease in lymphocyte SERT expression in depressive patients were reported and it may be related to an increase in inflammatory cytokines with a role in the pathophysiology of depression (Rivera-Baltanas *et al.*, 2012). SERT is relevant to the neuroanatomy of depression, given its localization in cortical, striatal, and limbic areas (Smith *et al*., 2011). *In vivo* imaging and *post-mortem* studies showed a lower SERT binding in brain regions, such as prefrontal cortex, of depressed patients, maybe due to a decrease in SERT gene expression (Arango *et al*., 2001). In animals neonatally exposed to citalopram, SERT expression was found reduced in the medial prefrontal cortex, primary somatosensory cortex (Maciag *et al*., 2006) and throughout the entire hippocampus (Weaver *et al*., 2010), and these effects persisted in adulthood.

4.2 Material and methods

4.2.1 Animals

Pregnant Sprague-Dawley rats (Harlan), were put on a 12:12h light/dark cycle (lights on at 8:00 am) and maintained at room temperature (20±2°C) with food and water *ad libitum*.

4.2.2 Treatment groups

At birth, animals were divided into three treatment groups: CLI, Sal, and untreated (Untr) rats. CLI and Sal animals were subcutaneously injected twice daily, from postnatal day (PND) 5 to 21, with 20 mg/kg clomipramine and saline solution, respectively. Injections were administered outside of the animals' home cage, which implied repeated, albeit short, maternal separations. Untr rats were left undisturbed for the entire period, until weaning. After weaning, animals were housed individually in standard rat cages and adapted to the laboratory's inverted 12:12h light/dark cycle (lights-on at 7 pm). The choice to adopt the inverted light/dark cycle was considered important to test animals in their natural wake phase, the dark phase, and measuring a spontaneous performance. Diversely, in both Experiment 1 and 2 animals were tested during the light phase.

In Fig. 4.1 a diagram of the experimental procedures to which animals were subjected is reported. Each experimental assessment was staggered from the previous of at

least one week to allow animals to recover. These pauses were also necessary to avoid any interference in the outcome of experimental testing due to the stress that procedures themselves induce in animals.

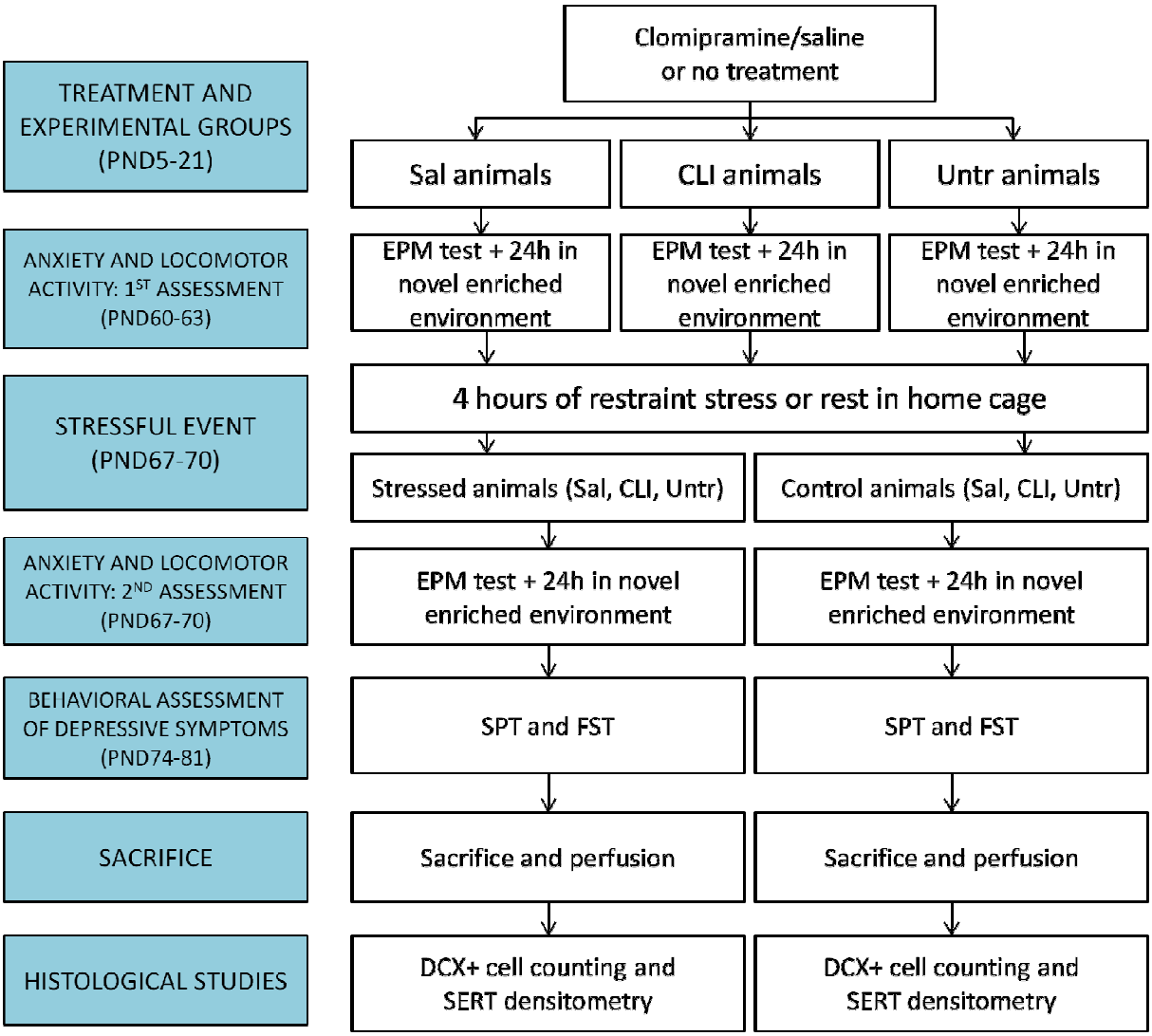

Fig. 4.1 - Diagram summarizing the experimental activities and animal groups involved in Experiment 3.

4.2.3 Pre-stress testing

4.2.3.1 Elevated Plus Maze Test

At PND60, all rats were subjected to the Elevated Plus Maze (EPM) test. Each rat performed a 5-min test, without any training session. Their performance on the instrument, which has been already described in section 4.1, was videotaped by a camera positioned at the centre of the ceiling of the testing room and then, with the help of the Ethovision software (Noldus®), which detected and recorded the positions of animals on the instrument with the frequency of 25 frames per second, parameters like distance moved, velocity, and time spent in specific zones were calculated.

4.2.3.2 Novel enriched environment

Immediately after the 5-min EPM session, animals were transferred to a novel, environment represented by Phenotyper cages, that were enriched with pieces of paper, bottle tops, and other stimulating objects for animals. Phenotyper cages are cages with plexiglas walls provided of holes for the airing of the cage, with interchangeable black or white bottoms, according to the fur color of the animals, and with a videocamera positioned at the top of the cage supplied with adjustable infrared lights. The Ethovision software (Noldus®) can analyze video-recordings with the frequency of 25 frames per second, thus allowing the experimenters to know with a high precision animals' movements inside the cage. Animals stayed inside the cage for 24h and 24h-videotapes are still in a preliminary stage of elaboration and analysis and in the present thesis they will not be presented.

4.2.4 Acute stress

One week after the above described assessement, half of each group of animals underwent an acute stressful event (stressed), while the other half stayed undisturbed in their cages (controls). Specifically, each stressed animal was put in a wire mesh restrainer for 4h and left undisturbed in its home cage. The condition did not allow the animals, to move, to feed and drink water.

4.2.5 Post-stress testing

Immediately following the acute stress, all the animals were re-tested for anxiety trait in the EPM test and for locomotor activity in the Phenotyper cages as described in sections 4.2.3.1 and 4.2.3.2, respectively.

After 1 week, animals were tested for depression with SPT and then with FST.

4.2.5.1 Sucrose Preference Test

The procedure adopted for SPT was the same described in section 3.2.4.2

4.2.5.2 Forced Swim Test

The procedure adopted for FST was the same described in section 3.2.4.1.

4.2.6 Perfusion and brain processing

At ~PND90 rats were deeply anesthetized, transcardially perfused with a NaCl 0.9% solution followed by a tissue fixation with cold paraformaldehyde (4% in PBS), and their brains were removed and stored in 30% sucrose at 4°C until they sank. Brains were then frozen at the microtome and cut in 40 μm coronal sections, which were preserved in 0.1M PBS with 0.1% NaN_3 until histological processes.

4.2.7 DCX immunohistochemistry

The procedure adopted was the same described in section 2.2.7, with the exception that counts were performed by visual inspection in the entire dentate gyrus of three evenly spaced section spanning the whole hippocampus.

4.2.8 SERT immunoistochemistry:

After incubation in 3% H_2O_2 and 10% MetOH in 0.1M PBS for 10 min for peroxidase inactivation, sections were blocked in 5% NHS + 0.3% Triton X-100 in PBS for 1h. Then sections were incubated overnight with a monoclonal mouse anti-SERT (Chemicon; 1:1000) diluted in PBS containing 3% NHS+ 0.3% Triton X-100 overnight at room temperature; incubation in secondary antibody (biotinylated horse anti-mouse IgGs; 1:200, Vector) diluted in PBS containing 3% NHS + 0.3% Triton X-100 was performed for 1h at room temperature. Then the staining was developed with the ABC kit (incubation for 1h at RT) followed by an incubation in 0.1M PBS containing 0.05% NiCl + 0.025% DAB + 0.003% H_2O_2 for 5 min. All the washes were done with 0.01M PBS containing 0.1% of Triton X-100.

4.2.8.1 SERT densitometry

Photographs at high magnification were taken with a digital camera connected to a microscope (objective 40X) and background signal (non specific SERT i-r signal) was calculated for each frame chosen from the areas of interest in the selected coronal sections (Table 4.1) (relative to Bregma according to Paxinos and Watson, 1998). SERT i-r values were normalized to OD values with the following formula:

$$1-(OD_x/OD_b)$$

where ODx is the OD of the brain area considered, and OD_b is the OD of the background measured in the same section of the brain area analyzed.

Brain area	frames per side per animal	Bregma
Cingulate cortex	4	0.00
Entorhinal cortex	2	-5.40
Hippocampal CA2	4	-3.48
Hippocampal hilus	4	-3.48

Table 4.1 - Number of frames selected from the brain areas of interest. Bregma levels are relative to those reported by Watson and Paxinos, 1998.

4.3 Results

4.3.1 Body weight

Body weight was monitored daily throughout the period of treatment in CLI and Sal treated rats: as shown in chart in Fig. 4.2 and as reported in previous experiments, CLI animals showed a significant decrease in body growth rate compared to Sal animals (Repeated measures ANOVA with $F_{(1,50)} = 93.621$, $p<0.001$). No weight monitoring was performed in Untr animals to avoid maternal separation.

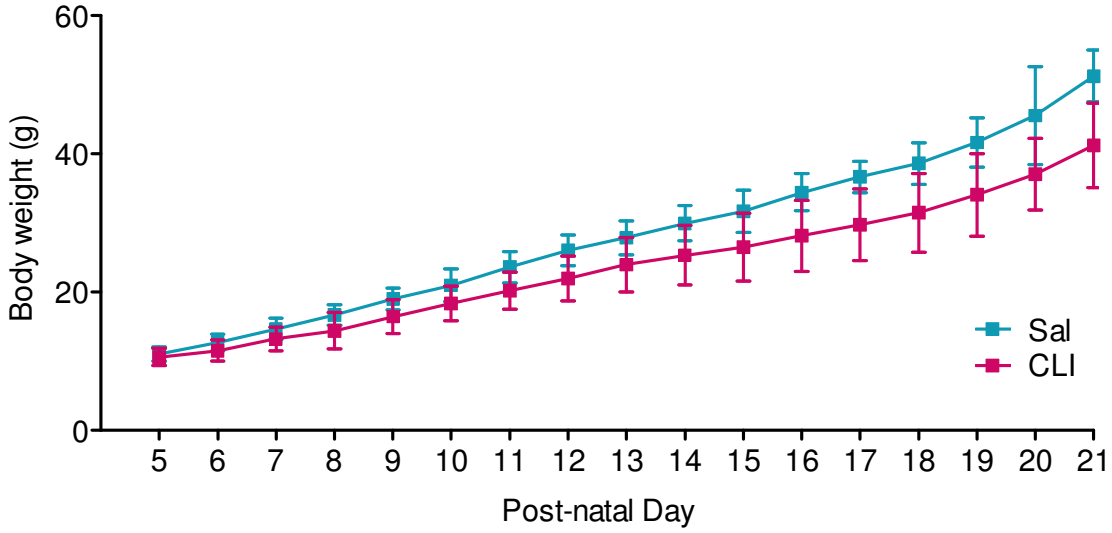

Fig. 4.2 - Body growth rate of CLI and Sal animals during the 17-day period of treatment. Untr animals were not weighed to avoid any stress and maternal separation.

4.3.2 EPM test

We evaluated the amount of locomotor activity in the EPM by measuring the total distance moved by the animal during the 5-minute test. In the session that preceded the restraint stress, no significant differences among the treatment groups were observed. After this first observation, we analyzed data as comparison between the two performances (preceding and following restraint) of each rat in the EPM test: to make this possible, data were elaborated as normalized index of the after- versus before-stress locomotor activity. The chart in Fig. 4.3 shows the differences in the performances of control and stressed animals: control animals did not show any significant difference in the performance of EPM test, while stressed animals showed a decrease in locomotor activity in the second session compared to the first (Univariate ANOVA, $F(1,61) = 3.943$, $p=0.05$). A treatment effect was observed ($F(2,61) = 4.648$, $p=0.014$) and post-hoc analysis showed that CLI animals moved significantly less than Untr animals ($p=0.018$).

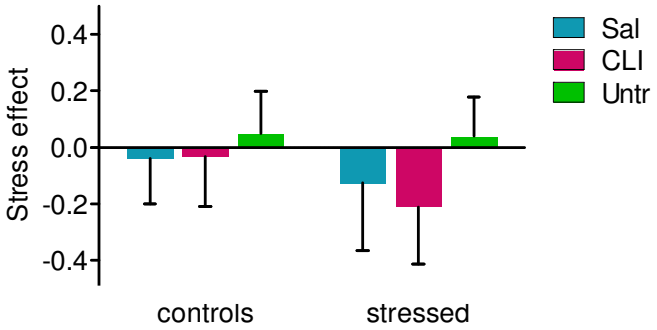

Fig. 4.3 - EPM test: normalized index of locomotor activity of after- (A) versus before- (B) stress performance ((A-B)/(A+B)).

In Fig. 4.4, representation of the negative effect of stress on the performance of EPM test is reported: distance moved by b) stressed animals was significantly diminished in the 2nd assessment, while no differences were detected in the performances of a) control animals.

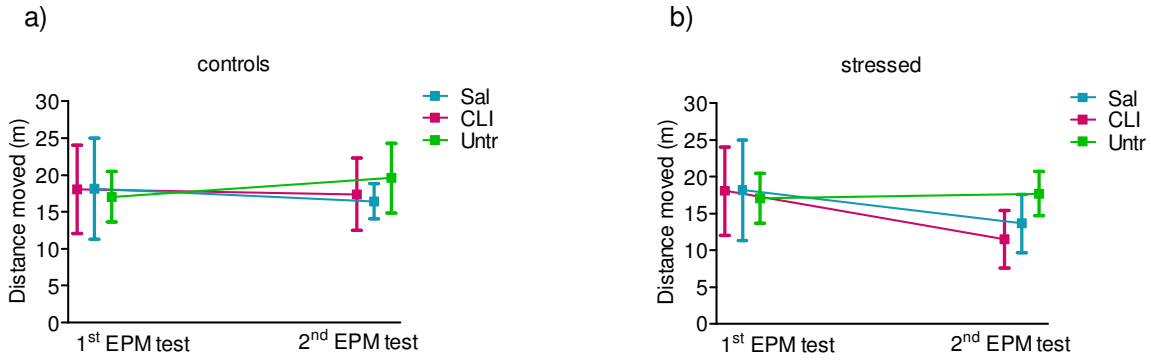

Fig. 4.4 - Performance in the two sessions of EPM test in a) control animals and b) stressed animals.

Charts reported in Fig. 4.5 show time spent in closed arms of EPM in the a) first session and b) second session. Animals seem not to be influenced by treatment in basal conditions as shown in graph a), and the repetition of the same test after one week did not induce any difference in the performance of controls. Furthermore, the repetition of the EPM test after a stressful event does not seem to affect the exploratory activity. Further analyses are ongoing to refine the measurement of exploratory versus "anxious" behavior as function of animal's distance from maze centre.

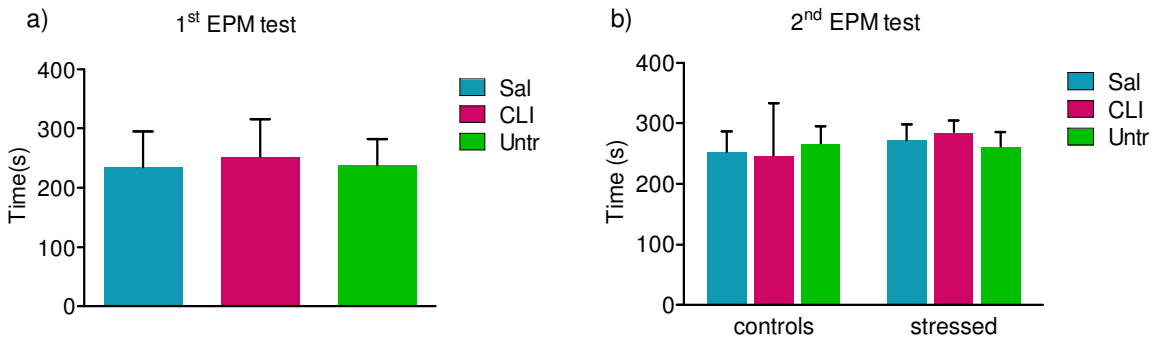

Fig. 4.5 - Time spent in closed arms in the a) 1st EPM test and b) 2nd EPM test among the experimental groups.

4.3.3 Assessment of depressive symptoms

4.3.3.1 Sucrose Preference Test

The chart in Fig. 4.6 shows the percent of sucrose consumption over the total liquid intake during the 2h test. Even if, in a certain degree, CLI animals of both control and

stressed groups showed a decrease in sucrose consumption, Univariate ANOVA did not show either treatment or stress effect.

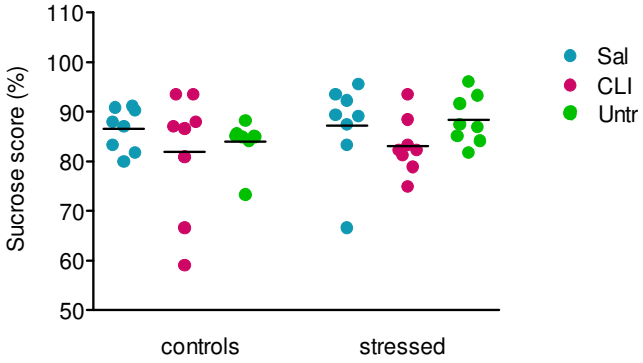

Fig. 4.6 - Sucrose preference score in experimental groups; sucrose score was calculated as sucrose consumption over the total liquid intake.

4.3.3.2 Forced Swim Test

In Fig. 4.7, graph a) reports the amount of time spent in immobility and graph b) the amount of time spent in climbing during the FST. From the control group, Untr animals showed an expected decrease in time spent in immobility, but statistical analysis showed no differences due to treatment. In the analysis of stress effect, no differences were found among groups in the amount of time spent in immobility. For what concern climbing activity, CLI animals spent less time than the other treatment groups in climbing activity and, in general, stress seem to basically affect the performance of Untr animals. Multivariate ANOVA showed treatment ($F_{(2,50)} = 14.868$, $p<0.001$) and stress ($F_{(1,50)} = 9.971$, $p=0.003$) effects. Furthermore a treatment*stress interaction ($F_{(2,50)} = 3.117$, $p=0.05$) effect was shown. Post-hoc analyses of climbing activity showed that the three treatment groups significantly differ from each other.

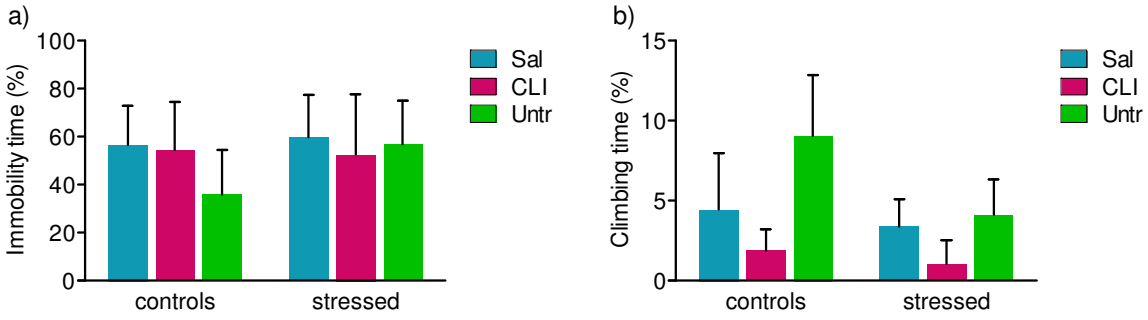

Fig. 4.7 - Time spent in a) immobility and b) climbing during the FST by the experimental groups of animals: time was expressed as percent of total test duration.

4.3.4 Neurogenesis study

Sacrifice of animals was executed in a period quite far from the one in which restraint stress was administered to animals, anywhere the investigation on a possible effect on neuronal proliferation was performed. The chart in Fig. 4.8 show DCX+ cell counts performed in 3 sections evenly spaced spanning the entire dentate gyrus of hippocampus of each animal. The chart shows that Untr animals exhibited the lowest number of DCX+ cells of all the treatment groups, and that stress seemed to affect treated animals but not Untr group. Multivariate ANOVA showed a significant effect of treatment on the number of DCX+ cells ($F_{(2,23)} = 6.127$, $p<0.01$), and post-hoc analysis (Bonferroni) showed that Untr animals had a significant lower number of cells compared to both Sal ($p=0.009$) and CLI ($p=0.05$) animals. Stress significantly decreased the number of cells in animals ($F_{(2,23)} = 23.716$, $p<0.001$)

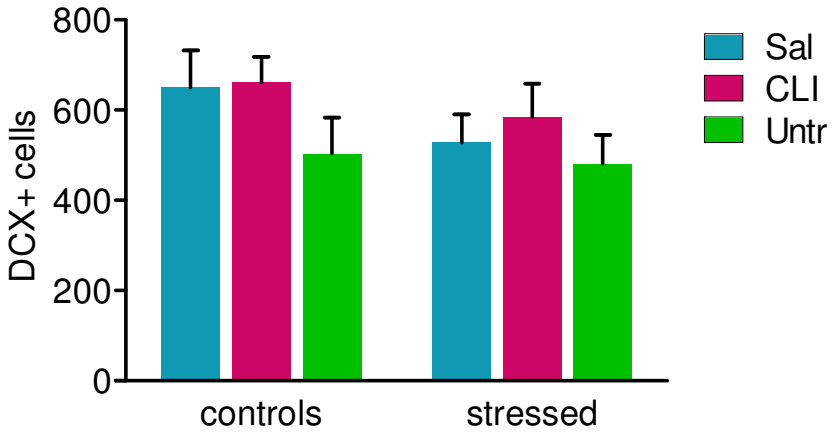

Fig. 4.8 - Number of DCX+ cells in the hippocampal subgranular zone of the experimental groups of animals.

4.3.5 SERT densitometry

In Fig. 4.9, representative microphotographs from SERT immunohistochemistry show qualitative distribution of the protein within the cingulate cortex, hippocampal CA2 and hilus, and the entorhinal cortex.

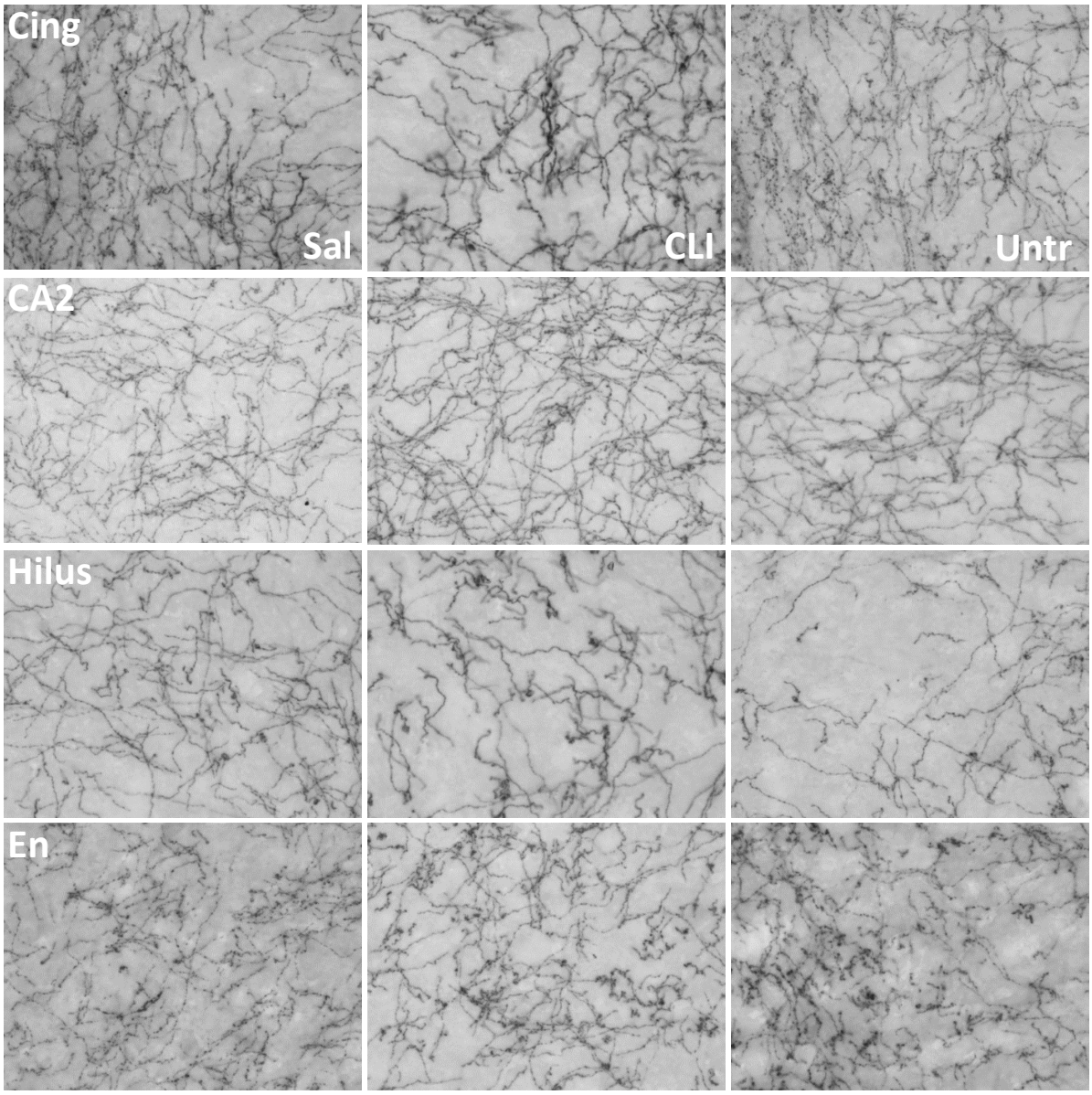

Fig. 4.9 - Representative microphotographs of SERT-labeled fibers from the different areas of each treatment group of animals are reported.

Graphs in Fig. 4.10 show quantitative SERT analysis in a) hippocampal hilus and b) cingulate cortex performed with densitometry. CLI animals show a reduction in SERT i-r in the region of hippocampal hilus and in the cingulate cortex the reduction in CLI animals seem to be potentiated by stress. Statistical results from Multivariate ANOVA reported in Table 4.2 revealed a significant treatment effect in both the hippocampal areas analyzed, hilus and CA2, and in cingulate cortex. As expected, no treatment effects were found in entorhinal cortex. Neither effect of stress nor stress*treatment interaction was found.

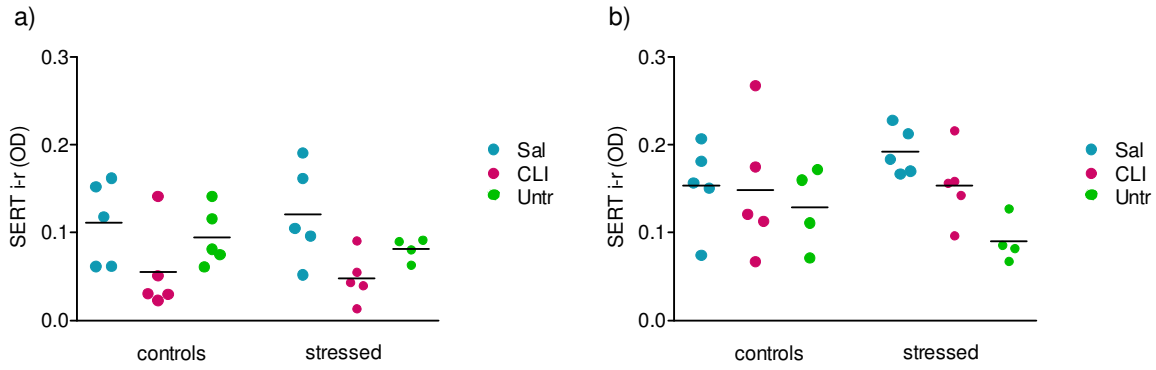

Fig. 4.10 - SERT i-r in hippocampal hilus and cingulate cortex of all animal groups. Post-hoc analysis of treatment effect (Bonferroni) showed significant differences in SERT i-r in hippocampal hilus between CLI and Sal animals (p=0.006) and in the cingulate cortex between Sal and Untr animals (p=0.035).

Brain region	Treatment		Stress		Stress*treatment	
	F	*p* value	F	*p* value	F	*p* value
Cingulate cortex	3.850	0.037*	0.009	0.925	1.364	0.277
Hippocampal CA2	8.018	0.002*	0.509	0.483	1.257	0.483
Hippocampal hilus	6.186	0.007*	0.167	0.687	0.339	0.716
Entorhinal cortex	1.302	0.292	0.002	0.961	0.820	0.453

Table 4.2 - Statistical results of Multivariate Anova of SERT i-r in different brain regions. N=28, 10 CLI, 10 Sal, and 8 Untr rats; 14 controls and 14 stressed animals.

4.4 Conclusions

Results from EPM test suggest an interaction between early life treatment and stress in adulthood. As in previous experiments, SPT did not show any significant effect of treatment and stress seemed not to influence anhedonic behavior, but results from FST confirmed the presence of despair behavior in CLI animals, with stress as an influencing factor.
Neurogenesis study confirmed preliminary results, showing no differences between CLI and Sal animals in the number of DCX-labeled cells; curiously, both CLI and Sal animals showed a higher number of cells compared to Untr animals, maybe for a compensatory effect from the early-life insult. The decrease of cell number in hippocampus after a stressful event has been already described in literature (Paizanis *et al*., 2007).
SERT expression was found to be affected by treatment in a region-dependent way: results obtained can be paralleled with those found by Weaver and colleagues ((Weaver *et al.*, 2010), who found a reduction in SERT expression in the hippocampus of rats neonatally treated with citalopram, suggesting the profound impairment in the neuro-

transmitter circuitry due to neonatal treatment with antidepressants. The absence of a stress effect I controversial if compared to the observations made in the neurogenesis study: it is still matter of discussion the effect that a stressful event can still exert in an epoch far from the sacrifice time on biological substrates such as cell proliferation and neurotransmitters' circuitry.
Ongoing analyses of locomotor activity in a novel enriched environment and Parvalbumin circuitry in specific brain areas are fundamental to complete the picture of the possible interaction between stress and treatment.

5 Discussion

The results of the investigations carried out and herein reported show that neonatal exposure to the tricyclic antidepressant clomipramine induce in adult rats a complex picture of behavioral and neurobiological abnormalities that could be correlated to a depressive syndrome or, in a simplistic way, to a 'neonatal antidepressant exposure syndrome', using the definition of Maciag (Maciag *et al*., 2006) to indicate the "pattern of maladaptive behaviors that are evident long after drug discontinuation and persist into adulthood" (cited from (Maciag *et al*., 2006).

Exposure to antidepressants has been reported to induce effects of different persistence and entity according to age of administration: adults exposed to antidepressant therapy exhibited no more behavioral effects after drug discontinuation; on the contrary, if women were cured during pregnancy and lactation with antidepressants for the treatment of affective disorders, their children showed signs of antidepressant withdrawal in the first weeks of life and some of them displayed behavioral abnormalities in motor development and control in their first few years of age (Casper *et al*., 2003; Sanz *et al*., 2005), influenced also by the gestational period and duration of treatment (Casper *et al*., 2011).

We did not perform a study on the effects of antidepressant treatment in rats during the period of administration or in the first days after drug discontinuation, but a longitudinal study at different ages from adulthood, through middle-age, until old age, was carried out, in order to assess the persistence of behavioral and neurobiological impairments. Vogel reported about a gradual, and maybe independent of live events, development of the set of behavioral abnormalities, with a peak at age 6-7 months followed by a spontaneous remission of the symptoms after several months (from age 10-11 months) from drug discontinuation, even if it was observed only in a subset of behavioral contexts such as REM sleep study and open field tests (Vogel *et al*., 1990a; Vogel *et al*., 1990b). Here we showed that neonatal clomipramine treatment significantly affected locomotor activity in the FST and self-reward behavior in the SPT in young animals (age 3-5 months), but CLI aged animals did not show any differences when compared to Sal aged animals (age 17-19 months). Furthermore, we conducted our behavioral analyses in an additional age group of animals, the middle aged-group (age 9 months), which may represent an intermediate phase between the early treatment effects and the late age recovery. The longitudinal study performed analyzed possible treatment effects also in contexts other than behavior such as volumetric examination and GFAP immunoreactivity of brain areas possibly involved in depression, and hippocampal cell proliferation. No more treatment effects were detected in hippocampal neurogenesis, of course significantly diminished in later life (Rao *et al*., 2005), and in PFC and hippocampal volumetric analysis in aged animals. GFAP densitometry revealed a treatment effect correlated with age in all brain areas analyzed: CLI aged animals showedan an increased GFAP i-r compared to SAL aged animals, but the opposite trend was found in young animals. While in CLI young animals a reduction of GFAP density in hippocampus was expected, because of the observations in depressed patients (Drevets *et al*., 2008) and animal models (Czeh *et al*., 2006; Gosselin *et al*., 2009), it is of difficult interpretation the trend found in aged rats: it is known that glia is highly activated in the old age (Unger, 1998; Middeldorp and Hol, 2011), but our results seem to indicate that early-life exposure to clomipramine potentiates astrocytic activation in later life, suggesting a rebound, maybe as a mechanism of recovery still in act.

Animals' behavior has been studied in different paradigms, evaluating despair and helplessness in the FST, anhedonia in the SPT, and anxiety trait in the EPM test, in both basal and challenging conditions. Despite several studies reported about a motor hyperactivity in CLI animals (Hartley *et al*., 1990; Andersen *et al*., 2002; Maciag *et al*., 2006), in our hands CLI animals, compared to Sal animals, showed a higher immobility and a lower climbing activity in the FST, as already observed by other authors (Velazquez-Moctezuma *et al*., 1993; Bhagya *et al*., 2008; Feng *et al*., 2008), and a lower distance moved assessed with the EPM test performed by stressed animals. It is not strange that contrasting results have been found in the evaluation of locomotor activity, considering that Hartley found that the performance of CLI rats differed in two distinct procedures of the open field test (Hartley *et al*., 1990). These observations cast some doubts on the predictability of tests for depressive symptoms, since behavioral characterization is biased by the procedure and by the test adopted. Results herein reported are comparable throughout the three studies performed anyway: CLI animals showed motor hypoactivity and no anhedonic behavior. For what concern the absence of anhedonic behavior, our results confirmed the observations made by Vogel (Vogel *et al*., 1990a). SPT and FST were useful not only to measure anhedonic and despair behavior respectively, but also to observe and evaluate animals' reaction to factor "novelty": all animals in a new context reacted in a certain degree and in a certain way. In the sucrose preference assessment, all animals, in the presence of two bottles of liquid, showed curiosity for the new setup of the cage environment, and showed an aggressive behavior when liquid and food were removed for the start of the 18h-deprivation period, trying to bite the experimenter. In the FST, animals, in both training and test sessions, vigorously struggle to escape from the cylinder during the first seconds in the water; only a very little group of animals, that included both Sal and CLI rats, when put in the water in the test session stayed immobile for the very first seconds, showing a sort of learned helplessness behavior, but maybe it was due to the previous experience of training. These observations may suggest that CLI animals are animals that preserve a reactive instinct as Sal animals when put in a challenging situation, and consequently this could be the reason why we did not find strong behavioral abnormalities. Coming to these considerations, we wanted to test the reaction of animals in extreme conditions, that were more stressful than the implicit stress that each behavioral test involves, and that could mime real life. We thought that laboratory animals are often too protected by operators from environment, with all that environment can includes: odors, noises, visual stimulators, handling carried out by more than one or two experimenters, sociality, predators and common dangers. If we want to build a valid animal model of depression, we possibly have to reproduce for animals conditions similar to daily human life, that is full of stressful events . In order to fairly study animals' reaction to a single stressful event, of simple and clean realization, we subjected animals to an acute event of restraint stress. As already described in section 4.1, restraint stress is a type of mild stressor, considered to be primarily a psychological stressor because it does not produce pain or direct physical insult (Girotti *et al*., 2006). Restraint stress was administered for a period of 4h in animals' home cage in order to reduce possible spurious stress due to a different environment. Animals, when put in the restrainer, tried to escape from the trap without success and then moaned during the first minutes of the stressful event. With a sense of giving up, then all animals spent time staying immobile. The EPM test carried out in basal conditions and after stress represented the ideal tool to assess, in a short period of time and without the need of training, the spontaneous behavior of animals. The investigation of anxiety trait in animals showed, in basal conditions, no differences due to early-life treatment with clomipramine in the several parameters evaluated, such as the amount

of time spent or entry frequency in open and closed arms or locomotor activity measured as distance moved on the maze. Animals spent the highest amount of time in closed arms, exhibiting a higher level of anxious than exploratory behavior, as previously described by Andersen (Andersen *et al.*, 2002; Andersen *et al.*, 2010). In the second session, rats that did not undergo acute stress showed no differences in the performance compared to the first session, as expected, while stress negatively affected locomotor activity of restrained animals, even if animals did not show differences in amounts of time spent in the different zones of EPM. CLI animals resulted the most affected by stress, with a lower locomotor activity compared to Sal and Untr rats. This result shows that stress may induce the emersion of behavioral correlates, such as hypoactivity, of a latent depressive syndrome. Other depressive symptoms were found in the behavioral assessment of the FST, with CLI animals that underwent restraint stress showing a decrease in climbing activity, another result that suggest a role for stress in extrapolating behavioral symptoms of depression, that in normal conditions are somehow hidden. The introduction of a third experimental group in Experiment 3 had its rationale in consideration of the need of a real group of control, for both the treatment condition and the early-life manipulation. Untr animals were bred as more as possible as "wild" animals, the only handling that they received was due to litter change and supply of food and water. Their body weight was not monitored during the period of treatment of CLI and Sal animals to avoid maternal separation even for some minutes per day. Thus Untr animals were not only animals that did not receive pharmacological treatment, they did not undergo handling, maternal separation, temporary environmental switching, and pain due to injections. All these factors are considered by many authors as stressful events that can affect normal development of animals if occurred during the first post-natal days (Sterley *et al*., 2011). In an unexpected way, our study became more complex when these remarks came out: were the symptoms found in adult animals realistically due to neonatal clomipramine treatment rather to a series of chronic stressful events during early-life? We cannot answer this question yet, because the experiment should have comprised one or more groups of animals to allow the evaluation of every single stressful factor that the reproduction of this animal model includes. It is widely known that neonatal manipulation, besides the way it is performed, has often reverberations on the normal development of both humans and animals.

About behavioral differences between females and males, the neonatal clomipramine model showed that females, in the two paradigms in which they were realted to males, that is to say FST and SPT, exhibited treatment effects, with CLI females, compared to Sal females, spending a larger amount of time in immobility and a lower amount of time in climbing in the FST, and consuming less sucrose in the habituation phase of the SPT. At the light of these observations, three hypotheses are possible: 1) female rats have a higher a predisposition than males to develop depression within the paradigm here reported; 2) female rats have a higher tendency to express depressive-like symptoms than males; 3) in this animal model of depression, females show a slower mechanism of recovery compared to males. We did not perform a longitudinal study for both males and females, but from our study, we learnt that females, observed at three different ages, have a gradual recovery from symptoms found in the young age. Unfortunately, we do not know anything about the recovery rate of males, thus we cannot cast further hypothesis on gender differences in this animal model of depression.

Neurogenesis studies performed were aimed at the investigation of a possible effect of early-life clomipramine treatment on hippocampal cell proliferation: neurogenesis is known to be decreased in stress-induced models of depression in rodents, as already said previously and, even if it has not been found decreased in postmortem stud-

ies in the brain of depressed patients (Stockmeier *et al.*, 2004), antidepressant treatment has been found to increase the number of newborn granule cells in rodents, as well as the number of progenitor cells in humans (Boldrini *et al.*, 2009). The 'neurogenic' hypothesis, although the few supporting data, is nowadays one of the guiding lines of research on depression. With the awareness that early-life administration of clomipramine is a validated method to induce depression in animals, and realizing that no authors up to now have ever published in literature any data describing cell proliferation rate, differentiation and survival in the hippocampus of CLI animals, we carried out an investigation that was aimed at the observation of neuronal proliferation in CLI animals compared to Sal animals. With BrdU immunostaining we could evaluate the proliferation rate of all cells in the hippocampus: in Experiment 2 it was found that the total proliferation rate in the dentate gyrus of the hippocampus of CLI animals, both females and males, was decreased. DCX+ cells, that represent the total number of neurons born in the last three weeks of animal's life, were not reduced in CLI animals. These observations suggest that even if total cell proliferation is decreased, the fraction represented by neurons is intact, but, paradoxically, it seems increased, if we consider results achieved in Experiment 3: animals that did not receive any manipulation in the early life, thus representing the real control group, showed a lower number of DCX+ cells compared to both Sal and CLI animals. Since no neurogenesis studies were performed before animals were 3 months old, thus no data about neurogenesis of the first days after the period of treatment are available, we can only hypothesize that a mechanism of rebound is in act when animals are in that age.

Morphological studies on DCX+ cells carried out with Neurolucida® in Experiment 2 showed that females exhibited, in the parameters herein measured, smaller neurons and in a certain degree neonatal clomipramine treatment induced in their cells an effect of decrease in the measure of somatic characteristics. Further, neonatal clomipramine treatment seems to affect the total dendrite length of neurons of females and males in an opposite way: total dendritic length of DCX+ cells of CLI females showed to be shorter than Sal females. Thus, even if all CLI animals presented dendrites with dystrophic features at a first inspection, only in females there was a quantitative confirmation.

The investigation of GFAP i-r, to evaluate if the decreased hippocampal cell proliferation is caused in part by a block of glial proliferation, as already observed in depressed patients (Drevets *et al.*, 2008), showed that astrocytic density was decreased in the hippocampal hilus of CLI young females, but not of males. As in the evaluation of behavioral results and neuronal morphology, CLI young females showed the effect of treatment occurred in the neonatal age, with all the regions analyzed affected, but CLI males did not exhibit the same results.

Imaging studies herein reported did not corroborate the observations of a decreased hippocampal volume described in humans (Bremner *et al*., 2000; Sheline, 2000; MacQueen *et al*., 2003; Neumeister *et al*., 2005), but the result of the enlargement of lateral ventricles in CLI animals, both females and males, maybe to the detriment of total brain volume, as in depressed, bipolar (Kempton *et al.*, 2011), and schizophrenic (Kempton *et al.*, 2010; Meduri *et al.*, 2010) patients, may suggest that, in depression, cell loss occurs not only in hippocampus but also in other brain areas, and in addition it could be not the only mechanism by which ventricular volume gets bigger.

Results from BDNF analysis, even if not novel (Cassano *et al.*, 2006) constitute one of the few issues in which male rats showed the effect of neonatal clomipramine treatment. The reduced levels of this important growth factor in the hippocampus, in-

volved in neuronal growth and differentiation, and associated with plasticity and survival of adult nerons and glia, is a confirmation, in this animal model, of the presence of some correlates of depression. The decrease in BDNF levels may be the cause of the qualitative morphological observations oof the presence of dystrophic features in hippocampal neurons of CLI rats. In light of the results above discussed, it could be interesting to perform an analysis of BDNF levels in females and in aged animals, in order to investigate gender and age differences in this context.

A very important result, that can be correlated to data in literature about a study on the neonatal citalopram administration in rats and the consequent undefined syndrome induced by treatment (Maciag *et al.*, 2006; Weaver *et al.*, 2010), is represented by the finding that SERT i-r is decreased in the hippocampus and cingulate cortex of CLI animals, two important regions of the limbic system, that is known to be particularly involved in the pathophysiology of depression. Early-life exposure to clomipramine may affect, as already said, brain circuitry of serotonin of an organism that is not able to respond because it has not enough defence mechanisms. Disruption of SERT circuitry in these regions consequently can induce an impairments in cognitive processes that are not strongly reflected in behavioral abnormalities in our model.

In conclusion, the present work provides evidence to some novel aspects of the neonatal clomipramine model of depression:

- neurobiological issues herein investigated gave important indications about the correlation of the effects of early-life exposure to clomipramine with depression: no authors have ever described in literature data about hippocampal neuronal proliferation and morphology, astrocytic and SERT density, and MRI volumetry of brain are all issues that;
- gender differences: no authors reported studies that involved both female and male rats. From behavioral to neurochemical investigations, significant differences were found about a major expression of correlates of depression in females than males, for reasons not clearly understood;
- remission of symptoms in the elderly age: besides behavioral investigations, that were not completely novel (Vogel *et al.*, 1990a; 2000)), astrocytic and neuronal investigations are novel important results that can further support the hypothesis of a mechanism of recovery that induce the remission of tha abnormalities observed;
- response to stressors, even if analyses of locomotor activity in the novel enriched environment are still ongoing, showed to be controversial and cast some doubts on the real effector of the totality of symptoms up to now described in this animal model, if it is the pharmacological treatment itself or the stressful events implicit in the treatment routine.

6 Acknowledgements

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7 Abbreviations

ACTH	adreno-cortico-tropin hormone
BBB	blood-brain barrier
BDNF	brain-derived nerve factor
BrdU	5'-bromo-2'-deoxyuridine
BrdU+	BrdU-labeled
CLI	clomipramine treated
CNS	central nervous system
CRF	corticotropin-releasing factor
CRH	corticotrophin releasing hormone
DCX	doublecortin
DCX+	DCX-labeled
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders lV edn. Text Revised
ECT	electroconvulsive therapy
EPM	elevated plus maze
FST	forced swim test
GFAP	Glial fibrillary acidic protein
GFAP i-r	GFAP immunoreactivity
HDACs	histone deacetylases
HPA	hypothalamic pituitary adrenal
IFN-α	interferon-α
IL	interleukin
MAOIs	monoamino oxidase inhibitors
MDD	major depressive disorder
MRI	magnetic resonance imaging
NPY	neuropeptide Y
OCD	obsessive-compulsive disorder
OD	optical density
PND	post-natal day
SD	Sleep deprivation
SPT	sucrose preference test
RSD	REM sleep deprivation
SAL	saline treated
SERT	serotonin transporter
SERT i-r	SERT immune-reactive
SSRI	selective serotonin reuptake inhibitors
TNF- α	tumor necrosis factor α
tPA	tissue Plasminogen Activator
TST	tail suspension test
WHO	World Health Organization

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