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Effect of antidepressants and adolescent stress on catecholamine transmission

in the rat bed nucleus of stria terminalis (BNST): a microdialysis study

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Abstract

Rationale: Antidepressants include a relatively wide spectrum of drugs that increase the synaptic concentration of monoamines, mostly through neurotransmitter reuptake blockade. The bed nucleus of stria terminalis (BNST) is considered a relay station in mediating the activation of stress response but also in the acquisition and expression of emotions. Chronic stress, particularly at developmental stage, has long-lasting deleterious effects on several cortico-limbic areas. Abnormal catecholamine transmission in the BNST evoked by chronic stress exposition at peri-adolescent age may be interpreted as relevant neurochemical marker at the onset of adolescent or adult depression. BNST is richly innervated by monoamines and sends back projections to the nucleus of origin. We previously showed that the administration of selective blockers of norepinephrine transporter (NET) increases the extracellular concentration (output) of dopamine, suggesting that dopamine could be captured by NET in the BNST. Among new experimental strategies investigated for the theraphy of depression, the treatment with sub-anesthetic doses of ketamine has been suggested to be one of the most promising.

Objectives: The aims of this study, carried out by means of in vivo microdialysis, were: (i) ascertain the acute effects that antidepressants with varying mechanisms of action have on dopamine and norepinephrine output in the BNST; (ii) ascertain the acute effects that sub-anesthetic doses of ketamine have on dopamine and norepinephrine output in the BNST; (iii) ascertain whether peri-adolescent unpredictable chronic stress could produce changes in dopamine and norepinephrine transmission in the BNST.

Results: We observed the following: (i) all the antidepressants tested (5-20 mg/Kg i.p.) increased the output of catecholamines, dose dependently; (ii) ketamine (10-40 mg/Kg i.p.) increased the output of catecholamines, dose dependently; (iii) peri-adolescent unpredictable chronic stress determined changes of basal and stimulated catecholamine output.

Conclusions: These results suggest that catecholamine transmission in the BNST may

be part of a common downstream pathway that is involved in the action mechanism of antidepressants, and in the effects of stress. Consequently, it is hypothesized that a dysfunction of neuronal transmission in this brain area may have a role in the etiology of affective disorders.

PREFACE

The content of this scientific report, is based on part of the three year experimental research activity performed at the Department of Biomedical Sciences, Section Neuropsychopharmacology of the University of Cagliari, for accomplishing the scientific track associated with the earning of the University of Cagliari Doctorate in Toxicology, under the tutoring of Prof. Ezio Carboni. The research activity of the Section of Neuropsychopharmacology, and in particular that carried out by Prof. Carboni, has been mostly focused on investigating the role of neurotransmission in the expression of the physiological brain activity as well as in the expression of effects of neuropsychiatric drugs and of substances of abuse. Thus, this research has been carried out by assessing levels of neurotransmitters in specific brain area, namely dopamine, norepinephrine, and serotonin, either in basal condition or under environmental challenges, or after the administration of drugs or substances of abuse. On the other hand, a substance of abuse as amphetamine, has been used for its toxic effects for exposing neurotransmission modifications that could be involved in physiological responses. Biogenic amines have been monitored in freely moving animals by the microdialysis technique or in brain areas dissected postmortem. The observed changes in the levels of biogenic amines have been discussed for improving the knowledge of brain circuitry and its involvement on the effects of drugs, abuse drugs, and toxic substances. In particular, this research has been focused on the study of the effect of different antidepressants on catecholamine transmission in the bed nucleus of stria terminalis (BNST), a brain area that has an important role in stress overseeing and therefore may be involved in the generation of depression and in the therapeutic effects of antidepressants. The data obtained have been compared with the effect of ketamine, a multifaceted drug that besides being a common veterinarian anesthetic drug is abused for its psychotomimetic effects, and surprisingly has been recently found capable to produce rapid antidepressant effect, although the mechanism of action of this effect is obscure. Furthermore, this research has investigated the effect of adolescent stress on catecholamine transmission in the BNST by using specific blockers of dopamine uptake or norepinephrine uptake such as GBR 12909 and reboxetine, or the psycho-stimulant amphetamine as tools to highlight neurotransmission changes that could be involved in the effect of chronic stress, and therefore in the etiology of depression. The results obtained by this research are discussed and presented together with a brief introduction on the catecholamine and

serotonin system, on the features and role of the brain area investigated, on the relationship of stress and depression, and lastly with a brief summary of the mechanism of action of the antidepressants investigated. Methods used have been also described.

INTRODUCTION

1. Stress

1.1. *The Concept of Stress*

The term "stress" includes a sequence of endogenous or exogenous unpleasant events that may alter the homeostatic balance of the organism. Upon stress exposition, our body triggers different responses in order to reestablish the lost/altered homeostasis. Just to name but a few, we can observe behavioral (alertness, vigilance, attention), vegetative (increased cardiovascular and respiratory activity, availability of energy, decreased activity of the gastro-intestinal system), and endocrine responses (production of corticosteroids and catecholamines). Stressing conditions trigger different stress responses, which varies depending on the individual ability to respond adaptively. Indeed, adaptive responses are characterized by a continuous physiological interaction between three basic systems involved in the stress response: hormonal, immune and nervous systems, as mentioned above. These systems are closely interconnected, and regulate each other by the means of feedback mechanisms. Moreover, cognitive stimuli that may have the features of stressful events, can trigger a stress reaction, and may be integrated in these cascade reactions. The adaptive response is intended to overcome the obstacles that may arise during life, promoting individuals survival: physiological responses associate with negative emotions serve to prepare to face rivals, or for the fight-or-flight reaction that occurs in response to harmful event, attack, or threat to survival. Besides, it is recognized as the first stage of a general adaptation syndrome that regulates stress response (Gozhengo *et al.*, 2009). In general, during physiological conditions, when the stressing stimulus is gone and the response occurred, the system reaches basal conditions again, thus these physiological responses have not any long term negative impact on our health if they remain transitory. However, sometimes, threatening situations are continuous, rather than episodic, producing a stress response more or less uninterrupted.

1.2. *Physiology of the Stress Response*

The hypothalamus-pituitary-adrenal (HPA) axis is the main coordinator of the body's metabolic and behavioral response to several stressful situations by means of feedback mechanisms. Various stressful stimuli (infection, trauma, inflammatory responses, psychological stress) can stimulate the production of corticotropin releasing factor (CRF) in the hypothalamus, followed by its entrance into the pituitary portal circulation. Cells in the anterior pituitary gland respond to CRF with the production of adrenocorticotropic hormone (ACTH), a single chain polypeptide resulting from pro-opiomelanocortin (POMC). In the cortical gland, ACTH activates the enzyme adenylate cyclase (AC) with consequent increase in intracellular cyclic adenosine monophosphate (cAMP) and activation of a specific protein kinase A (PKA). In turn PKA phosphorylate, activating, several enzymes, including an esterase releasing the precursor of corticosteroids cholesterol from intracellular stores where it is esterified, making it available for subsequent metabolism. Free cholesterol is transported into the mitochondrial membrane for the first reaction of the corticosteroids biosynthesis: the oxidation to pregnenolone by the cytochrome P-450. The adrenal gland can produce two different groups of corticosteroids, namely the glucocorticoids (cortisone, or corticosterone in rodents, and cortisol, active in carbohydrates metabolism) and the mineralocorticoids (active in electrolytes metabolism). Because of their lipophilic structure, glucocorticoids can easily cross the biological membranes and the blood brain barrier, therefore being the limiting factor of the feedback mechanism regulating HPA activity: the increase of circulating glucocorticoids also inhibits the synthesis and the effects of CRF and ACTH, causing a prolonged and rapid inhibitory effect on the production of ACTH. The rapid inhibition is probably due to mechanisms independent by protein synthesis, through which, by binding to specific membrane receptors, modulates neurotransmitter function. In addition, experimental studies have demonstrated how some steroidal compounds can alter neuronal excitability through interaction with certain receptors for neurotransmitters (Majewska *et al.*, 1986; Paul and Purdy, 1992). The prolonged inhibition is caused by different mechanisms of gene depression on both hypothalamic and pituitary level: inhibition of CRF synthesis, reduction of CRF pituitary receptors, inhibition of cAMP production stimulated by CRF, decreased levels of mRNA encoding for ACTH and POMC. These processes finely

regulate secretion of ACTH, and thus corticosteroids, by the balance between excitatory and inhibitory neuronal glucocorticoids which is essential for physiological homeostasis.

CRF and norepinephrine are, among the others, the most important brain mediators coordinating the stress responses of neuroendocrine, vegetative sympathetic, and immune systems. Due to its morphological and anatomical features, the sympathetic system has a prevalent physiological and suitable activity to prepare the body for strenuous startle responses induced by emergency situations, such as stress, danger, and/or fear. Norepinephrine is the main neurotransmitter released from postganglionic nerve terminals of the sympathetic system, while epinephrine is mainly produced by the adrenal medulla and released into the bloodstream. Further, norepinephrine is released in the brain as a neurotransmitter. Some of the behavioral and physiological responses produced by aversive stimuli appear to be mediated by noradrenergic neurons. For instance, microdialysis studies have shown an increase of norepinephrine release due to stressful situations in the hypothalamus, in the frontal cortex and in the basolateral forebrain (Yokoo *et al.*, 1990; Cenci *et al.*, 1992).

1.3. *Effect of Chronic Stress*

During chronic stress situations, the negative feedback regulating the HPA axis is impaired. Therefore, the overproduction of glucocorticoids may lead to modifications responsible for neuronal degeneration in the central nervous system (CNS), particularly in the hippocampus. In the CNS, stress causes cortisol excitotoxicity, altered calcium (Ca^{2+}) homeostasis, increased synthesis of free oxygen radicals (Sapolsky, 2000), and decreased expression of trophic factors, including brain-derived neurotrophic factor (BDNF) (Smith *et al.*, 2005). In depressed patients, a number of evidence showed HPA function alteration (Carroll *et al.*, 1976; Nemeroff, 1988) by means of increased blood stream levels of ACTH and cortisol (Arborelius *et al.*, 1999; Holsboer, 2000). Since these prominent features of stress exposure, these two conditions have been put in relation to each other. Highly stressful situations and events during childhood, adolescence, and throughout life, may have not only psychological, but also physical and biological consequences, representing risk factors

for the development of mood disorders (Brown *et al*., 1994). Depressed patients showed reduced coping strategies, normally aimed to promote the individual reaction, the domination of negative events, and the control of the emotional state. Evidences show also an increased pituitary volume and reduced functionality of glucocorticoids receptors, which would explain the inability of circulating cortisol to inhibit the HPA axis activity. Antidepressant administration normalizes the HPA function, bringing together the remission of symptoms (Heuser and Holsboer, 1992).

2. Depression

2.1. Definition

Major Depression, as classified by the "Diagnostic and Statistical Manual of Mental Disorders" (DSM-V), is a chronic disabling condition affecting the population each year with high incidence, accounting for 5-12 % and 10-25 % in men and in women, respectively, in a ratio of 1:2 (Kessler *et al.*, 1994). The symptoms of psychiatric illness concern different aspects, namely the affective-emotional (feelings of worthlessness and guilt), vegetative (sleep disturbances, lost, or in some cases increased appetite, alteration of the normal circadian rhythm activity), cognitive (impaired memory and concentration), and the psycho-motor activity (emptiness of mind). These abnormalities may lead to a mood tone reduction – long-lasting and independent from unpleasant events – and to decreased interest or pleasure in daily activities (anhedonia). A patient is considered depressed when a number of the symptoms mentioned above are observed for periods longer than 2 weeks, and when such symptoms prevent the patient from performing the normal social and employment functions. Although the etiology of depression is for the most part unknown, it is currently accepted that the combination of genetic and epigenetic factors can determine the appearance of the disease (Agam and Belmaker, 2008). Several lines of evidence argue that among epigenetic factors, exposure to chronic stress, severe emotional trauma, viral infections, adverse events occurring during development, have a major role in the etiology of depression.

2.2. The Biological and Psychological Bases of Depression: Neurochemical and Molecular Mechanisms

Many and sometimes controversial are the findings, and therefore the hypothesis, concerning the neurochemical and pathophysiological basis for the onset of depression. In the following, some of these theories will be described in detail.

- *2.2.1. The Monoamine Hypothesis*

The monoaminergic hypothesis suggests that depression is directly related to a decrease of the serotonin (5-H) and norepinephrine neurotransmission. In the past decades, a number of evidences also suggested abnormalities of the dopamine transmission.

The first real indication that depression represented the outcome of a functional disorder of the diffuse modulatory systems, occurred in 1960 as a direct result of studies originated from the casual observation that reserpine, a natural alkaloid used in the treatment of hypertension which works by inhibiting the vesicular storage of several neurotransmitters, reducing the synaptic monoamine availability, induced depressive-like symptoms. This suggestion was further supported by the evidence that the first two classes of drugs proved to be effective in the treatment of depression (e.g., tricyclic antidepressants and monoamine oxidase inhibitors), enhanced particularly the serotonin and norepinephrine neurotransmission. Based on these observations, the researchers developed the hypothesis that the mood tone regulation is closely associated with the monoamine levels in the brain issued, and in particular, that depression may be the consequence of a deficiency of one of these neurotransmitters. Nowadays, the increase of the serotonin and norepinephrine levels in the extracellular space, is the main mechanism of action of many drugs used in the treatment of depression.

Chronic treatment with antidepressants induces a progressive desensitization (down regulation), probably due to the increase of monoamine concentration in the synaptic cleft. This happens particularly on 5-HT_{1A} and 5-HT_7 auto-receptors localized onto presynaptic serotonergic neurons in the raphe nucleus, aimed to inhibit the terminal function (neurotransmitter release) (Ceglia *et al.*, 2004), and onto postsynaptic $5\text{-HT}_{2A/1D}$ hetero-receptors expressed in other brain areas (Syyalahti *et al.*, 2006). Down regulation of inhibitory presynaptic $5\text{-HT}_{1B/1D}$ receptors is also responsible for neurotransmitter release facilitation. Presynaptic receptors undergo a rapid down regulation, whereas postsynaptic receptors display a bigger/stronger resistance. Mechanisms of down regulation have been moreover described in the prefrontal cortex for α_2 noradrenergic presynaptic receptors (Invernizzi *et al.*, 2001; Parini *et al.*, 2005).

Indeed, the monoamine hypothesis is too simplistic to explain a very complex disease

like depression. Despite the high effective circulating levels of drug observed in the blood and in the brain already few days following the antidepressant administration, therapeutic effects can be appreciated only after few weeks. It should also be considered that not all the drugs facilitating the serotonin and norepinephrine transmission belong to the antidepressant category (the most striking examples are made from cocaine and other stimulant drugs), and that drugs with antidepressant activity does not significantly inhibit the monoamine reuptake. These observations lead to the hypothesis that an effective drug can alleviate depression producing adaptive long-term changes in the brain involving alterations of the gene expression.

- *2.2.2. The Neurotrophic Hypothesis*

The altered availability of other molecules normally involved in neuronal function has been thus taken into account. Additionally to the mentioned events (characterized by rapid onset), antidepressant drugs induce chronic long-term adaptive changes of post-receptor signaling cascades and of the subsequent gene expression. These modifications include the cAMP cascade leading to PKA activation, and other mechanisms including the activation of the phospholipase C (PLC), resulting in increased levels of intracellular Ca^{2+}, activation of Ca^{2+}/calmodulin kinase, and activation of Mitogen-Activated Protein (MAP) kinase. cAMP Response Element Binding (CREB) transcription factor has been implicated in many psychiatric disorders. Its role consists in the activation of the transcription of several genes, including those coding for BDNF and for its binding site tropomyosin receptor kinase B (Trk-B). CREB phosphorylation increases BDNF expression, thus promoting neuronal survival, function, trophism, neurogenesis and plasticity. By means of these mechanisms, antidepressants increase BDNF expression after chronic administration (not acutely), confirming that the time needed for the restoration of its proper production may, at least in part, explain the latency of their antidepressant effect.

It has been described that patients with mood disorders show reduced concentration of BDNF at both central and peripheral level (Altar, 1999; Chen *et al.*, 2001; Shimizu *et al.*, 2003) compared to healthy patients. The neuronal atrophy and dysfunction characterizing depression is confirmed by studies in depressed patients showing a decreased brain volume of some hippocampal (Sheline *et al.*, 1996) and cortical (Rajkowska *et al.*, 1999; Manji *et al.*, 2000) region. The treatment with different classes

of antidepressants (selective serotonin and norepinephrine reuptake inhibitors, monoamine oxidase inhibitors) can increase BDNF levels in the hippocampus of depressed patients (Chen *et al.*, 2001), moreover preventing the decrease of BDNF observed after the exposition to stressful stimuli (Smith *et al.*, 1995; Nibuya *et al.*, 1995).

- ## *2.2.3. The Diatesis/Stress Model*

The diathesis/stress model has been proposed such as psychological theory that considers separately the individual vulnerability and stress arose from life experiences (Monroe and Simons, 1991). The diathesis is intended as the predisposition to develop depression. The related stress response includes biological, psychological, and psychosocial mechanisms (Ingram and Luxton, 2005). The onset of depression is often precipitated by exposition to strongly negative aversive events (Jeronimus *et al.*, 2013; Willner *et al.*, 2013), or by persistence of chronic mild stressors (Harkness and Monroe, 2006). The interaction between the environment and risk genes, might explain part of the individual vulnerability (Krishnan and Nestler, 2008). For instance, children who have a family history of depression and who underwent to particular stressors would be more vulnerable to develop the disorder (Oatley *et al.*, 2006b). In agreement, is reported that negative childhood life experiences such as abuse and neglect, may represent strong risk factors (Kendler *et al.*, 2006; Willner *et al.*, 2013). The diathesis/stress model may be a useful tool to investigate how the interaction between biological and genetic traits (diathesis), and environmental events (stress exposure), can increase the probability to develop mental disorders such as anxiety or depression (Belsky and Pluess, 2009; Willner *et al.*, 2013).

2.3. *Drug Theraphy of Depression*

In general, antidepressants increase the synaptic concentration of monoamines as a consequence of the blockade of their neuronal reuptake. However, also drugs leading to the inhibition of the enzymes responsible for their degradation show the same effect. Among the more frequently prescribed drugs there are selective serotonin reuptake inhibitors (SSRIs), and selective serotonin and norepinephrine reuptake inhibitors

(SNRIs). Compounds with a mixed serotonergic activity, selective norepinephrine reuptake inhibitors (NERIs) and antidepressants specifically acting on dopaminergic transmission have been also synthesized. More in detail:

- *2.3.1. Triciclic Antidepressants (TCAs)*

The main mechanism of action of TCAs is the non-selective block of the monoamine reuptake from the nerve terminal, probably due to the competition for the membrane transporter for serotonin (SERT) and norepinephrine (NET) (Amara and Kuhar, 1993). The final result is the increase of the serotonin and norepinephrine level in the extracellular space. TCAs also block serotoninergic, α_1 adrenergic, H_1 histaminergic, and M_1 cholinergic receptors. These actions are responsible for many of the side effects of TCAs, and are the main limitation for their use in disease's therapy. Imipramine is the most representative drug belonging to this class; the molecule is converted in the liver in its major active metabolite desipramine. Desipramine is a rather selective NET blocker, whereas imipramine shows a higher affinity for SERT (O´Donnell and Shelton, 2011). Other drugs belonging to the TCAs category are chlorimipramine (highly selective for SERT in vitro, but more selective on NET in vivo, being demethylated into desmethylclomipramine), and amitriptyline (equally active on NET and SERT).

- *2.3.2. Selective Serotonin Reuptake Inhibitors (SSRIs)*

SSRIs specifically inhibit the serotonin reuptake through the selective SERT blockade. Unlike TCAs, SSRIs exert a weak effect on NET. Furthermore, SSRIs have no significant interaction on M_1 muscarinic, α_1 adrenergic, and H_1-histaminergic receptors, thus showing lesser side effects than TCAs. On the other hand, SSRIs can induce side effects mainly due to their action on the $5-HT_2$ receptor, namely sexual dysfunction, loss of libido in both males and females, insomnia and/or sedation, tremors in sensitive people. Moreover, SSRIs may induce nausea and vomit acting on peripheral $5-HT_3$ receptors regulating intestinal peristalsis. Compounds like fluoxetine, citalopram, its enantiomer escitalopram, fluvoxamine, paroxetine, and sertraline belong to this category.

- *2.3.3. Selective Norepinephrine Reuptake Inhibitors (NERIs)*

Reboxetine is the first NERI used in the treatment of depression. Doubts have been raised on its efficacy, regarding the possibility of inducing comorbidity (Eyding *et al*, 2010), namely the ability to induce anxiety in a patient who is depressed at the same time. However, pharmacological studies in laboratory animals show that the acute administration results in the increase of the norepinephrine extracellular concentration. Moreover, in the rat prefrontal cortex, where the norepinephrine and dopamine amount is enough for the NET binding competition, reboxetine also increase the dopamine extracellular concentration (Carboni *et al*, 2000).

- *2.3.4. Selective Serotonin and Norepinephrine Reuptake Inhibitors (SNRIs)*

SNRIs block both NET and SERT without inducing the same side effects of TCAs, showing no significant affinity for M_1, H_1, and α_1 adrenergic receptors. Venlafaxine and duloxetine belong to this category. Low doses of venlafaxine preferentially block the serotonin reuptake, while higher doses are required to block the norepinephrine reuptake. Duloxetine blocks preferentially the NET (the SERT blockade is exerted at higher concentrations). Moreover, it is effective in the treatment of depression accompanied by pain (the improvement of this symptom occurs much earlier than that on mood). Among the advantages offered by SSRIs administration there is their dose-dependent substrate affinity: low doses in anxious patients exert the antidepressant effect without inducing comorbidity.

- *2.3.5. Atypical Antidepressants*

The drugs belonging to this heterogeneous category exert their effect through several mechanisms of action.

Mianserin and mirtazapine (Specific Noradrenergic Antidepressants, NASSAs) are antagonists of the α_2 adrenergic receptor located in serotonergic (hetero-receptor) and noradrenergic (auto-receptor) presynaptic terminals, where it normally inhibits the neurotransmitter release. By blocking its function, NASSAs increase the concentration of both serotonin and norepinephrine, enhancing their release. Mianserin and mirtazapine show a similar molecular structure consisting of three carbonium rings (two of which benzenic), and a nitrogen atom responsible for the receptor binding. Mirtazapine is provided of an additional nitrogen atom in the first benzene ring,

conferring the ability to block also $5\text{-HT}_{2A/C}$ and 5-HT_3 receptors.

Trazodone and nefazodone are considered serotonin receptors antagonists, since they exert their effects blocking the 5-HT_{2A} receptor; low concentrations inhibit the serotonin and norepinephrine reuptake, showing few side effects.

- *2.3.6. Antidepressants Interacting with Dopaminergic Transmission*

The most interesting molecule belonging to this category is the amisulpride, an antipsychotic drug which effectiveness is highly dependent on the dosage. Low doses of amisulpride show a good efficacy by increasing the dopamine release via the blockade of presynaptic dopamine D_2 receptors.

Bupropion is a rather selective dopamine transporter (DAT) and NET inhibitor with insignificant SERT affinity (Shelton and Stahl, 2004). By its interaction with the dopamine transmission, bupropion can increase the activity status of a depressed subject without affecting the mood. However, its effect is mild, and therefore, it is considered a "niche drug", mainly used in patients less sensitive to the treatment with the most prescribed antidepressants, or when dopamine system activation is required.

- *2.3.7. Monoamine Oxidase Inhibitors (MAOIs)*

MAOIs include a number of drugs sharing the ability to block the oxidative deamination of biogenic amines, prolonging their presence in the synaptic cleft. Depending on their mechanism of action, MAOIs are divided into: (i) irreversible non-selective: phenelzine and tranylcypromine, which bind both MAO-A and MAO-B permanently, avoiding their interaction with the substrate; (ii) reversible and selective for MAO-A: moclobemide, which blocks the enzyme only temporarily.

Although MAOIs are probably as effective as TCAs, sometimes, severe and unpredictable interactions between MAOIs and biogenic amines introduced with the diet (e.g., tyramine) make their clinical use difficult and potentially dangerous. Therefore MAOIs, are used only when therapies with other antidepressants are ineffective, representing drugs of last choice in the treatment of refractory depression.

- *2.3.8. Other Therapeutic Approaches*

Several antidepressant targets not related to monoaminergic neurotransmission have been proposed (Holmes *et al.*, 2003; Berton and Nestler, 2006; Connolly and Thase,

2012). Among these, CRF, neuropeptide P, vasopressin, neuropeptide Y, and galanin, have been suggested as modulators of monoaminergic neurotransmission. Other mechanisms involving intracellular signal transmission pathways, neurotrophic factors, ligands for CB_1 cannabinoid receptors, cytokines (Berton and Nestler, 2006), have been also suggested. The agonist for melatonin receptor agomelatine has been introduced in drug therapy for its ability to block 5-HT$_{2C}$ receptor, although it shows the same limits in effectiveness and speed of action of traditional antidepressants (Kennedy and Emsley, 2006).

The last two decades have seen an important boost of the knowledge about glutamatergic transmission, opening new perspectives in the development of neuropsychiatric drugs. In humans, two of the most recent relevant results in the field of antidepressants are related to changes of the glutamatergic neurotransmission, namely the antidepressant effects of deep brain stimulation (DBS) (Mayberg *et al.*, 2005; Puigdemont *et al.*, 2012), and of the non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist ketamine (Zarate *et al.*, 2006).

- *2.3.9. Deep Brain Stimulation*

DBS resulted effective for the treatment of drug-resistance Parkinsonian patients and for other psychiatric conditions, including Major Depression (Laxton *et al.*, 2013). Neuroimaging studies have shown excessive activation of cortical areas in depressed patients, normalized after psychological (cognitive behavioral therapy), and pharmacological treatments either with SSRIs or with DBS (Seminowicz *et al.*, 2004; Mayberg *et al.*, 2005). Interestingly, in rodents, the antidepressant-like effect of DBS is antagonized by serotonin (but not norepinephrine) depletion, suggesting an important role for the serotoninergic transmission in the onset of the disease (Hamani *et al.*, 2010).

- *2.3.10. N-methyl-D-aspartate Receptor Antagonists*

NMDA receptor antagonists such as phencyclidine, ketamine, and MK-801, have been extensively used as pharmacological models of schizophrenia (Javitt and Zukin, 1991; Krystal *et al.*, 1994). Interestingly, ketamine, at doses evoking psychotomimetic effects, is also able to evoke rapid and persistent (within 2 hours and up to a week) antidepressants effects in depressed patients resistant to other forms of treatment

(Zarate *et al.*, 2006). The mechanism of this action is still unclear, but cellular and molecular studies revealed that ketamine remodels brain networks involved in the action of antidepressants through the activation of the mammalian target of rapamycin (mTOR) pathway (Li *et al.*, 2010).

- *2.3.11. Clinical Studies on Ketamine Effect*

A role for ketamine as a new strategy for the treatment of depression is suggested by a number of clinical studies (Mathews *et al.*, 2008; Skolnick *et al.*, 2009; McCormick *et al.*, 2010; Hashimoto, 2011). Several reports in humans have shown that intravenous infusion of a single sub-anesthetic dose evoked fast-acting and long-lasting antidepressant effects (Berman *et al.*, 2000; Mathew *et al.*, 2005; Maeng and Zarate, 2007), also in treatment-resistant depressed patients (Berman *et al.*, 2000; Zarate *et al.*, 2006). Although these studies have also revealed that the acute administration is not sufficient to maintain the antidepressant effect, needing patients repeated the treatment when they experienced relapse (Zarate *et al.*, 2006; Murrough *et al.*, 2013), some other indicated that repeated injections sustained the antidepressant action with few mild side effects (aan het Rot *et al.*, 2010).

- *2.3.12. Studies on Animal Models of Depression*

Studies on rats have shown that ketamine's antidepressant effects may depend on the rapid induction of synaptic proteins in the prefrontal cortex (PFC) and hippocampus (Garcia *et al.*, 2008; Li *et al.*, 2010). In the rat PFC, ketamine rapidly reversed the stress-induced deficit in spine density (Li *et al.*, 2011) by the activation of the mTOR signaling pathway (Li *et al.*, 2010; 2011; Duman, 2014). In line with these observations, ketamine produced antidepressant effects in several animal models of depression (Garcia *et al.*, 2009; Rezin *et al.*, 2009; Li *et al.*, 2011). Moreover, ketamine antagonism at NMDA receptor increased glutamate levels in the rat medial PFC (Moghaddam *et al.*, 1997). Acting on non-NMDA receptors, glutamate promoted cellular mechanisms leading to behavioral effects reversed by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonists (Maeng *et al.*, 2008; Zhou *et al.*, 2014). This is in accordance with studies suggesting that also AMPA receptors may be implicated in the antidepressant effect of ketamine (Naughton *et al.*, 2014), as well as the increase of BDNF translational mechanisms (Autry *et al.*, 2011)

and following synaptogenic processes (Jourdi *et al.*, 2009; Akinfiresoye and Tizabi, 2013).

3. Animal Tests and Models of Depression

3.1. Definition of Animal Models of Psychopathologies

Animal models are very useful experimental approaches for the study of phenomena occurring in humans. The aim of the development of animal models of disease is to recreate a human syndrome, or at least some of its aspects, in animals, in order to understand the mechanisms underlying symptomatology, or phenotype, identifying a suitable drugs target. Taking advantage of these models allows a better understanding of the behavioral changes even for very long time periods, as well as biochemical, molecular, and electrophysiological modifications.

3.2. Stress-Based Tests and Models of Depression

Some of the most common stress-based model of depression-like behavior are the forced swim test (FST), the tail suspension test (TST), and the learned helplessness (LH) paradigm (Seligman, 1975; Porsolt *et al.*, 1977; Sherman *et al.*, 1979). These tests show predictive validity for several classes of antidepressants acting on monoaminergic transmission (Porsolt *et al.*, 1977). Other experimental paradigms such as the chronic mild stress (CMS) reproduce important features of face and construct validity (Willner, 1997a; Willner *et al.*, 2005). Some of these paradigms are described in the following.

  Forced Swim Test. This test can be used to evaluate or to induce the depressive-like behavior. Briefly, for the FST, animal is placed in a bucket filled with water from which escaping is not possible, and after a certain amount of time some behaviors are taken as read-out. Normally, after an initial brief period of hyperactivity, animals assume a characteristic immobile position with no attempts to escape. The immobility has been associated with a situation of behavioral despair, and it is considered as a measure of depressive-like behavior (Porsolt *et al.*, 1977). Other behaviors like climbing or swimming can be assessed (Porsolt *et al.*, 1977; Pollack *et al.*, 2010). In agreement with this observation, it has been proved as many antidepressant drugs tend to reduce the immobility time, while active behaviors seem

to be differentially affected by acute administration of antidepressants. Climbing behavior is affected by TCAs and noradrenergic compounds, while swimming behavior is particularly sensitive to SSRIs (Detke *et al.*, 1995).

Tail Suspension Test. In the TST, animals are suspended in the air by their tails (Steru *et al.*, 1985; Thierry *et al.*, 1986) for a certain amount of time. The immobility time is considered as a measure of depressive-like behavior. Antidepressants, acutely increase active behaviors, reducing immobility time.

However, several studies show different responses of animals exposed to both paradigms (Mombereau *et al.*, 2004). In addition, other studies describe different responses after prior antidepressants administration, depending on the paradigm used (Cryan *et al.*, 2002) and on the strain of mice tested in the study (Liu and Gershenfeld, 2001). These paradigms are characterized by the application of an acute stressor, while depression involves long-lasting interactions. Furthermore, in human patients, the antidepressant treatment needs a consistent time to be effective, despite the positive outcome found after a single administration in both tests. Therefore these observations limit the relevance of FST and TST.

Learned Helplesness. The LH is a condition-based test in which animals are exposed to inescapable and unpredictable shocks during a training phase. On the day of the test, when escape is possible, their ability to escape the same aversive stimuli is evaluated (Sherman *et al.*, 1979). A decreased number of escape attempts, or increased escape latencies characterized "helpless animals". These responses are reverted by both acute and sub-chronic treatment with antidepressants (Duman and Monteggia, 2006). Moreover, helpless animals show also other features associated whit depressive human symptoms, such as reduced locomotor activity, loss of appetite and weight, impaired performance in self-stimulation paradigms (Willner, 1990).

Novelty-Suppressed Feeding. The novelty-suppressed feeding test assesses the anxiety-like behavior (and comorbidity with depression) by evaluating the latency to eat pellet chow into a brightly lit novel cage for animals which were previously food-deprived, thus evaluating the conflict between an anxious situation and a need to feed (Dulawa *et al.*, 2004). Moreover, the latency to approach and/or eat the pellet chow is decreased after chronic, but not acute antidepressant treatment (Santarelli *et al.*, 2003).

Social Stress. Many animal models belong to this class of tests. In general,

animals can be exposed to different types of social stressors, like for example, proximity to a dominant male, or to the smell of predators. Changes in animal behavior are then taken as read-out. In the social defeat model, for instance, after the daily fighting with a novel and physically superior aggressor rodent, animals develop a depression-like behavior characterized by anhedonia and social avoidance (Berton *et al.*, 2006) that can be reverted by chronic, but not acute, antidepressant treatment (Krishnan *et al.*, 2007; Schintu *et al.*, 2012).

Early Life Stress. These models are based on exposing the animals to different stressors during a very early stage of life. Animals separated from their mothers during the first days of life (Maternal Separation), or pups of mothers stressed during pregnancy (Prenatal Stress) show behavioral and molecular abnormalities that in some cases can be reverted by treatment with antidepressant drugs.

Chronic Mild Stress. Animals are repeatedly exposed to different unpredictable stressors, like immersion in cold water, inverted light/dark cycle, restraint, deprivation of food and water, in order to induce different neuroendocrine and behavioral responses. Indeed, after being exposed to one, or to all these stressors, animals may develop anhedonia-like phenotype, showing a reduced preference for sweet solutions (sucrose) or for pleasant conditions (Willner *et al.*, 2005; Jayatissa *et al.*, 2006). Anhedonia can be reversed by chronic, but not acute, antidepressant treatment (Willner *et al.*, 2005).

3.3. Chronic Mild Stress

Nowadays, most of the animal models available are based on the assumption that Major Depression can be caused or facilitated by chronic stress exposure (Willner *et al.*, 2013).

In earlier observations, Katz described a reduced intake of sweet fluids in rats subjected over a prolonged period to a variety of relative sever stressors, suggestive of a decreased hedonic state (Katz, 1982). In order to avoid adaptation and predictability of repeated exposure to the same stimulus, Willner and colleagues developed the chronic mild stress (CMS) paradigm, in order to study depressive-like behavior and the neurobiological and neurochemical basis of affective disorders (Willner *et al.*, 1992;

Willner, 1997a; 2005). A standard procedure to define the CMS is not really existing, and different terms have been used along time by different research groups (e.g., "chronic mild stress", "unpredictable chronic mild stress", "chronic variable stress") to describe essentially the same approach. All these procedures share the use of a variety of mild physical stressors, scheduled in a relatively unpredictable sequence over a period of several weeks, which can include cold water immersion, food deprivation, cage-tilt, changes in the dark/light cycle, with a daily variation of the stressful stimulus (Willner *et al.*, 1987). The phenotype of animals exposed to CMS, indeed, resembles aspects of depression in many features, although the CMS cannot model all the clinical aspects, like suicidal tendencies of depressed patients.

As mentioned, it has been reported that chronic exposure to a variety of mild unpredictable stressors causes a gradual decrease of consumption and preference for a palatable sucrose solution (Willner *et al.*, 1987). CMS also causes impairments in other measures of hedonic reactivity such as preference conditioning (D'Aquila *et al.*, 1997; Valverde *et al.*, 1997), having no effect on aversive place conditioning (Papp *et al.*, 1996). This "sub-sensitivity" to rewards is thought to be an analogous state of anhedonia, a core symptom of the human depressive disorder (Willner *et al.*,, 1987; Nestler and Carlezon, 2006). Several physiological and behavioral alterations accompany the exposure to CMS. Both hypo-activity (Gorka *et al.*, 1996; Grippo *et al.*, 2003) and hyper-locomotion (Gronli *et al.*, 2005; Mineur *et al.*, 2006) have been observed in the open field test, in rodents. Symptoms like loss of appetite and weight resembling those of depressed patients, have been also described (Willner, 2005; Perrine *et al.*, 2013). Sleep abnormalities (particularly, increased sleep fragmentation, longer REM periods and shift of the circadian rhythms phases) have been detected after exposure to CMS (Gorka *et al.*, 1996; Gronli *et al.*, 2004), as well as decreased sexual activity and aggressive behaviors (Gambarana *et al.*, 2001). Learning and memory impairment following CMS exposition have been also reported (Kim *et al.*, 2006). Numerous neurochemical changes such as increased plasma levels of corticosterone (Perrine *et al.*, 2013) and increased serotonin turnover in the hypothalamus (Cox *et al.*, 2011) have been induced by CMS. Most of the behavioral and neurochemical abnormalities can be reversed by chronic, but not acute, treatment with clinically effective antidepressants belonging to different classes (Moreau *et al.*, 1993; Papp *et al.*, 1996), and drugs known to be ineffective as antidepressants show no

efficacy, suggesting a high degree of predictive validity (Willner, 2005). Finally, while some study described anxiety-like (Maslova *et al.*, 2002), some other reported anxiolytic-like behavior following CMS exposition (D'Aquila *et al.*, 1994; Ducottet *et al.*, 2003).

- *3.3.1. Chronic Mild Stress Effect on Catecholamine Systems*

Strongest support on affection of catecholamine transmission has been provided by several observations. CMS-induced anhedonia is associated with decreases in dopamine D_2 receptor binding and message in the nucleus accumbens (Willner *et al.*, 1991; Dziedzicka-Wasylewska *et al.*, 1997). A decreased inhibition of dopamine turnover by quinpirole has been reported in anhedonic animals (Stamford *et al.*, 1991). CMS-induced anhedonia is also associated with an increase in cortical β-adrenergic receptor binding (Klimiek and Papp, 1994; Papp *et al.*, 2002), and in the number of β-adrenoreceptors in the hippocampus (Harro *et al.*, 1999), whereas a decrease in cortical β-receptor binding has been observed in animals showing decreased immobility in the forced swim test (Haidkind *et al.*, 2003).

- *3.3.2. Chronic Mild Stress Studies During Adolescence*

It is widely accepted that chronic stress during developmental stage has long-lasting deleterious effects on several corticolimbic structures. Although many studies have investigated the effects of chronic stress during adolescence [see the next chapter], no many reports elucidate specifically how CMS impact on the adolescent brain.

Toth and colleagues (2008), indicated that young adult males subjected to the CMS procedure during adolescence did not exhibit reduced sucrose preference, indicative of the anhedonic phenotype. Interestingly, they also found increased BDNF levels and neurogenesis in the dentate girus of the hippocampus. Other studies investigated alteration produced by exposition to adolescent chronic restraint stress (Eiland *et al.*, 2013), chronic variable stress (Isgor *et al.*, 2004; Oztan *et al.*, 2011a; 2011b), or other chronic stress paradigms.

4. Adolescence

4.1. <u>Definition</u>

Adolescence is a critical period for the development of the central nervous system (CNS). During this time, many processes take place leading to its full maturity. Among them, just to name but a few, there is the continuous myelination of nerve fibers, the increase in white matter volume, the progressive increase in gray matter volume, reaching a peak of density during early adolescence, and the strong proliferation of new synapses (Dahl, 2004a; 2004b; Steinberg, 2008). These events are followed by processes of reduction and rearrangement of synaptic contacts occurring at specific time-points for each area (Dahl, 2004a), that lead to the redefinition of the brain circuits that become functionally efficient. However, the brain development does not end with adolescence, but continues in adulthood, although with less overflowing events (Dahl, 2004a; 2004b; Steinberg, 2008). The brain regions involved in stress response and emotion, undergo profound structural and functional changes following events and/or stressful stimuli. Thus, the alterations induced by stress in adolescence, may contribute to an increased vulnerability and susceptibility for the development of psychopathologies in adulthood (Davey *et al*., 2008).

4.2. <u>Adolescence in Humans</u>

The adolescent brain is partially developed and strongly linked to emotions. The limbic system development happens earlier than cortical structures. Indeed, the frontal and the prefrontal cortex (PFC), involved in cognition, social functions, language, rationality, and regulating the reaction to emotions, reach the complete maturation after the adolescence period, around 25 years of age (Casey *et al*., 2002; 2005; Galvan *et al*., 2007). Therefore, the immaturity of cortical brain regions, and their inability to exert a control on primary impulses, could explain the prevalence of impulsive risky and emotional behavior during adolescence (Casey *et al*., 2005). Risky and impulsive behaviors in adolescents are associated with function of sub-cortical areas involved in the evaluation of reward, like the nucleus accumbens (NAcc). Several imaging studies,

show that NAcc activation is increased during risky decision making (Montague and Berns, 2002; Kuhnen and Knutson, 2005; Galvan *et al.*, 2006; 2007), and that its activation is bigger in adolescents, than in children, or adults (Ernst *et al.*, 2005; Galvan *et al.*, 2006). According to these observations, and in line with animal studies (Laviola *et al.*, 1999; Spear *et al.*, 2000), adolescence is characterized by the imbalance between the functional maturity of the limbic system and the immaturity of cortical regions. This condition is unique in adolescence compared to childhood, where limbic and cortical systems are both in the developmental phase, and to adulthood, where both systems are fully mature (Hare *et al.*, 2007a).

4.3. Animal Models of Adolescence

Adolescence is generally associated with the development of the reproductive function and the acquirement of survival skills allowing to the maturation and the independence from parental care (Spear and Brake, 1983; Spear, 2000; Laviola *et al.*, 2002). It is moreover characterized by a marked transition in cognitive, psychological and social development (Spear, 2000), accompanied by age-specific changes in spontaneous behavior (Spear, 2000; Laviola *et al.*, 2002). Several studies suggest that adolescent rodents exhibits peaks in behavior resembling that of human adolescents (Burke and Miczek, 2014). Adolescent behaviors include high novelty exploration (Stansfield and Kirstein, 2006) often associated to high risk-tasking behavior (Adriani *et al.*, 1998; Spear, 2000; Laviola *et al.*, 2003), hyperactivity, novelty-seeking (Adriani *et al.*, 1998; Laviola *et al.*, 2003) and social playful interaction (Cirulli *et al.*, 1996; Spear, 2000). Thus, animal models have been developed in order to study neurobiological and neurochemical features underlying this critical period of brain maturation, trying to correlate the age of small mammals with that of humans.

Pre-pubertal/post-weaning stage usually happens around 21st days of age (Eiland and Romeo, 2013). Puberty is considered as a stage of development characterized by marked hormonal and somatic changes, often occurring earlier in females (Sisk and Foster, 2004; Eiland and Romeo, 2013). Adolescence includes the phase of development that begins with the onset of puberty, and ends with behavioral and sexual maturity (Sisk and Foster, 2004). The specific age (expressed in postnatal days - PND) when a

rodent is considered an adolescent is variable and sometimes controversial, between experimental studies (Yetnikoff *et al.*, 2013). In the broadest sense, the time between PND 21 and PND 59 is considered as adolescence in rats (Laviola *et al.*, 2003; Tirelli *et al.*, 2003). More in detail, this time period can be further subdivided into three phases: (i) early adolescence (PND 21-34), also called juvenile period (Brenhouse and Andersen, 2011), (ii) mid-adolescence (PND 34-46), and (iii) late adolescence (PND 46-59) (Tirelli *et al.*, 2003). In line with this classification, other reports indicate that these stages correspond to early (10-14 years), middle (15-17 years), and late (18-21 years) adolescence in humans (Braet *et al.*, 2013; Burke and Miczek, 2014). Rats are considered young adults at PND 60, according to the final maturation of reproductive behavior (Damassa and Smith, 1977; McCormick *et al.*, 2007), although some studies suggest that some aspects of brain development continue after PND 60 (Brenhouse and Andersen, 2011). A more specific classification, namely peri-adolescence, has been proposed and validated by Spear and Brake in rodents (1983), referring to the period between PND 28-42 (Spear and Brake, 1983; Spear, 2000; Laviola *et al.*, 2002). The use of peri-adolescent rodents have been suggested as a useful strategy to study the risk factors involved in the etiology of behavioral disorders in human adolescents (Laviola *et al.*, 2002), including drug abuse, schizophrenia, and attention deficit with hyperactivity disorder (ADHD) (Laviola *et al.*, 1999; Cirulli and Laviola, 2000). Abnormalities of the HPA function as well as an impaired stress response, have been in fact correlated with a strong incidence for the development of these disorders in children (Kaneko *et al.*, 1993).

4.4. *Stress and HPA Function During Adolescence*

Stressful experiences during childhood and adolescence have been associated with the development of psycho-pathological disorders later in life (Kessler and Magee, 1993; Fumagalli *et al.*, 2007). The HPA axis transition over adolescence (McCormick *et al.*, 2008), has been hypothesized to render the adolescent more vulnerable to the effect of stress, and to the development of stress-related disorders (Toledo-Rodriguez and Sandi, 2011). The morphology of the paraventricular nucleus of hypothalamus (PVN), the expression of glucocorticoid and mineralocorticoid receptors, CRF mRNA levels, are

similar, compared to adulthood, by PND 21 (Romeo *et al.*, 2007), whereas other parameters continue to change over adolescence (for a more detailed review see McCormick *et al.*, 2010). Exposition to acute stress induced a higher release and prolonged high plasma concentration of corticosterone in adolescents, compared to adults (Romeo *et al.*, 2004; Cruz *et al.*, 2008; McCormick *et al.*, 2010). Some studies reported differences in the response to acute stress exposure, depending on animal age: the stress response was reduced during post-pubertal period, compared to pre-pubertal age and adulthood (Goldman *et al.*, 1973; McCormick *et al.*, 2008). On the other hand, no significant differences were observed after CMS exposition on the circadian release of corticosterone (Toth *et al.*, 2008). Fos immunoreactivity, considered as a marker of neuronal activation, was observed in the PVN of both adolescent and adult rats subjected to restraint stress (Kellogg *et al.*, 1998), however the same stress induced a widespread Fos induction in several brain areas of adult animals, but limited to the PVN in adolescent rats (Kellogg *et al.*, 1998). Moreover, age-related differences were observed in several anxiety tests (Genn *et al.*, 2003; Doremus *et al.*, 2004), reflecting abnormal function of the HPA (McCormick *et al.*, 2008). Adolescents and adults did not differ in stress-induced regulation of glucocorticoid receptor mRNA in the hippocampus after exposition to either acute or repeated stressors (Romeo *et al.*, 2008).

Moreover, studies focusing on the long-term effects of peri-adolescent stress exposition, and on its impact on HPA function in adulthood show controversial evidences also depending on the heterogeneity of the animal strain used for the experiments, the kind of stress applied, the time window chosen for the application and for the subsequent testing in adulthood. Importantly, another parameter to take into consideration is the gender. The studies mentioned above were performed on adolescent and adult males, but studies on females are progressively increased over the years because both androgens and estrogens hormones play a fundamental role in the development and differentiation of the adolescent brain (Seeman *et al.*, 2001).

4.5. *Chronic Stress Effect on Dopamine System During Adolescence*

Experimental paradigms used to examine the effects of chronic stress, particularly in rodents, have commonly employed social defeat (repeated exposure to a dominant

aggressor), isolation, restraint, or exposure to aversive odors or environments. Among changes described on dopamine neurotransmission in the PFC in adulthood, have been reported decreased basal dopamine levels, decreased D_2 receptor expression, increased DAT binding, increased MAO-A gene expression, and increased MAO-A promoter histone acetylation (Watt *et al.* 2009; Novick *et al.*, 2011). In the striatum have been reported increased D_1 receptor binding (Novick *et al.* 2011), and abnormal behavior, such as increased aggression and anxiety-like behaviors (Wright *et al.* 2008). Chronic social defeat applied at PND 35-40 also altered responses to amphetamine, resulting in increased locomotion, decreased corticosterone secretion, decreased medial PFC dopamine levels, increased NAcc core dopamine levels, and impaired D_2 receptor down regulation in the NAcc core (Burke *et al.* 2010; 2011; 2013). These findings suggest that chronic stress exposition during adolescence may detrimentally impact on the developmental trajectory of dopaminergic circuits, leading to long-term molecular and behavioral maladaptations (Burke and Mikzek, 2014).

4.6. *Monoamine Systems Development During Adolescence*

Changes in dopamine tissue concentration are related to the density of dopamine fibers (Wahlstrom *et al.*, 2010). Dopamine innervation of the medial prefrontal cortex (PFC) showed and enduring and progressive increase throughout adolescence, until post natal day (PND) 60 (Kalsbeek *et al.*, 1988). In particular, dopaminergic activity seems to growths from early to mid-adolescence (Burke and Miczek, 2014), probably because of a greater dopamine synthesis and turnover in the latter stage (Teicher *et al.*, 1993; Andersen *et al.*, 1997a). Thus, the high density of dopamine fibers observed in early adolescence in the PFC (Spear, 2000) may be balanced later in life by a decline in dopamine synthesis (Andersen *et al.*, 1997) and/or dopamine turnover (Teicher *et al.*, 1993). In the nucleus accumbens (NAcc) and in the dorsal striatum, dopamine levels increased particularly between PND 28-42 (Andersen and Gazzara, 1993). However, Mathews and colleagues (2009) found that tyrosine hydroxylase levels were higher in adulthood than adolescence in the NAcc core, although not in striatum. Studies investigating the density of the dopamine transporter (DAT) as index of dopamine innervation, revealed its higher expression until PND 21, compared to adulthood, in the

anterior striatum, ventral tegmental area, substantia nigra and bed nucleus of stria terminalis (Coulter *et al.*, 1996; Spear, 2000). Similarly, Tarazi and colleagues (1998a) reported that DAT increased rapidly until PND 21, then gradually until PND 35, in the NAcc and striatum, suggestive of an earlier maturation of striatal and accumbal dopamine fibers, compared to cortical (Burke and Miczek, 2014).

Scattered evidence supports the idea that also the serotonin system undergoes developmental alterations during time period subsuming adolescence (Spear, 2000). It has been suggested that abnormalities of serotonin transmission in the PFC may be responsible for impulsive behavior (Adriani *et al.*, 2003; Cardinal *et al.*, 2004; Staiti *et al.*, 2011).

5. *Brain Area Investigated*

5.1. *Extended Amygdala*

The so called complex of the extended amygdala (ExtA) is a large basal forebrain macrostructure encompassing contiguous and heterogeneous subnuclei, interconnected and functionally related between each other, extending from the centromedial amygdala to the bed nucleus of stria terminalis (BNST) (Alheid, 2009). The ExtA is thus composed by the BNST, the substantia sublenticolare, the central nucleus of the amygdala (CeA) and the intercalated masses. The CeA is extended in the shell of the nucleus accumbens (NAcc), a transition area occupying the ventral part of the striatum (Alheid and Heimer, 1988; Heimer *et al.*, 1991).

The ExtA nuclei and its interconnections allow the integration of inputs from different sensory systems, regulating the analysis of both internal and external information, thus providing an interpretive and emotional context for the initiation and control of appropriate behavioral and emotional responses related to motivation (Di Chiara, 2002).

5.2. *Bed Nucleus of Stria Terminalis*

BNST is the site where converge information from brain regions associated with the stress response, and the control of emotional, cognitive, autonomic, and behavioral functions. In particular, BNST plays an important role in the regulation of the HPA axis activity (Choi *et al.*, 2007; 2008), since it is the largest station between the paraventricular nucleus of the hypothalamus (PVN), the amygdala and the hippocampus (Forray and Gysling, 2004). It is also suggested a role for BNST as a key mediator of the interaction between stress and addiction, by virtue of the reciprocal innervation with the ventral tegmental area (VTA) (Dong and Swanson, 2004), that is crucial for the expression of behaviors related to motivational state, reward, and relapse linked to negative and/or stressful events. The involvement of BNST in mechanisms associated with fear, anxiety, post-traumatic stress disorder (PTSD) has been widely reported.

BNST receives glutamatergic inputs from cortical, thalamic and amygdala regions, GABAergic inputs from the amygdala, and modulatory inputs from brainstem and hypothalamic regions, projecting back to many of these areas (Kash, 2012). BNST neurons are known to be mainly GABAergic (Sun and Cassell, 1993), expressing neuropeptides, namely CRF and neuropeptide Y, exerting opposite effects in the influence of neuronal signaling and behavior (Heilig, 2004; Gafford *et al.*, 2012). The presence of these neuropeptides in the BNST supports the involvement of several neuronal population differently engaged by stress or drugs of abuse (Day *et al.*, 1999; Kash, 2012).

- *5.2.1. Noradrenergic and CRFergic Inputs to the BNST*

A high density of noradrenergic fibers has been found in the BNST (Phelix *et al.*, 1992; Freedman and Cassell, 1994). Noradrenergic terminals originate mainly from cell bodies of neurons expressed in the brainstem areas A1 and A2 (Riche *et al.*, 1990; Woulfe *et al.*, 1990; Roder and Ciriello, 1994; Forray *et al.*, 2000), the same cell groups responsible for the innervation of the PVN, the CeA, and to a lesser extent the medial nucleus of the amygdala (Riche *et al.*, 1990; Roder and Ciriello, 1994; Xu *et al.*, 1999; Wong *et al.*, 2000). Several scientific evidences show that repeated exposure to stress (Pardon and Morilak, 2003), as well as the removal of the noradrenergic input to the BNST (Banihashemi and Rinaman, 2006), or specific lesions (Herman *et al.*, 1994), produced alterations of the HPA axis function and of the regulation of anxiety state, leading to a deregulated stress response.

The anterior-lateral portion of BNST contains a high density of CRF-expressing neurons (Moga *et al.*, 1989; Phelix and Paull, 1990) and receptors besides CRH_{1-2} (Chalmers *et al.*, 1995). CRF (and GABAergic) projection arise mainly from the CeA (Forray and Gysling, 2004).

- *5.2.2. Serotoninergic Inputs to the BNST*

BNST receives serotoninergic inputs from the dorsal raphe nucleus (Phelix *et al.*, 1992). Uncontrolled stress, and anxiogenic drugs, have proved to be able to activate subclasses of dorsal raphe neurons targeting limbic regions such as the BNST (Hammack *et al.*, 2009). In line with this, several studies show that serotonin receptors located in the BNST can modulate anxiety behavior in rodents. Inhibition of

5-HT$_{1A}$ receptor in the BNST would have an anxiolytic effect (Levita *et al.*, 2004). In contrast, activation of excitatory receptors 5-HT$_{2A,2C,7}$, would have the opposite effect (Guo *et al.*, 2009). In 5-HT$_{2C}$ receptor knockout mice has been observed reduced stress-induced activation of CRF neurons in the BNST. Immunohistochemical studies also suggested that 5-HT$_{2C}$ receptors can regulate the activity of these neurons (Heisler *et al.*, 2007). In addition, the serotonin transporter availability in the BNST and amygdalo-hippocampal areas, seems to be positively correlated with individual differences in anxiety-like behavior (Oler *et al.*, 2009).

- *5.2.3. Dopaminergic Inputs to the BNST*

BNST is surrounded both laterally (caudate putamen/globus pallidus) and dorsally (nucleus accumbens shell) by dopamine-rich areas. In particular, the dorsal-lateral BNST is heavily interconnected with the VTA (Dong and Swanson, 2004), moreover receiving dopaminergic inputs from the A10 cell group located in the periacqueductal grey area (Hasue and Shammah-Lagnado, 2002; Meloni *et al.*, 2006). Multiple drugs of abuse share the property to increase dopamine transmission in the BNST (Carboni *et al.*, 2000). Recent studies from Park and colleagues (2012a; 2012b) have shown that dopamine release in the BNST follows natural rewards and predictive cues exposition associated with intra-cranial self-stimulation of the medial forebrain bundle.

- *5.2.4. Glutamatergic Inputs to the BNST*

BNST receives glutamatergic projection from the prefrontal cortex (PFC). These excitatory inputs arise mainly from the caudal portion of the infralimbic and prelimbic cortices, but also from the insula and the enthorhinal cortex, and from the caudal orbital PFC (Mc Donald, 1998). Other excitatory inputs include projections from the hippocampus via the ventral subiculum, and from the basolateral amygdala (Cullinan *et al.*, 1993; Mc Donald, 1998). Recent studies confirmed these important interactions, demonstrating that anterolateral BNST relays information from the medial PFC and the hippocampal formation, thus inhibiting the HPA axis activity (Radley and Sawchenko, 2009; 2011). Thus, BNST seems to play a crucial role for the regulation of anxiety behavior and HPA function.

- *5.2.5. BNST Projections to the Ventral Tegmental Area*

BNST sends mainly GABAergic (Kudo *et al.*, 2012), but also glutamatergic projections to the VTA (Cullinan *et al.*, 1993; Georges and Aston-Jones, 2001; Jalabert *et al.*, 2009). The target of these projections are GABAergic neurons (Kudo *et al.*, 2012; Jennings *et al.*, 2013), in turn responsible for a robust inhibition of VTA dopamine neurons (van Zessen *et al.*, 2012). Thus, GABAergic and glutamatergic BNST projections exert, respectively, an indirect excitatory and inhibitory role on dopamine neurons. Jennings and colleagues (2013) showed moreover that activation of BNST GABAergic terminals in the VTA has a rewarding and anxiolytic effect, whereas the activation of BNST glutamatergic terminals is aversive and anxiogenic.

- *5.2.6. Other BNST Efferents*

The lateral hypothalamus, an area involved in the regulation of drug-seeking behavior (Aston-Jones and Harris, 2004), receives as well a robust innervation from the BNST (Dong and Swanson, 1994). As previously mentioned, another BNST target is the PVN (Swanson and Sawchenko, 1983), a key brain region for the regulation of the HPA axis function. The PVN receives GABAergic and CRFergic inputs from the BNST (Roland and Sawchenko, 1993; Dong and Swanson, 2006). Based on the role of CRF signaling within the PVN in the maintenance of the HPA axis homeostasis (Rivier and Lee, 1994; Laorden *et al.*, 2000; Rotllant *et al.*, 2007), several reports indicate that stressful and abuse conditions could increase the BNST CRFergic drive onto PVN CRF neurons (Stamatakis *et al.*, 2014), confirming its role in stress and aversion related behavior.

6. Neurotransmitters Systems

6.1. *Catecholaminergic Transmission*

The term "catecholamine" refers to a group of substances of low molecular weight, sharing a common chemical structure that consists of an aromatic ring with two hydroxyl side groups in position 3 and 4, and a side-chain of ethylamine. Norepinephrine and dopamine primarily act as neurotransmitters in the peripheral and central nervous system (CNS), while epinephrine is highly localized in the adrenal medulla, where it is released from the bloodstream to act as a hormone in anger, fear and stress.

- *6.1.1. Biosynthesis*

The starting point for catecholamine synthesis is the availability of the essential aminoacids phenylalanine, and tyrosine. After a series of enzymatic steps they are converted into dopamine, norepinephrine, and finally epinephrine. Introduction of tyrosine with diet is sufficient for catecholamine synthesis; however, even phenylalanine can be converted into tyrosine by the enzyme phenylalanine hydroxylase. Tyrosine is transported into catecholaminergic neurons by a specific transporter. The first step of catecholamine synthesis takes place in the cytosol of the nerve terminal and in the chromaffin cells, where tyrosine hydroxylase (TH) catalyzes the hydroxylation of the tyrosine´s phenol into dihydroxyphenylalanine (DOPA). The speed of this reaction, however rather slow, represents the limiting factor of catecholamine synthesis, and is under tight control of neuronal activity. DOPA does not accumulate into catecholaminergic neurons, as it is rapidly converted into dopamine by a non-specific cytosolic DOPA decarboxylase (also called aromatic amino acid decarboxylase). Dopamine is then transported into the synaptic vesicle by specific vesicular transporters for monoamines. Within the vesicles of adrenergic and noradrenergic neurons, and chromaffin cells of the adrenal gland, is highly localized dopamine-ß-hydroxylase, the enzyme converting dopamine into norepinephrine. In the cytosol of chromaffin cells and adrenergic neurons, norepinephrine is finally methylated into epinephrine by the enzyme phenylethylamin-N-methyl transferase.

- *6.1.2. Regulation of Catecholamine Synthesis*

Catecholamine content in the sympathetic nervous system and in the adrenal gland remains fairly constant even under marked changes in activity. This is due to the regulation of catecholamine biosynthesis, release, and responsiveness. Fast adaptive changes of catecholamine synthesis take place in minutes, whereas slower changes occurs over hours and/or days. Rapid catecholamine regulation is directed mainly on the enzyme TH, that is inhibited by the presence of free catecholamines in the cytosol. Thus, with decreased nerve activity, catecholamine cytosolic levels are reduced, and TH inhibition is removed. The higher levels of more hydroxylated tyrosine lead to an increased catecholamine synthesis. Changes in phosphorylating conditions appear to rapidly regulate TH activity. Increased nerve activity over long period of time, is accompanied by an increase in the amount of TH and dopamine-ß-hydroxylase.

- *6.1.3. Vesicular Storage and Release*

Into the nerve terminal and chromaffin granules, catecholamines can be free in the cytosol or stored into synaptic vesicles, where they can either be free or bounded to macromolecular complexes. Macromolecular complexes reduce the free neurotransmitter concentration within the vesicle, with advantage for the efficiency of vesicular transporter that uses the pH gradient between vesicle and cytosol created by a vesicular proton pump consuming ATP as an energy source.

Upon a suitable stimulus, catecholamines are released into the synaptic cleft after presynaptic membrane depolarization, increased cytosolic Ca^{2+}, and fusion of synaptic vesicles with the presynaptic membrane. At this point, neurotransmitters act on postsynaptic cells binding to specific receptors that mediate the biological response. These actions present limited duration. When neurotransmitter level reach high concentration in the sinaptic space, further release is inhibited by presynaptic receptors expressed on nerve terminal.

- *6.1.4. Signal Inactivation*

Catecholamines released in the synaptic space are inactivated through two main mechanisms: reuptake into the nerve terminal, and metabolism by action of the enzymes monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT).

- *6.1.5. Catabolism*

The main routes of catecholamine degradation are represented by oxidative deamination operated by MAO, and COMT. The oxidative deamination exerted by mitochondrial MAO is the major norepinephrine and epinephrine metabolic pathway into the nerve terminal. The correspondent aldehydes are in turn rapidly converted into the corresponding carboxylic acids or alcohols, and subsequently O-methylated by COMT action, with the final conversion into 3-methoxy-4-hydroxyphenyletanol acid (VMA) or 3-methoxy-4-hydroxyphenylglycol acid (MHPG). One of the dopamine metabolic pathways involves the deamination by means of the MAO and subsequent oxidation into dihydroxyphenylacetic acid (DOPAC). The latter metabolic COMT substrate is converted into homovanillic acid (HVA).

- *6.1.6. Reuptake*

The major mechanism terminating catecholamine effects is their reuptake into the nerve terminal. This mechanism can remove from the extracellular space about the 80% of neurotransmitter released after depolarization. Reuptake is an active, saturable, and Na^+-dependent process, due to specific membrane transporters expressed at presynaptic level, specific for their neurotransmitter. The specificity is imposed by their expression, that is typically cell-specific: noradrenergic neurons selectively express the NET, while dopaminergic neurons express the DAT. However, the NET has an affinity for dopamine four times higher than that for norepinephrine, thus increasing dopamine extracellular concentration in the prefrontal cortex of rats treated with drugs that selectively block the NET (Carboni *et al.*, 1990).

6.2. *Catecholamine Receptors*

After catecholamine release from either the nerve terminal or the adrenal gland, they bind specific receptors located on the surface of the effector cells. Depending on the nature of the receptor, this interaction triggers membrane and intracellular cascade events leading the cell performs its specific functions. Catecholamine receptors belong to the superfamily of G-coupled protein receptors, characterized by an extracellular N-terminal, followed by seven transmembrane α-helices, connected by three intracellular

and three extracellular loops, and finally an intracellular C-terminal. The third cytoplasmic loop and the COOH terminal portion are crucial for recognition of specific G-proteins. G-coupled protein receptors mediate slow responses, and their system is subject to considerable amplification of the signal, involving second messengers mediating the multiple responses of target cells. Catecholamine receptors are classified into adrenergic (for epinephrine and norepinephrine) and dopaminergic receptors.

- *6.2.1. Dopaminergic Receptors*

Dopamine receptors are divided into two main families: D_1-like family including D_1 and D_5 dopamine receptors, and D_2-like family that includes D_2, D_3 and D_4 dopamine receptors. D_1-like receptors activate stimulatory G proteins, whereas D_2-like receptors activate inhibitory G proteins (Beaulieu and Gainetdinov, 2011).

The activation of $G\alpha_{s/olf}$ by D_1-like receptors stimulates adenylate cyclase (AC), that induces cAMP production leading to protein kinase A (PKA) activation. PKA generates the cellular signaling cascades necessary for long-term plasticity, and also induces dopamine and cAMP-regulated neuronal phosphoprotein (DARPP-32) phosphorylation, which inhibits protein phosphatase-1 (PP-1). Inhibitory interactions between DARPP-32 and PP-1 regulate neural plasticity through extracellular signal-regulated kinase (ERK) pathways (Valjent *et al.*, 2005). The activation of $G\alpha_{i/o}$ proteins on D_2-like receptors inhibits AC, regulating cAMP activity.

The phospholipase C (PLC) pathway can be induced by activation of both D_1 and D_2 receptors. The subsequent production of inositol 1,4,5-triphosphate (IP-3) and diacylglycerol (DAG) induce increased Ca^{2+} levels leading to activation of protein phosphatase-2B (PP-2B) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) signaling cascades that have been identified as regulators of long-term plasticity (Valient *et al.*, 2005).

D_2 receptors are expressed presynaptically as well as postsynaptically. D_2 receptors located on the soma and dendrites of dopaminergic cells act as auto-receptors, decreasing firing frequency, whereas D_2 receptors located on nerve terminals reduce dopamine synthesis and release. In the CNS, dopamine receptors are widely expressed because they are involved in the control of locomotion, cognition, emotion, affecting as well as neuroendocrine secretion (Missale *et al.*, 1998). Dopamine receptors are expressed in regions that receive dopaminergic innervations.

Commonly, the most expressed subtypes are the D_1 and the D_2 receptors. In sub-cortical areas, their expression is approximately equal, whereas in the cortex the D_1 type expression is prevalent (Tritsch and Sabatini, 2012). D_3 receptors are predominantly localized in limbic regions. D_4 and D_5 receptors are expressed in both cortical regions and limbic areas (Tritsch and Sabatini, 2012).

- *6.2.2. Adrenergic Receptors*

Adrenergic receptors are classified into two distinct classes, namely α- and β-adrenoceptors, further subdivided into subgroups (α_1 and α_2; β_1, β_2 and β_3).

α_1 adrenergic receptors are coupled to $G_{q/11}$ proteins, thus responsible for mobilization of cytosolic Ca^{2+} and stimulation of phosphatidyl inositol (PI) turnover. α_1 adrenergic receptors are widely distributed in the CNS (Nicholas *et al.*, 1996), predominantly located on postsynaptic neurons (Santana *et al.*, 2013). They are abundant in the rat cerebral cortex, thalamic nuclei, and dorsal raphe, where they mediate slow depolarization and facilitation of neuronal excitation. Presynaptic α_1 adrenergic receptors have been found in the nucleus accumbens, where they may regulate dopamine release (Mitrano *et al.*, 2012).

Stimulation of central α_2 adrenergic receptors leads to decreased cAMP production, hyperpolarization and neuronal inhibition, mostly increasing potassium (K^+) conductance, or inhibiting Ca^{2+} channels. α_2 adrenergic receptors act as auto-receptors controlling norepinephrine release from the nerve terminal, or as hetero-receptors, being also located postsynaptically, thus regulating serotonin and dopamine transmission (Gobert *et al.*, 1998; Devoto *et al.*, 2001). They are predominantly expressed is in the nucleus of the solitary tract, the locus coeruleus, the cerebral cortex, the hippocampus, the cerebellum, the pituitary gland, the spinal cord, the midbrain and the basal ganglia.

β receptors (β_1, β_2, β_3) are coupled with a G_s protein, thus activating AC and promoting cAMP-dependent intracellular mechanisms. Only β_1 and β_2 receptors are expressed in the CNS (Nicholas *et al.*, 1996).

β_1 adrenergic receptors are heterogeneously localized with high expression peaks in the cerebral cortex and the caudate. Their distribution seems to be unrelated to catecholamine content.

β_2 adrenergic receptors are located on neuronal and glial elements, with the highest density in the cerebellum.

Epinephrine and norepinephrine are equipotent at β_1, whereas epinephrine is considerably more potent than norepinephrine at β_2.

6.3. Catecholamine Systems

- *6.3.1. Noradrenergic Projections*

In the CNS have been identified two main groups of noradrenergic neurons, which cell bodies are located in the brainstem, particularly in the locus coeruleus and the lateral tegmental nuclei (Dahlström and Fuxe, 1964). Neurons located in the locus coeruleus project diffusely to the cerebral cortex, hippocampus, amygdala, thalamus, hypothalamus, and cerebellum. Neurons of the lateral tegmental nuclei project to other regions such as the hypothalamus, part of the amygdala, and the spinal cord.

Particularly, in the lateral tegmental system have been identified three main groups of neurons: (i) the dorsal motor nucleus of the vagus, (ii) the nucleus of the solitary tract, and (iii) the lateral tegmental nucleus. From the nucleus of the solitary tract originate noradrenergic neurons involved in the regulation of the CNS activity.

Noradrenergic transmission is involved in the evaluation of external stimuli, attention, behavioral reorientation, and activation of vegetative, metabolic, and endocrine systems, in order to achieve optimal behavioral responses, moreover enhancing signal to noise ratio in target areas. This neurotransmission is particularly sensitive to stressful stimuli, and represents a target for a number of different psychoactive drugs including antidepressants, antipsychotics, and drugs used in the treatment of ADHD. In depression, a reduced function of these neurons would be responsible for the hypersensitivity to environmental stimuli, making depressed subjects unable to generate appropriate behavioral responses.

- *6.3.2. Dopaminergic Pathways*

In the CNS, dopamine neurons innervate different brain areas forming four different pathways: (i) nigrostriatal, (ii) mesocortical, (iii) mesolimbic, and (iv) tuberoinfundibular (Dahlström and Fuxe, 1964).

The nigrostriatal pathway is characterized by long projecting neurons, which cell bodies are located in the substantia nigra pars compacta (A9), projecting

predominantly to the dorsal striatum (particularly to the caudate putamen). The extrapyramidal system is involved in motor coordination, learning of complex movements, and in the regulation of muscle tone. Neuronal degeneration in this area is at the onset of Parkinson's disease and all related symptoms.

By means of long projections, dopamine cells located in the VTA (A10) innervate the PFC constituting the mesocortical pathway, involved in the regulation of emotional affective behavior and mood, modulation of cognitive activities, planning abilities, and social activity. It is also involved in the mechanisms of reward and incentive learning. Decreased dopaminergic activity in the PFC is associated with negative symptoms of schizophrenia.

Neuronal projections from the VTA to sub-cortical brain regions such as the accumbens, the amygdala, the hippocampus, and the olfactory tubercle, constitute the mesolimbic pathway, involved in primordial functions such as emotional responses, behavior, motivation, stimuli processing and reward mechanisms.

Short projecting dopaminergic neurons which cell bodies are located in the arcuate and periarcuate nucleus of the hypothalamus (A12) innervate the median eminence - strictly connected with capillaries of the hypothalamic-pituitary portal circulation - constituting the tuberoinfundibolar pathway. Dopamine released into the portal circulation, reaching the anterior pituitary gland inhibits prolactin release via activation of D_2 receptors.

6.4. Serotoninergic Transmission

About the 90% of 5-hydroxytriptamine (5-HT, serotonin) is localized in the enterochromaffin cells of the intestinal tract; rich in serotonin are also platelets that, not being able to synthesize serotonin, catch it from the blood, accumulate it in their granules, and release it when they aggregate. Finally, serotonin is produced by neurons in the central and peripheral nervous system, where serotonin exerts an important role in different psychobiological processes.

- *6.4.1. Synthesis and Metabolism*

The main source of serotonin is the amino acid L-tryptophan, ingested with diet and transported into the cells through the transporter for neutral aminoacids. L-tryptophan is hydroxylated and converted into 5-hydroxytryptophan (5-OH-tryptophan) by the tryptophan hydroxylase, which activity is positively modulated by stimulation of serotonergic neurons. 5-OH-tryptophan is therefore decarboxylated and converted into serotonin by an aromatic amino acid decarboxylase (DOPA decarboxylase).

The main serotonin catabolic pathway provides its oxidation to 5-hydroxy-3-indolacetaldeide by MAO-A localized in the mitochondria within the nerve terminal; aldehyde is then immediately converted into 5-hydroxy-3-indoleacetic acid (5-HIAA) by the enzyme aldehyde dehydrogenase. A minor formation of serotonin metabolites is due to serotonin conversion into O-sulfate by a sulfotransferase.

- *6.4.2. Release and Termination of the Signal*

Neurotransmitter release occurs by Ca^{2+}-dependent mechanism which promotes vesicles exocytosis and serotonin release in the synaptic cleft. Serotonin extracellular concentration is reduced mainly through two processes: i) dilution in the extracellular space and passage in the blood vessels; ii) reuptake by the SERT, which affinity for its substrate is modulated by the concentration of Na^+ and Cl^- co-transported within the cell.

- *6.4.3. Role of Serotonin in the Central Nervous System*

Serotonin distribution is widespread in the brain (Jans *et al.*, 2007). Serotonergic neurons are located in the caudal and rostral raphe nuclei of the brainstem, projecting to different brain areas such as the hippocampus, the PFC, the amygdala, the hypothalamus, the basal ganglia and the cingulate cortex (Jans *et al.*, 2007). Through these projections, serotonin participates in the control of many important functions including sleep, mood, aggression, cognition, temperature, and feeding (Ogren *et al.*, 2008; Nichols and Nichols, 2008). In line with this, changes of normal serotonin activity appear to be involved in psychiatric and neurological diseases such as depression, schizophrenia, anxiety disorders, Down's syndrome, Alzheimer's disease, autism, attention deficit disorder, alcoholism, sleep, apnea. Indeed, the cerebral

serotonin system is a target for the treatment of several psychiatric disorders, such as depression and anxiety, moreover playing an important role in the control of behavioral, autonomic, and endocrine responses to stressful stimuli. The serotonergic system is tightly connected both anatomically and functionally with other neurotransmitter systems, so the activity of drugs active on a system is generally followed by changes in the functional status of the others.

6.5. *Serotonin Receptors*

Serotonin receptors are expressed in the CNS, but their distribution and functional significance varies depending on the receptor type. Seven different classes of serotonin receptors have been identified, further divided into several subtypes. Except for the 5-HT_3 receptor that is an excitatory ligand-gated ion channel permeable to Na^+, K^+, and to a less extent Ca^{2+} (Hannon and Hoyer, 2008), all the others are G-coupled protein receptors, activating several signaling pathways (Nichols and Nichols, 2008).

- ### 6.5.1. 5-HT_1 Receptors

5-HT_1 receptors family is mainly coupled to $G_{i/o}$ proteins, determining AC inhibition, decreased cAMP intracellular levels, stimulated K^+ channels activity, and inactivation of Ca^{2+} channels. They are divided into different isoforms: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F}. They are abundant in both the dorsal and the medial raphe nuclei, exerting an important role as mood regulators in the hippocampus. They are also found in the cortex (especially in the PFC), the amygdala nuclei, and the lateral septum, all areas involved in the regulation of emotional states.

In particular, 5-HT_{1A} are somato-dendritic auto-receptors expressed onto raphe neurons, where they mediate the reduction of firing frequency. They are also expressed postsynaptically in other brain regions such as the hippocampus and cortical areas (Jans *et al*., 2007). The activity of these receptors appears to be particularly important in some psychiatric illnesses such as depression and anxiety, and its pharmacological modulation may play a role in the therapeutic action of antidepressant drugs (SSRIs) (Ohno, 2011).

Also 5-HT_{1B} receptors may be involved in depression, as shown by studies in animal

models (Svenningson *et al.*, 2006) and humans (Murrough *et al.*, 2011).

- *6.5.2. 5-HT$_2$ Receptors*

They are coupled to $G_{q/11}$ proteins, thus leading to an increase in IP-3 and DAG formation. Three distinct receptors, namely 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$, belong to this class.

In particular, 5-HT$_{2A}$ receptors are expressed on cortical pyramidal cells and interneurons as well as in the brainstem, limbic areas, and basal ganglia. They are found at postsynaptic level (Miner et al., 2003), but also presynaptically, suggesting a modulation of the excitatory activity (Jakab and Goldman-Rakic, 1998).

- *6.5.3. 5-HT$_3$ Receptors*

5-HT$_3$ receptors are ligand-operated ion channel resulting from the assembly of different subunits, which activation promotes cell depolarization. 5-HT$_3$ receptors are expressed in neuronal cell types at both presynaptic and postsynaptic level. Their activation modulates the release of several neurotransmitters; in limbic areas, presynaptic 5-HT$_3$ receptors are also present on dopaminergic terminals, and their activation produces an increase of dopamine release. Studies in animals and humans have identified 5-HT$_3$ receptor antagonists as potential targets for anxiety disorders for their promising anti-anxiety effects (Gupta *et al.*, 2015).

- *6.5.4. 5-HT$_4$ Receptors*

They are G_s protein coupled receptors, responsible for increased cAMP formation. They are abundant in the limbic system (hippocampus, accumbens, amygdala), in the striatum, and in the cortex. A role in learning and memory processes has been suggested (Chapin *et al.*, 2002).

- *6.5.5. 5-HT$_5$, 5-HT$_6$, 5-HT$_7$ Receptors*

Poor informations are available for these receptors. While 5-HT$_5$ receptors are primarily coupled to G_i proteins and responsible for decreased cAMP levels, 5-HT$_6$ and 5-HT$_7$ receptors exert the opposite effect.

7. Microdialysis Coupled with Electrochemical Detection

7.1. *Introduction*

Brain microdialysis technique coupled with electrochemical detection (ED) is a method that allows to monitor in vivo levels of substances such as neurotransmitters and neuromodulators from several brain areas, and has proven to be a valuable tool for the study of the mechanisms of action and preclinical profile of centrally-acting drugs. This method involves the implantation of special dialysis fibers (probes) in a specific area, and perfusing it with artificial cerebrospinal fluid (Ringer´s solution). Molecules that are sufficiently small to pass through the dialysis membrane then diffuse across the membrane from an area of high concentration (the extracellular compartment) to an area of low concentration (the inner space of the dialysis membrane). Substances recovered are collected and assayed by high pressure liquid chromatography (HPLC) to evaluate their concentration in the dialysate, that is closely related to their extracellular concentration in the area investigated. After recover from surgery, therefore, the effects of drugs or other treatments on the assayed substance can be evaluated in freely moving animals, thus comparing the observed variations with the related behavior.

7.2. *Fields of Application*

Given their role in the control and regulation of principal functions and behaviors, the biogenic amines dopamine, norepinephrine, and serotonin, together with their precursors and metabolites, have been implicated in the pathophysiology of several neurological and psychiatric conditions, as biomarkers for disease development or progression, and targets for the development of novel therapeutic strategies. The ease of estimating biogenic amines is mainly due to their chemical structure, whereas the growing facility with which their role in the brain has been ascertained is due to their unique function as neurotransmitters. The possibility of detecting dopamine, norepinephrine, and serotonin by ED in extracts such as dialysate from freely moving animals has given an extraordinary advantage to researchers. No comparison can be

made with previous techniques in which a time course of a drug effect on a specific neurotransmitter could be made only by sacrifying groups of animals at given time-points and assessing the total amount of a transmitter, without being able to distinguish the fraction that is actively released upon depolarization. The possibility of detecting an increasing number of neurotransmitters almost everywhere in the brain, offers a wide spectrum of applications. Moreover, considering the large number of pharmacological tools that can be used to modify the level of a neurotransmitter or its metabolites in a specific brain area, the number of possible applications increases exponentially.

A technical explanation of the experimental procedure is described in the section Materials and Methods. Here follow a brief description of the main principles of the technique.

7.3. *Basis of Monoamine Electrochemical Detection*

The possibility of detecting biogenic amines such as dopamine, norepinephrine, and serotonin and their metabolites is due to their readiness to be oxidized. Although monoamines oxidation occurs spontaneously in solution, ED is based on application of an electrical potential to a carbon-based electrode on which monoamines flow after separation on a chromatographic column. The oxidation produces a derivative with the giving up of two electrons per molecule to the electrode. Because the applied potential to the electrode immersed in an electrical conducting solution (mobile phase) results in an electrical current, the giving up of electrons to the electrode produces alteration of the basal current which is proportional to the amount of substance that is oxidized. The electronic elaboration of this signal through a detector allows the quantitative detection of oxidable substances present in the sample injected in the HPLC. The variation of current produces a chromatogram in which the peak detected correspond to a substance, and its height or area is proportional to its concentration in the sample. By use of a standard curve, the absolute amount of substance can be assessed.

7.4. Dialysis Probes: Geometry and Time After Implant

Based on their characteristics the probes used for the dialysis experiments are classified into three categories: (i) transcerebral, (ii) U-shaped, (iii) concentric.
The main disadvantage of transcerebral probes is due to their inability to reach deep brain tissues as midbrain structures, or dialyze symmetrical brain areas, simultaneously. The main advantage of the vertical probes, compared to the previous ones, is instead due to the possibility to be used for the study of any brain area (also deep structures such as hypothalamic regions). The development of the vertical probes provided a means of reducing the extent of surgically induced injury. This probe consists of a loop of dialysis membrane which is implanted vertically into the brain via a single hole in the skull. Still, less damage is produced by a vertical concentric style dialysis probe.
A recovery period after the probe implantation is moreover necessary to clean up the excess of neurotransmitter due to the leakage from terminals damaged by the probe insertion. In the majority of studies in rats, a period of recovery of about 24 hours is used, although depending on the probe, 6-8 hours might be sufficient to get Ca^{2+} dependent tetrodotoxin-sensitive neurotransmitter release. When the microdialysis probe is implanted in mice, 48 hours recovery determines a more stable baseline of extracellular concentration of neurotransmitter. Two probes can be implanted in an animal when the difference of the drug effect in two different regions of brain has to be ascertained. Moreover, microdialysis can be performed by implanting a guide cannula 1-2 mm above the brain areas of interest, well in advance, to allow the animal to recover from the surgery. Thus it is intuitive that also careful surgical procedure can be a critical factor in ensuring the success of this method.

7.5. Membrane Properties

- *7.5.1. Membrane "cut-off"*

The "cut-off" is an expression of the permeability of the membrane constituting the probe, limiting the diffusion of molecules whose dimensions go beyond a certain maximum value. In the case of commonly used probes, is allowed the diffusion of

solutes of low molecular weight (400-600 Dalton), and accordingly is prevented the passage of protein molecules, thus obtaining relative cleaned samples. The solution that is collected and analyzed reflects the composition of the extracellular fluid: since the iso-osmotic Ringer perfusing the probe equilibrates with the extracellular diffusion in both directions according to the concentration gradient, compounds of low molecular weight spread second gradient from cerebral fluid to Ringer perfusion that escapes through the probe at a constant flow. Thus, the dialysis membrane also acts as a filter to prevent the diffusion of large molecules from extracellular fluid into the perfusion medium. This provides certain advantages for the analysis of transmitter content in the dialysate. First, the membrane can prevent large molecules such as enzymes from entering the perfusion solution and thereby halt the continuous enzymatic degradation of neurotransmitters once they have entered the perfusion solution. Also, by virtue of its ability to exclude molecules from the perfusion solution, the membrane partially purifies samples prior to their analysis. Additionally, because the dialysis membrane prevents the perfusion solution from directly contacting the tissue, this provides a barrier to turbulence and infection.

- *7.5.2. Neurotransmitter Recovery*

The neurotransmitter release assessed with microdialysis can be considered the result of physiological events reflecting the neuronal activity. This process depends (i) on the neuronal "firing" depolarization of the nerve terminal through the voltage-dependent Na^+ channels, and (ii) the entry of Ca^{2+} (also in this case through the opening of the channels caused by voltage dependent depolarization-induced opening of Na^+ channels). In addition to the above criteria is expected that the release of neurotransmitters is affected by drugs that modify the synthesis, metabolism and compartmentalization. For small molecules such as monoamines, the limiting factor will be the recovery, usually, the rate of diffusion through the extracellular fluid and not through the membrane. In fact, the neurotransmitter released in the synaptic compartment diffuses in a space in which there are neurons and glial cells that tend to reuptake it, and a series of enzymes responsible for its metabolism.

The presence of the dialysis membrane also provides certain technical advantages. The recovery of the neurotransmitters may depend on many factors such as: (i) the length of the dialysing surface (the longer is the active surface, the greater is the recovery;

the size of the perfused area can be controlled by limiting the active surface of the membrane); (ii) the flow of the perfusion fluid (the higher is the flow rate, the greater is the recovery; the perfusion is greatly simplified because it is not necessary to adjust inflow and outflow accurately in order to prevent build up of pressure or clogging of the cannula); (iii) the rate of diffusion of substances in the extracellular fluid; (iv) the dialysis membrane properties.

7.6. _Relative and Absolute Recovery_

It is important distinguishing between the relative recovery and the absolute recovery. In the first case, the concentration of a particular substance in the perfusate when leaving the probe is expressed as a percentage of the concentration of the surrounding medium, whereas in the second case, the total amount of the recovered substance in a given period of time, is expressed in moles /liter.

It has been debated whether expression of microdialysis data should be in % of basal or in absolute amount of neurotransmitter detected. Variability of basal neurotransmitter recovery is very high even when the working conditions are keep unchanged. It is difficult to obtain statistical significance using the raw data when the change of output induced by drug treatment is minimal. On the contrary, when the change of output induced by drug treatment is expressed as % of basal (considering the mean of the last three samples before treatment as a basal), reproducibility of results is very high and a drug effect can be statistically significant even when few animals are used.

7.7. _Applications_

Microdialysis has found its most important application in the field of neuroscience, and particularly in the field of neuropharmacology. This technique make possible to study not only the mechanism of drugs acting at central level, but also the changes induced by natural stimuli, and then deduce important information about the physiology of the central nervous system.

Among applications of microdialysis, the most important are: (i) studies of functional neuroanatomy; (ii) study of the mechanism of action of drugs known, or in the development of new drugs and their pharmacological screening; (iii) pharmacokinetic studies and distribution of drugs (as the blood-brain barrier may be considered intact, by administering the drug systemically, can get feedback on the pharmacokinetics of the compound of interest); (iv) behavioral studies (biochemical responses of behaviors induced by physiological or pharmacological treatment); (v) hormonal studies and monitoring of metabolic events (in fact, by implanting dialysis probes in other tissues it is possible to monitor any substance compatible with the "cut-off" membrane); (vi) applications to clinical neurosciences (for studies related to ischemia, trauma, and epileptic episodes).

AIM OF THE STUDY

Investigation of monoamine neurotransmission in experimental animals has contributed to the understanding of the function of many mammalian brain areas, in physiological as well as in pathological condition. On the basis of this evidence, the main aim of the research described in this thesis was to investigate catecholamine neurotransmission in order to better understand the role of the bed nucleus of stria terminalis (BNST) in the etiology of depression and in the pharmacological effect of antidepressants. Among psychiatric disorders, depression has a significant impact on the population and a high social cost (Pincus and Petit, 2001), thus we wanted to investigate this issue to contribute to a better understanding of this disease. To this end we have first evaluated the effect of antidepressants on catecholamine transmission in the BNST (Cadeddu *et al.*, 2014). The BNST is a part of the complex of the extended amygdala (ExtA) (Heimer *et al.*, 1993, de Olmos and Heimer, 1999), an area involved in the acquisition and the expression of emotions and motivated behavior (Hernandez and Hoebel, 1988). Recently, it was hypothesized that sub-cortical areas involved in the expression of motivated behavior and reward, such as the ventral striatum and the nucleus accumbens (NAcc) may have an important role in the pathophysiology of depression (Nestler and Carlezon, 2006). Thus, being the ExtA a complex that includes NAcc shell and BNST, it was of interest investigating the role of catecholamines in the BNST in depression and in the mechanism of action of antidepressants. Considering that the BNST receives a significant noradrenergic (Forray and Gysling, 2004) and serotoninergic (Phelix *et al.*, 1992) innervation, besides the dopaminergic one, and considering that noradrenergic and serotonergic transmissions are the main therapeutic target of antidepressant drugs, we investigated the role of different classes of antidepressants on catecholamine transmission in the BNST to compare their effect in this area. The BNST receives also a dense innervation from the basolateral amygdala and dense dopaminergic projections from the ventral tegmental area, and projects, among other brain nuclei, to both the lateral hypothalamus and the periacqueductal grey (de Olmos and Ingram, 1972; de Olmos and Heimer, 1999; Phelix *et al.*, 1992); importantly, BNST is strictly connected via CRF innervation to the paraventricular nuclei (PVN). Therefore it may play an important role in the control and adaptation to the stress response. Considering that depression is in part due to an unfavorable adaptation to the stress response, understanding the role of catecholamines in the BNST may also contribute to the understanding of etiology of

depression.

Once we realized that the catecholamine transmission is this area could play a relevant role in the therapeutic effect of antidepressants and likely in the etiology of depression, we investigated the effect of ketamine, a multifaceted drug that besides being a common veterinarian anesthetic and a drug that is abused for its psychotomimetic effects, surprisingly, it has been recently found capable to produce a rapid antidepressant effect. In fact, a clinical effect of ketamine can be observed within 2 hours from administration (Zarate *et al.*, 2006), whereas typical antidepressants take 2-3 weeks to produce their effects. The low doses of ketamine that were used in clinical trials produced moderate psychotomimetic and dissociative effects 30-40 minutes after administration. Interestingly, these doses of ketamine, were able to evoke rapid and persistent antidepressants effects also in depressed patients resistant to other forms of treatment (Zarate *et al.*, 2006; Kavalali and Monteggia, 2015). The mechanism of this action is still unclear, but cellular and molecular studies revealed that ketamine can remodel brain networks involved in the therapy of depression (Duman *et al.*, 2012; Duman, 2014). Considering that molecular mechanism of action of ketamine is rather different than the antidepressants investigated in the BNST, it was of great interest to compare its effect on catecholamine transmission in the BNST.

The third part of this thesis, attempts to evaluate the possibility that changes in catecholamine transmission in the BNST may be involved in the etiology of depression. In particular, the knowledge of depression specific neuronal circuitry alterations can allow to gain insights in depression etiology and in the development of new antidepressants. Although depression etiology is mostly unknown, there is general agreement that both genetic and epigenetic factors may contribute to its appearance. Among epigenetic factors, the role of stress is considered crucial, and in particular, exposition to chronic stress at pre-pubertal or adolescence age may be of fundamental importance in the appearance of this disease later in life (Belmaker and Agam, 2008). On these basis several animal model of depression have been developed, and among them the unpredictable chronic mild stress (UCMS) proposed by Willner (1997a) has been recognized to have high validity because it is associated with neurochemical changes and a behavioral expression that parallels human depression (Willner, 2005). An interesting feature of this model is unpredictability and variability that prevent rapid adaptation to the applied stressors, thus crafting this model of depression

efficacious and reproducible (Bowman *et al.*, 2003; Herman, 2013). We utilized UMCS in adolescent rats, because we wanted to evaluate the long term effect on brain neurotransmission in the BNST. In fact, we think that UMCS could produce its effect by altering the last part of the brain development, thus determining an alteration of the physiological process of neurochemical changes that occur in adolescence, a period in which individuals go through the assumption of the adult personality (McEwen, 2008). Moreover, it has been recently demonstrated that neuro-physiological changes that occur during pre-pubertal and adolescent age have a role in the appearance of depression (Davey *et al.*, 2007). Taken into account the role of mesolimbic dopamine transmission in motivation (Berridge, 2007) and the role of chronic stress in depression pathology (Hammen, 2005), the role of norepinephrine in modulating brain circuits that are activated by stress (Morilak *et al.*, 2005), the aim of this study is the evaluation of dopamine and norepinephrine transmission in the BNST of rats that have been previously exposed to unpredictable chronic stress at peri-adolescent age (post natal days - PND 28-42).

In order to assess long term effects of chronic stress, dopamine and norepinephrine will be evaluated through the microdialysis technique in freely moving rats (Carboni, 2003) at the PND 72-90. Microdialysis experiments allow the detection of the extracellular dopamine and norepinephrine basal concentration in the area investigated, but also allow to assess the modification of the dopamine and norepinephrine extracellular concentration (output) induced by the administration of drugs (Carboni and Silvagni, 2004). Therefore treated rats, once assessed the basal dopamine and norepinephrine output, will be administered with a challenge low dose (0.5 mg/Kg i.p.) of D-amphetamine, in order to assess changes in stimulated dopamine and norepinephrine transmission even in absence of changes in basal transmission (Carboni and Silvagni, 2004). Moreover, treated rats will be administered with a challenge dose of the selective norepinephrine transporter (NET) blocker reboxetine (10 mg/kg i.p.), or with a challenge dose of GBR 12909 (10 mg/kg i.p.), a selective blocker of dopamine transporter (DAT). The evaluation of the response of dopamine and norepinephrine output, after the administration of NET or DAT blockers, in stressed and control rats, will allow gain insights on the modification of the reuptake, and thus of the catecholamine transmission in the BNST.

MATERIALS AND METHODS

1. Animals

Male Sprague-Dawley rats purchased from Harlan (S. Pietro al Natisone, Italy) and bred in the animal facility of the Institute of Biomedical Science, in Cagliari. At the postnatal day (PND), a total of 58 animals were separated from their mother and group-house caged (six per cage) under standard conditions of humidity (60%), temperature (22°C) and artificial light (12 hours light/dark, from 8 A.M. to 8 P.M.). Food and water were available *ad libitum*.

All experimental procedures were approved by the local Ethical and Animal Care Committee and performed according to the guidelines for care and use of experimental animals of the European Union (EEC Council 86/609; D.L. 27/01/1992, n. 116). All efforts were made to minimize animal suffering and reduce the number of animal used.

2. Unpredictable Chronic Mild Stress Procedure

The Unpredictable Chronic Mild Stress (UCMS) is a protocol for the chronic stress exposure of rats consisting of various randomized and scheduled stressors of social and environmental low-intensity. It is a variation of the chronic stress procedure described in rats by Willner and colleagues as a realistic animal model of depression (1992).

At PND 28 rats were randomly divided into two groups, namely a control non-stressed, and stressed (UCMS) group. The PND 28 was chosen as day to start the protocol because it represents pre-pubertal peri-adolescence period (Spear and Brake, 1983; Spear, 2000). The stressed group was subjected to 14 consecutive days of UCMS, from PND 29 to 42, in order to include the entire peri-adolescence period of life. PND 42 (beginning of mid-adolescence) represents a stage when pubertal changes happen and male social behavior switches to adult-like behavior (Spear, 2000; Bingham *et al.*, 2011).

The UCMS group, depending of the light/dark period was daily exposed to 3 stressors randomly chosen from the following list:

During the light phase:

Tube restraint (TRe) for 60 minutes in a novel room environment. Animals, still in their home cage, were individually placed in transparent plexiglass cylindrical restrainers (internal diameter 6 cm) provided of a movable lid to adjust the length to the animal body and achieve the restrain condition. Each restrainer had holes for ventilation.

Forced swim (FSw) for 5 minutes in a novel room environment. Animals were placed in a bucket (40 cm high, diameter of 20 cm) filled for approximately 25 cm with water at 25°C.

During the dark phase:

Light/dark cycle inversion (CyI), e.g., a dark phase with lights on, in a novel room environment.

Food and water deprivation (FWD) for 12 hours.

Social isolation in small cages (SIs) for 12 hours.

Water bedding (WBed), for 12 hours. Animals were housed with 1 cm of water bedding in their home cages.

Daily stressors were applied at different time points every day in order to prevent habituation and to provide an unpredictable feature to the stressors as previously described (Santarelli *et al.*, 2003; Yalcin *et al.*, 2005). Moreover, in order to apply a mild protocol, some particular rules were followed, namely, the animals were not exposed to the first daily stressor before 11.00 A.M., when FWD was applied the night before; animals were kept dry and warm after FSw, and furthermore, to increase unpredictability, overnight-stressors could be applied either alone or combined to others, except FWD which was always applied alone.

Control animals were kept in a separate room with free access to food and water, and they were handled two or three times per day at the same time in which the stressors were applied to the UCMS group.

The body weight of the animals from both groups was daily monitored, and during all the duration of the experiments.

3. Microdialysis

Experiments were carried out in adult animals (PND 72-90) subjected to UCMS at peri-adolescent age (PND 29-42), and their relative controls. PND 72-90 were chosen because correspond to the entrance in early adulthood, when male sexual and social behaviors are completely developed (Panksepp, 1981; Spear, 2000). Moreover, this life period has been associated with the onset of stress-related psychiatric symptoms and addiction-related disorders, that often occur in humans, as well, at the equivalent period of life (Andersen, 2003; Watt *et al.*, 2009).

Separate groups of rats were housed under standard conditions in the animal facility until PND 70. The day of the experiment they were simply injected intraperitoneally (i.p.) with the antidepressants tested or ketamine. Dialysis experiments were performed between PND 72-90, when rats reached young adulthood.

3.1. *Probes and Surgery*

The day of the surgery rats were anaestetized with an i.p. injection of Equithesin (0.97 g pentobarbital, 2.1 g $MgSO_4$, 4.25 g chloral hydrate, 42.8 ml of propylene glycol, 11.5 ml of 90 percent ethanol, distilled water up to 100 ml, 5 ml/Kg), and placed in a stereotaxic apparatus (Kopf, Germany). A small hole was drilled on the side of the exposed skull in correspondence of our region of interest. Animals were implanted with in-house constructed vertical probes [AN 69 dialysis fiber, OD: 310 μm, ID: 220 μm, cut-off 40.000 Dalton (Hospal-Dasco, Bologna, Italy)] in the BNST (AP -0.4; L ±1.2; V -8.0 from dura mater, 2 mm active-membrane length). The coordinates used were expressed in millimeters from Bregma, according to The Atlas by Paxinos and Watson (2007). Then, the probes were fixed to the skull with dental cement (Shofu Cx-Plus, GmbH, Ratingen, Germany), and the skin sutured. The rats were housed in a transparent plexiglas hemispheric cage, covered with a top hemisphere, with food and water available.

3.2. Dialysis Experiments

Experiments were performed on freely-moving animals 24 hours after the probe implantation. Artificial cerebrospinal fluid (Ringer's solution, NaCl 147 mM, $CaCl_2$ 2.2 mM, KCl 4 mM, pH 6/6.5) was pumped through the dialysis probe at a constant rate of 1 µl/min via a microinjection pump. Samples were collected every 20 minutes and immediately analyzed by high-performance liquid chromatography (HPLC) coupled with electrochemical detection in order to evaluate dopamine and norepinephrine sample content. Dialyzes samples (20 µl) were injected without any purification into an HPLC system equipped with reversed-phase column (C-18, 15 cm x 4.6 mm, 3.5 µm, Supelco, Milan, Italy) and a coulometric detector (ESA Coulochem II, Bedford, MA, USA), as previously described (Carboni, 2003). The sensitivity of the assay allowed for the detection of 5 fmoles of dopamine and norepinephrine. When the basal concentration (output) of dopamine and norepinephrine reached stables values, rats were given a challenge dose of the drug tested or saline. The dopamine and norepinephrine output was firstly collected four times and it was considered stable when the concentration calculated during the fourth trial differed less than 10% from the mean concentration obtained from the previous three samples. Stable levels of neurotransmitters were usually obtained after the first 2-3 hours of dialysis. Each implanted rat received a single acute i.p. injection of the tested drug. In animals subjected to peri-adolescent UCMS and their relative controls, D-amphetamine was administered at the dose of 0.5 mg (controls, n=4; UCMS, n=6); reboxetine at the dose of 10 mg (controls, n=4; UCMS, n=4); GBR12909 at the doses of 10 mg (controls, n=4; UCMS, n=4). Eight rats were administered with saline (controls, n = 4; UCMS, n = 4). In naive rats, each antidepressant was tested at doses of 5, 10, and 20 mg, respectively, as follows: desipramine (5, 7, and 8 times), reboxetine (5, 10, and 10 times), imipramine (6, 7, and 7 times), citalopram (5, 6, and 7 times), fluoxetine (5, 5, and 6 times), bupropion (5, 5, and 6 times). Ketamine was tested at doses of 10, 20, and 40 mg, (4 times for each dose administered). Four rats were administered with saline.

3.3. *Histology*

Histological analysis was performed in order to verify the anatomical position of the probe. At the end of the experiment, rats were anaesthetized with Equitesin overdose and decapitated. The brain was removed and stored in formaldehyde (10%). Brains were then cut on an oscillating microtome (Campden Instruments, Lafayette, IN, USA) producing consecutive coronal slices containing the regions of interest following the coordinates according to The Atlas of Paxinos and Watson (2007). Results from rats implanted outside the area of interest were discarded.

3.4. *Drugs*

Desipramine HCl, imipramine HCl, fluoxetine HCl, bupropion HCl, and D-amphetamine sulfate were purchased from Sigma (by Salars, Como, Italy). Ketamine HCl was purchased from Ketalar (by Farmaceutici Gellini, Milan, Italy). Citalopram HCl was a gift from Innova Pharma (Milan, Italy), reboxetine HCl a gift from Pharmacia Upjohn (Milan, Italy), and GBR12909 a gift from Novo A/S (Baagsværd, Denmark). Drugs were dissolved in saline and administered i.p., immediately.

3.5. *Statistics*

All data are expressed as mean ± SE. Statistical analysis was carried out by STATISTICA (Statsotf, Tulsa, OK, USA). Two-way or three-way analysis of variance (ANOVA) for repeated measures was applied to the data expressed either as absolute fmoles or as a percentage of basal norepinephrine or dopamine concentration. Results from treatments showing significant overall changes were subjected to post hoc Tukey´s tests with significance for $p < 0.05$. Basal values were the mean of three consecutive samples before treatment, differing less than 10% each other.

RESULTS

1. Effect of Desipramine on Dopamine and Norepinephrine Output in the BNST

Figures 2a and 2b show that desipramine (administered at 5, 10, and 20mg/Kg i.p.), increased both dopamine (150, 206, and 237 % of basal levels, for the three doses, respectively) and norepinephrine output (217, 255, and 339 % of basal levels, for the three doses, respectively). Two-way ANOVA of the results obtained showed a significant treatment effect ($F_{3,20}$ = 3.83, $p < 0.05$ and $F_{3,20}$ = 5.44, $p < 0.05$), time effect ($F_{9,180}$ = 16.75, $p < 0.001$ and $F_{9,180}$ = 13.71, $p < 0.001$), and time × dose interaction ($F_{27,180}$ = 3.12, $p < 0.001$ and $F_{27,180}$ = 2.87, $p < 0.005$) for dopamine and norepinephrine, respectively.
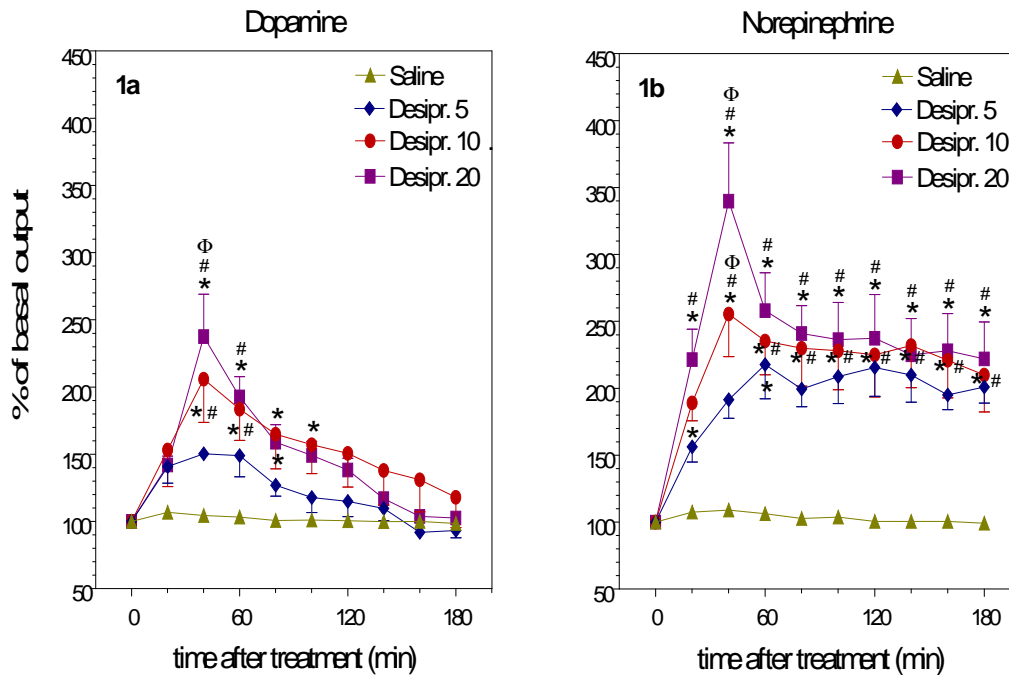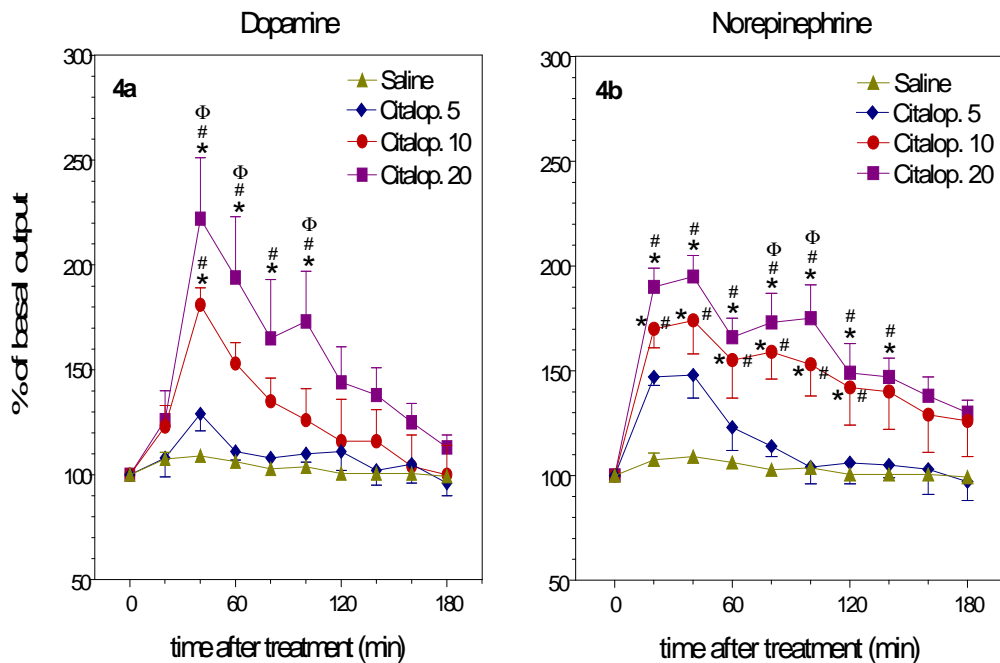

Figg. 1a and 1b. Effect of desipramine (5, 10 and 20 mg/Kg i.p.) on BNST dialysate dopamine (1a) or norepinephrine (1b), expressed as a percentage of basal output. Each point is the mean (±SE) of at least four determinations. *$p < 0.05$ from basal values; #$p < 0.05$ versus the corresponding time point after saline; ø$p < 0.05$ versus the corresponding time point of 5 mg/Kg.

2. Effect of Reboxetine on Dopamine and Norepinephrine Output in the BNST

Figures 2a and 2b show that reboxetine (administered at 5, 10, and 20 mg/Kg i.p.), increased both dopamine (106, 206, and 228 % of basal level, for the three doses, respectively) and norepinephrine output (129, 204, and 274 % of basal levels, for the three doses, respectively). Two-way ANOVA of the results showed a significant treatment effect ($F_{3,25}$ = 5.35, $p < 0.01$ and $F_{3,25}$ = 6.36, $p < 0.05$), time effect ($F_{9,225}$ = 6.46, $p < 0.001$ and $F_{9,225}$ = 8.99, $p < 0.001$), and time × dose interaction ($F_{27,225}$ = 1.86, $p < 0.01$ and $F_{27,225}$ = 3.93, $p < 0.001$) for dopamine and norepinephrine, respectively.
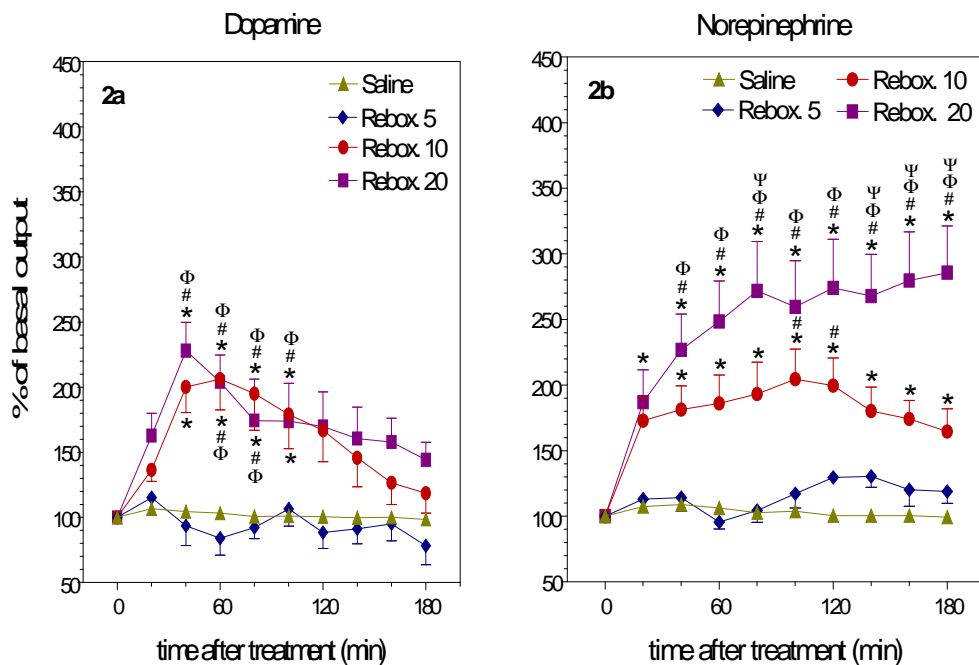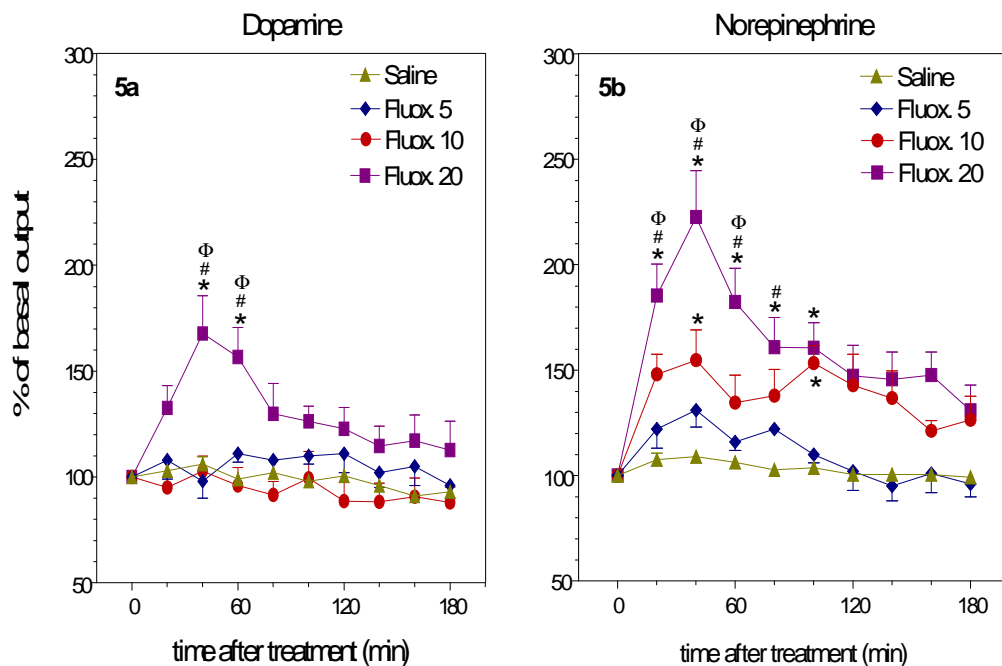

Figg. 2a and 2b. Effect of reboxetine (5, 10, and 20 mg/Kg i.p.) on BNST dialysate dopamine (2a) or norepinephrine (2b), expressed as a percentage of basal output. Each point is the mean (±SE) of at least four determinations. *$p < 0.05$ from basal values; #$p < 0.05$ versus the corresponding time point after saline; ø$p < 0.05$ versus the corresponding time point of 5 mg/Kg; ψ$p < 0.05$ versus the corresponding time point of 10 mg/Kg.

3. Effect of Imipramine on Dopamine and Norepinephrine Output in the BNST

Figures 3a and 3b show that imipramine (administered at 5, 10, and 20 mg/Kg i.p.), increased both dopamine (169, 203, and 459 % of basal level, for the three doses, respectively) and norepinephrine output (202, 235, and 613 % of basal levels, for the three doses, respectively). Two-way ANOVA of the results obtained showed a significant treatment effect ($F_{3,20} = 3.83$, $p < 0.05$ and $F_{3,20} = 13.49$, $p < 0.001$), time effect ($F_{9,180} = 16.75$, $p < 0.001$ and $F_{9,180} = 29.91$, $p < 0.01$), and time × dose interaction ($F_{27,180} = 8.97$, $p < 0.001$ and $F_{27,180} = 8.48$, $p < 0.001$) for dopamine and norepinephrine, respectively.
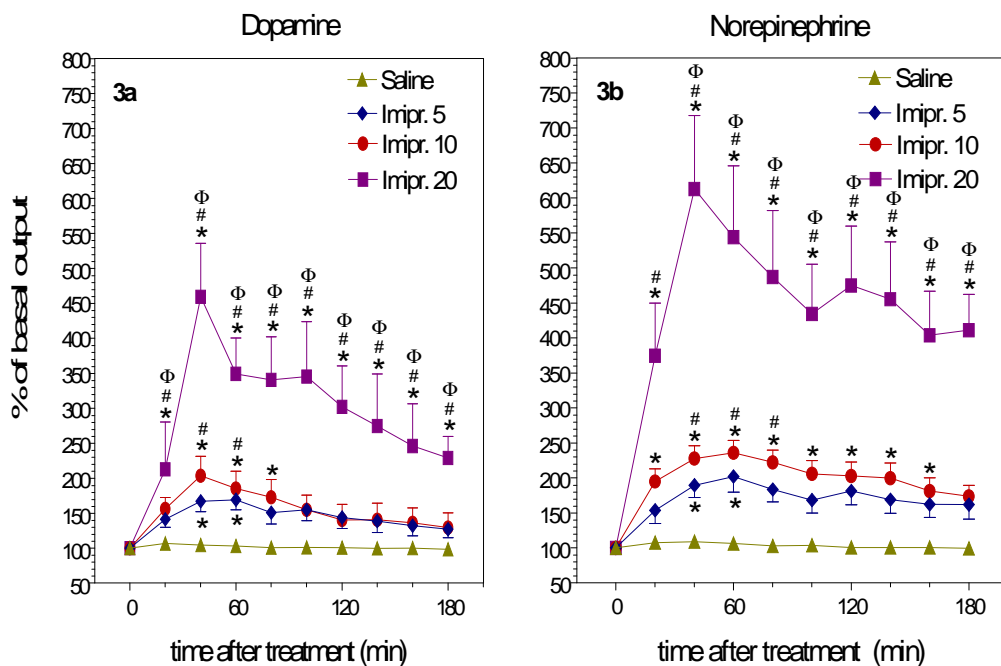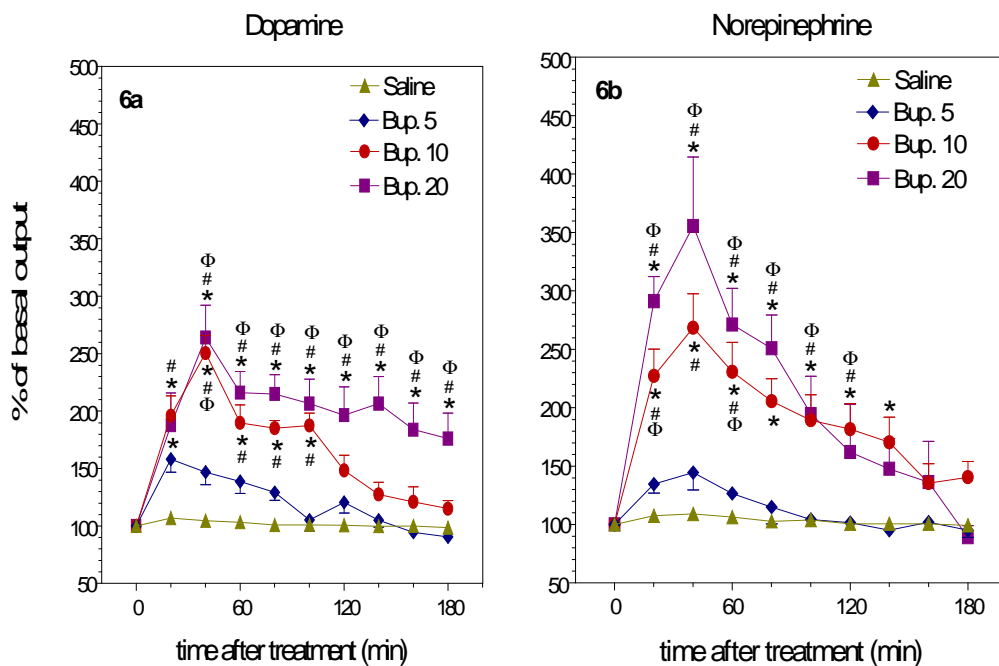

Figg. 3a and 3b. Effect of imipramine (5, 10, and 20mg/Kg i.p.) on BNST dialysate dopamine (3a) or norepinephrine (3b), expressed as a percentage of basal output. Each point is the mean (±SE) of at least four determinations. *$p < 0.05$ from basal values; #$p < 0.05$ versus the corresponding time point after saline; $ø$$p < 0.05$ versus the corresponding time point of 5 mg/Kg.

4. Effect of Citalopram on Dopamine and Norepinephrine Output in the BNST

Figures 4a and 4b show that citalopram (administered at 5, 10, and 20 mg/Kg i.p.), increased both dopamine (129, 181, and 222 % of basal level, for the three doses, respectively) and norepinephrine output (148, 174, and 195 % of basal levels, for the three doses, respectively). Two-way ANOVA of the results obtained showed a significant treatment effect ($F_{3,20}$ = 14.7, p < 0.001 and $F_{3,20}$ = 7.26, p < 0.005), time effect ($F_{9,180}$ = 28.88, p < 0.001 and $F_{9,180}$ = 17.05, p < 0.001), and time × dose interaction ($F_{27,180}$ = 2.86, p < 0.001 and $F_{27,180}$ = 2.53, p < 0.001) for dopamine and norepinephrine, respectively.
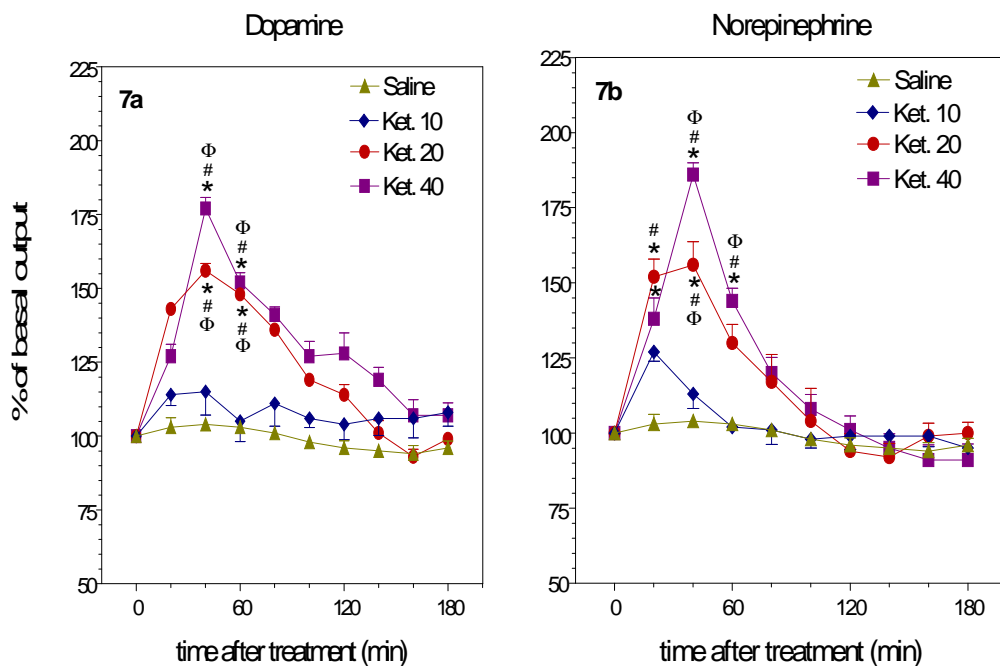

Figg. 4a and 4b. Effect of citalopram (5, 10, and 20 mg/Kg i.p.) on BNST dialysate dopamine (4a) or norepinephrine (4b) expressed as a percentage of basal output. Each point is the mean (±SE) of at least four determinations. *p < 0.05 from basal values; #p < 0.05 versus the corresponding time point after saline; øp < 0.05 versus the corresponding time point of 5 mg/Kg.

5. Effect of Fluoxetine on Dopamine and Norepinephrine Output in the BNST

Figures 5a and 5b show that fluoxetine (administered at 20 mg/Kg i.p.), increased dopamine output by 167 % of basal levels. Fluoxetine (administered at 5, 10, and 20 mg/Kg i.p.), increased norepinephrine output (131, 154, and 222 % of basal levels, for the three doses, respectively). Two-way ANOVA of the results obtained showed a significant treatment effect ($F_{3,16} = 6.48$, $p < 0.005$ and $F_{3,16} = 3.17$, $p < 0.001$), time effect ($F_{9,144} = 2.39$, $p < 0.05$ and $F_{9,144} = 11.30$, $p < 0.001$), and time × dose interaction ($F_{27,144} = 2.35$, $p < 0.001$ and $F_{27,144} = 2.41$, $p < 0.001$) for dopamine and norepinephrine, respectively.

Figg. 5a and 5b. Effect of fluoxetine (5, 10, and 20 mg/Kg i.p.) on BNST dialysate dopamine (5a) or norepinephrine (5b), expressed as a percentage of basal output. Each point is the mean (±SE) of at least four determinations. *$p < 0.05$ from basal values; #$p < 0.05$ versus the corresponding time point after saline; ø$p < 0.05$ versus the corresponding time point of 5 mg/Kg.

6. Effect of Bupropion on Dopamine and Norepinephrine Output in the BNST

Figures 6a and 6b show that bupropion (administered at 5, 10, and 20 mg/Kg i.p.), increased both dopamine (158, 250, and 264 % of basal level, for the three doses, respectively) and norepinephrine output (144, 266, and 355 % of basal levels, for the three doses, respectively). Two-way ANOVA of the results showed a significant treatment effect ($F_{3,16} = 20.55$, $p < 0.001$ and $F_{3,16} = 13.07$, $p < 0.001$), time effect ($F_{9,144} = 16.73$, $p < 0.001$ and $F_{9,144} = 43.14$, $p < 0.001$), and time × dose interaction ($F_{27,144} = 3.72$, $p < 0.001$ and $F_{27,144} = 9.58$, $p < 0.001$) for dopamine and norepinephrine, respectively.

Figg. 6a and 6b. Effect of bupropion (5, 10, and 20 mg/Kg i.p.) on BNST dialysate dopamine (6a) or norepinephrine (6b) expressed as a percentage of basal output. Each point is the mean (±SE) of at least four determinations. *$p < 0.05$ from basal values; #$p < 0.05$ versus the corresponding time point after saline; ø$p < 0.05$ versus the corresponding time point of 5 mg/Kg.

7. Effect of Ketamine on Dopamine and Norepinephrine Output in the BNST

Figures 7a and 7b show that ketamine (administered at 10, 20, and 40 mg/Kg i.p.), increased both dopamine (114, 156, and 176 % of basal level, for the three doses, respectively) and norepinephrine output (127, 155, and 186 % of basal levels, for the three doses, respectively). Two-way ANOVA of the results showed no significance for both dopamine and norepinephrine when ketamine was administered at 10mg/Kg ($p > 0.05$). Two-way ANOVA of the results showed, for doses of 20 and 40 mg/Kg, a significant treatment effect ($F_{3,19} = 4.63$, $p < 0.01$ and $F_{3,19} = 3.76$, $p < 0.02$), time effect ($F_{9,171} = 12.72$, $p < 0.001$ and $F_{9,171} = 31.41$, $p < 0.001$), and time × dose interaction ($F_{27,171} = 3.04$, $p < 0.001$ and $F_{27,171} = 6.29$, $p < 0.001$) for dopamine and norepinephrine, respectively.

Figg. 7a and 7b. Effect of ketamine (10, 20, and 40 mg/Kg i.p.) on BNST dialysate dopamine (7a) or norepinephrine (7b) expressed as a percentage of basal output. Each point is the mean (±SE) of at least four determinations. *$p < 0.05$ from basal values; #$p < 0.05$ versus the corresponding time point after saline; ø$p < 0.05$ versus the corresponding time point of 10 mg/Kg.

8. Basal Output of Dopamine and Norepinephrine in the BNST

Figure 8 shows that peri-adolescent UCMS did not affect basal dopamine output in the BNST of adult rats. Basal output of dopamine (expressed in fmol/20 µl sample ± SE) in stressed (UCMS) rats (10.6 ± 3.1, n = 12) was not significantly different from those of relative controls (10.1 ± 2.7, n = 14). Two-way ANOVA of dopamine estimation showed a no significant UCMS effect ($F_{1,44} = 0.99$, $p = 0.32$).

Basal output of norepinephrine (expressed in fmol/20 µl sample ± SE) in stressed (UCMS) rats (30.0 ± 8.1, n = 11) was significantly higher than those of relative controls (22.2 ± 5.9, n = 14). Two-way ANOVA of norepinephrine estimation showed a significant UCMS effect ($F_{1,23} = 4.61$, $p < 0.04$). Post hoc analysis (Tukey) showed that BNST norepinephrine levels in UCMS rats were significantly higher than those of relative controls.
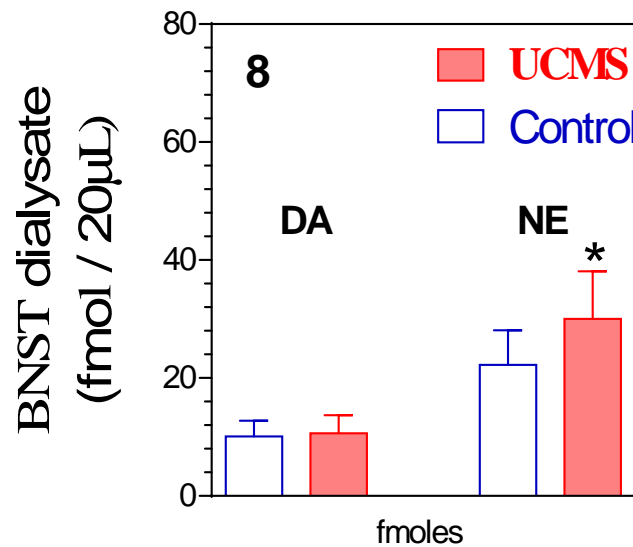
Fig. 8. Dopamine and norepinephrine basal output in the BNST of UCMS and control adult rats. Each column is the mean (± SE) of at least 11 determinations.

9. Effect of Amphetamine on Dopamine and Norepinephrine Output in the BNST

Figure 9a shows that amphetamine (0.5 mg/Kg i.p.) maximally increased dialysate dopamine levels by 313% and 419% above basal level in control and stressed (UCMS) rats, respectively, as recorded at 40 minutes after treatment. Three-way ANOVA of the results obtained showed a significant: (i) treatment effect ($F_{1,16}$ = 33.60, p < 0.01); (ii) time effect ($F_{8,128}$ = 8.83, p < 0.01); (iii) treatment x time interaction ($F_{8,128}$ = 7.26, p < 0.01), and a non significant: (i): stress effect ($F_{1,16}$ = 3.05, p = 0.09); (ii) stress x treatment interaction ($F_{1,16}$ = 2.95, p = 0.10); (iii) stress x time interaction ($F_{8,128}$ = 1.39, p = 0.20); (iv) stress x treatment x time interaction ($F_{8,128}$ = 1.32, p = 0.23). Post hoc analysis (Tukey) showed that amphetamine increased dopamine output significantly more in UCMS treated rats at 60 and 80 minutes after the treatment.

Figure 9b shows that amphetamine (0.5 mg/Kg i.p.) maximally increased dialysate norepinephrine levels by 414% and 459% above basal level in control and stressed (UCMS) rats, respectively, as recorded at 40 minutes after treatment. Three-way ANOVA of the results obtained showed a significant: (i) treatment effect ($F_{1,16}$ = 68.87, p < 0.01); (ii) time effect ($F_{8,128}$ = 28.21, p < 0.01); (iii) treatment x time interaction ($F_{8,128}$ = 24.65, p < 0.01), and a non significant: (i): stress effect ($F_{1,16}$ = 0.13, p = 0.71); (ii) stress x treatment interaction ($F_{1,16}$ = 0.11, p = 0.74); (iii) stress x time interaction ($F_{8,128}$ = 0.57, p = 0.79); (iv) stress x treatment x time interaction ($F_{8,128}$ = 0.48, p = 0.86). Post hoc analysis (Tukey) showed no significant differences in amphetamine-increased norepinephrine output in UCMS and control rats.

Figure 9c shows the effect of amphetamine (0.5mg/Kg i.p.) or saline on norepinephrine extracellular concentration (fmol/20 µl) in stressed (UCMS) rats and relative controls. Three-way ANOVA of the results obtained showed a significant (i) stress effect ($F_{1,13}$ = 5.47, p < 0.01); (ii) treatment effect ($F_{1,13}$ = 38.53, p < 0.01); (iii) time effect ($F_{8,104}$ = 17.18, p < 0.01); (iv) treatment x time interaction ($F_{8,104}$ = 17.23, p < 0.01), and a non significant (i) stress x treatment interaction ($F_{1,13}$ = 1.44, p = 0.25); (ii) stress x time interaction ($F_{8,104}$ = 1.05, p = 0.39); (iii) stress x treatment x time interaction ($F_{8,104}$ = 1.07, p = 0.38). Post hoc analysis (Tukey) showed that

amphetamine increased norepinephrine output significantly more in UCMS treated rats at 40 minutes after the treatment.
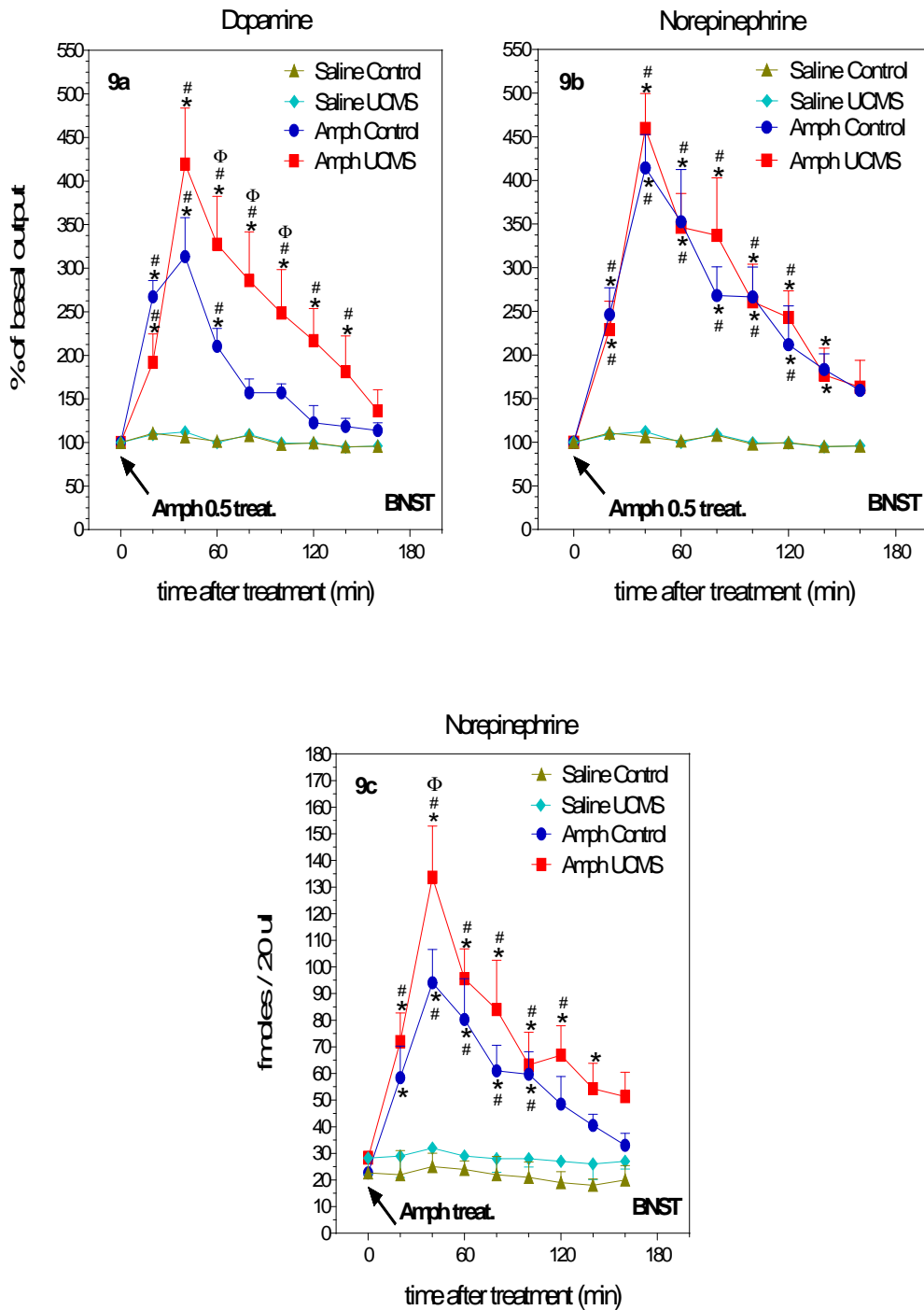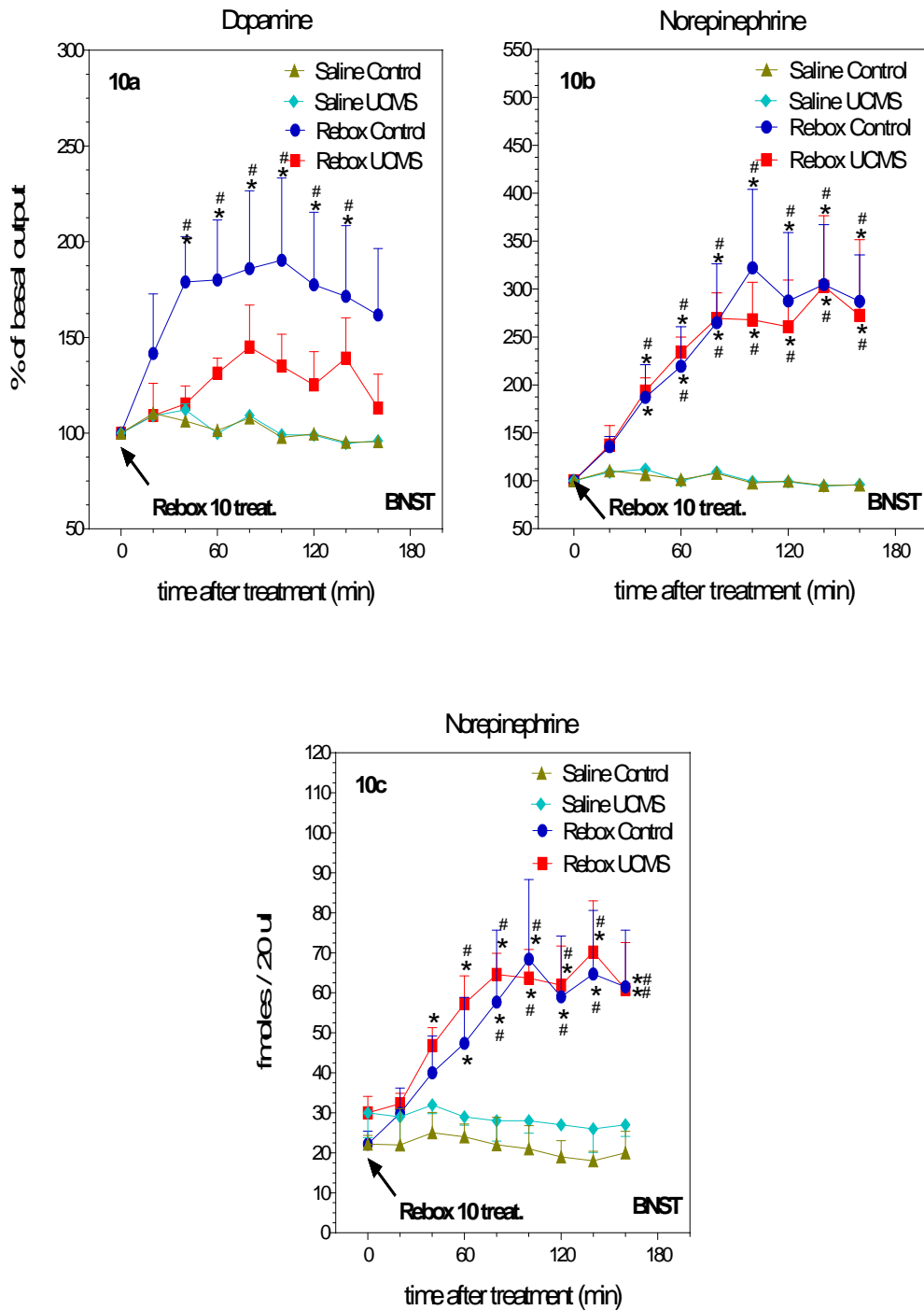

Figg. 9a, 9b, and 9c. Effect of amphetamine (0.5 mg/Kg i.p.) and saline on dialysate dopamine (9a) or norepinephrine (9b) (data expressed as a % of basal levels), and norepinephrine (9c) (data expressed as fmol/20 µl), from the BNST of Control rats. (Control n=4; UCMS n=6). $*p < 0.05$ from basal values; $\#p < 0.05$ versus the corresponding time point after saline; $øp < 0.05$ versus the corresponding time point of Control.

10. Effect of Reboxetine on Dopamine and Norepinephrine Output in the BNST

Figure 10a shows that reboxetine (10 mg/Kg i.p.) maximally increased dialysate dopamine levels by 190% and 145% above basal level in control and stressed (UCMS) rats, respectively, as recorded at 100 and 80 minutes after treatment, respectively. Three-way ANOVA of the results obtained showed a significant: (i) treatment effect ($F_{1,14}$ = 8.20, $p < 0.01$); (ii) time effect ($F_{8,112}$ = 3.74, $p < 0.01$); (iii) treatment x time interaction ($F_{8,112}$ = 4.44, $p < 0.01$), and a non significant: (i): stress effect ($F_{1,14}$ = 1.59, p = 0.22); (ii) stress x treatment interaction ($F_{1,14}$ = 1.69, p = 0.21); (iii) stress x time interaction ($F_{8,112}$ = 1.02, p = 0.42); (iv) stress x treatment x time interaction ($F_{8,112}$ = 1.23, p = 0.28). Post hoc analysis (Tukey) showed no significant differences in reboxetine-increased dopamine output in UCMS and control rats.

Figure 10b shows that reboxetine (10 mg/Kg i.p.) maximally increased dialysate norepinephrine levels by 322% and 303% above basal level in control and stressed (UCMS) rats, respectively, as recorded at 100 and 140 minutes after treatment, respectively. Three-way ANOVA of the results obtained showed a significant: (i) treatment effect ($F_{1,14}$ = 29.22, $p < 0.01$); (ii) time effect ($F_{8,112}$ = 13.12, $p < 0.01$); (iii) treatment x time interaction ($F_{8,112}$ = 15.34, $p < 0.01$), and a non significant: (i): stress effect ($F_{1,14}$ = 0.02, p = 0.88); (ii) stress x treatment interaction ($F_{1,14}$ = 0.03, p = 0.86); (iii) stress x time interaction ($F_{8,112}$ = 0.30, p = 0.96); (iv) stress x treatment x time interaction ($F_{8,112}$ = 0.30, p = 0.96). Post hoc analysis (Tukey) showed no significant differences in reboxetine-increased norepinephrine output in UCMS and control rats.

Figure 10c shows the effect of reboxetine (10 mg/Kg i.p.) or saline on norepinephrine extracellular concentration (fmol/20 µl) in stressed (UCMS) rats and relative controls. Three-way ANOVA of the results obtained showed a significant: (i) treatment effect ($F_{1,14}$ = 22.16, $p < 0.01$); (ii) time effect ($F_{8,112}$ = 14.28, $p < 0.01$); (iii) treatment x time interaction ($F_{8,112}$ = 14.24, $p < 0.01$), and a non significant: (i) stress effect ($F_{1,14}$ = 0.80, p = 0. 38); (ii) stress x treatment interaction ($F_{1,14}$ = 0.06, p = 0.80); (iii) stress x time interaction ($F_{8,112}$ = 0.26, p = 0.97); (iv) stress x treatment x time interaction ($F_{8,112}$ =

0.26, *p* = 0.97). Post hoc analysis (Tukey) showed no significant differences in reboxetine-increased norepinephrine output in UCMS and control rats.
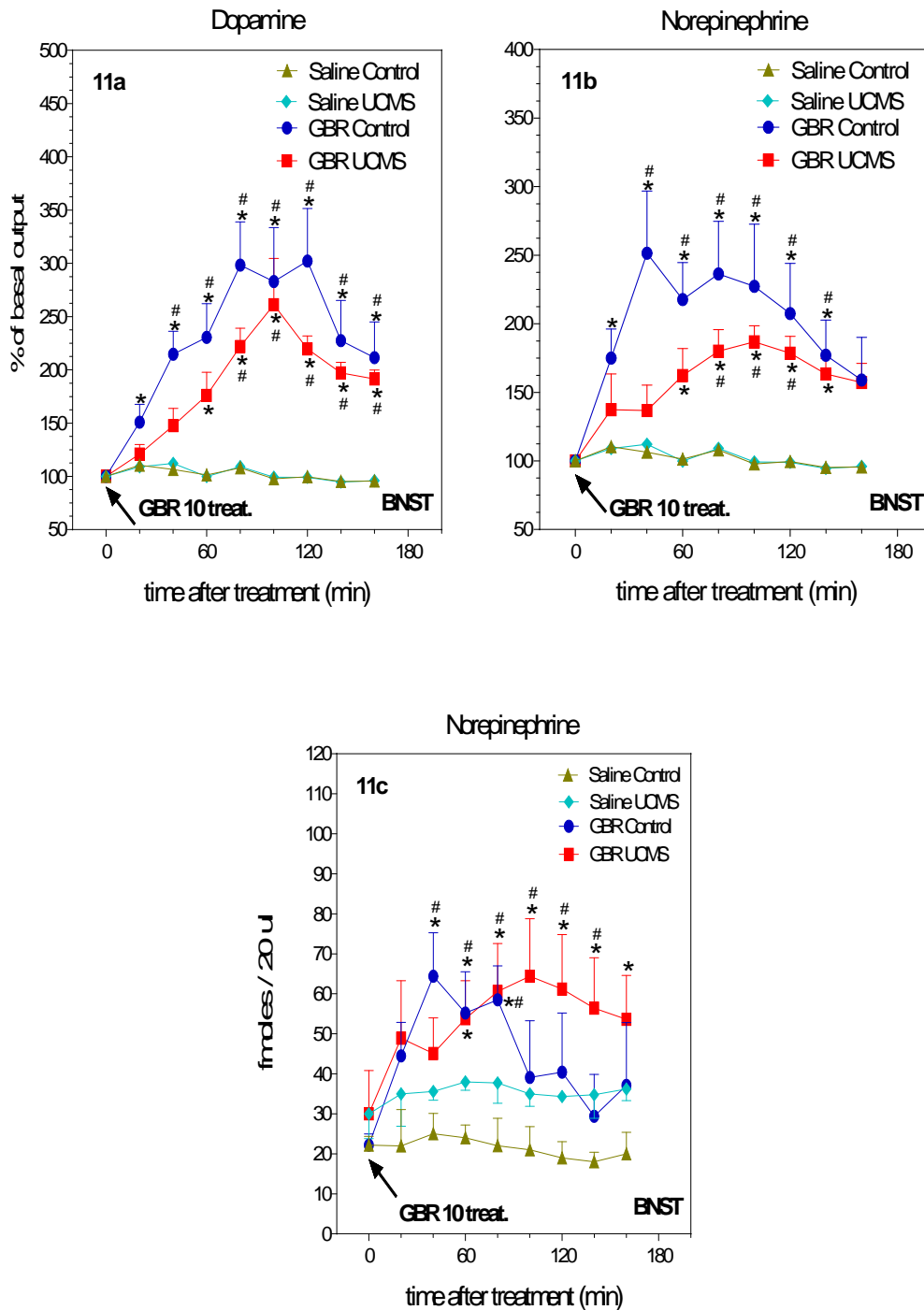

Figg. 10a, 10b, and 10c. Effect of reboxetine (10 mg/Kg i.p.) and saline on dialysate dopamine (10a) or norepinephrine (10b) (data expressed as a % of basal levels), and norepinephrine (10c) (data expressed as fmol/20 μl), from the BNST of Control or UCMS rats. (Control n=4; UCMS n=4). *p* < 0.05 from basal values; #*p* < 0.05 versus the corresponding time point after saline.

11. Effect of GBR 12909 on Dopamine and Norepinephrine Output in the BNST

Figure 11a shows that GBR 12909 (10 mg/Kg i.p.) maximally increased dialysate dopamine levels by 302% and 261% above basal level in control and stressed (UCMS) rats, respectively, as recorded at 120 and 100 minutes after treatment, respectively. Three-way ANOVA of the results obtained showed a significant: (i) treatment effect ($F_{1,14} = 89.89$, $p < 0.01$); (ii) time effect ($F_{8,112} = 15.00$, $p < 0.01$); (iii) treatment x time interaction ($F_{8,112} = 16.25$, $p < 0.01$), and a non significant: (i): stress effect ($F_{1,14} = 3.87$, $p = 0.06$); (ii) stress x treatment interaction ($F_{1,14} = 4.06$, $p = 0.06$); (iii) stress x time interaction ($F_{8,112} = 0.90$, $p = 0.51$); (iv) stress x treatment x time interaction ($F_{8,112} = 0.97$, $p = 0.45$). Post hoc analysis (Tukey) showed no significant differences in GBR 12909-increased dopamine output in UCMS and control rats.

Figure 11b shows that GBR 12909 (10 mg/Kg i.p.) maximally increased dialysate norepinephrine levels by 251% and 187% above basal level in control and stressed (UCMS) rats, respectively, as recorded at 40 and 100 minutes after treatment, respectively. Three-way ANOVA of the results obtained showed a significant: (i) treatment effect ($F_{1,14} = 40.91$, $p < 0.01$); (ii) time effect ($F_{8,112} = 8.86$, $p < 0.01$); (iii) treatment x time interaction ($F_{8,112} = 7.72$, $p < 0.01$), and a non significant: (i): stress effect ($F_{1,14} = 2.78$, $p = 0.11$); (ii) stress x treatment interaction ($F_{1,14} = 2.93$, $p = 0.10$); (iii) stress x time interaction ($F_{8,112} = 2.01$, $p = 0.05$); (iv) stress x treatment x time interaction ($F_{8,112} = 2.40$, $p = 0.01$). Post hoc analysis (Tukey) showed no significant differences in GBR 12909-increased norepinephrine output in UCMS and control rats.

Figure 11c shows the effect of GBR 12909 (10 mg/Kg i.p.) or saline on norepinephrine extracellular concentration (fmol/20 μl) in stressed (UCMS) rats and relative controls. Three-way ANOVA of the results obtained showed a significant: (i) treatment effect ($F_{1,14} = 17.48$, $p < 0.01$); (ii) time effect ($F_{8,112} = 8.94$, $p < 0.01$); (iii) stress x time interaction ($F_{8,112} = 2.21$, $p < 0.03$); (iv) treatment x time interaction ($F_{8,112} = 8.97$, $p < 0.01$); (v) stress x treatment x time interaction ($F_{8,112} = 2.18$, $p < 0.03$), and a non significant: (i) stress effect ($F_{1,14} = 1.55$, $p = 0.23$); (ii) stress x treatment interaction

($F_{1,14} = 0.01$, $p = 0.90$). Post hoc analysis (Tukey) showed no significant differences in GBR 12909-increased norepinephrine output in UCMS and control rats.

Figg. 11a, 11b, and 11c. Effect of GBR 12909 (10 mg/Kg i.p.) and saline on dialysate dopamine (11a) or norepinephrine (11b) (data expressed as a % of basal levels), and norepinephrine (11c) (data expressed as fmol/20 μl), from the BNST of Control or UCMS rats. (Control n=4; UCMS n=4). *$p < 0.05$ from basal values; #$p < 0.05$ versus the corresponding time point after saline.

DISCUSSION

1. Effect of Acute Antidepressants on Catecholamine Transmission in the BNST

This study shows that antidepressants, when administered acutely (i.p.), increase the extracellular concentration of catecholamines in the BNST. Below is the discussion of the data as it has been published the paper that is included in the appendix of this document: Cadeddu R., Ibba M., Sadile A., Carboni E.: "Antidepressants Share the Ability to Increase Catecholamine Output in the Bed Nucleus of Stria Terminalis: a Possible Role in Antidepressant Therapy?" Psychopharmacology (Berl). 2014 May; 231(9):1925-33. doi: 10.1007/s00213-013-3335-y. Epub 2013 Nov. 13. PubMed PMID: 24221827.

What this study has shown is that various antidepressants share the property of increasing norepinephrine and dopamine output in the BNST. Because of the complexity of BNST monoamine innervations and projections (Dong and Swanson, 2006), the results observed will be discussed separately. Moreover, given that the observed effects are produced by the acute administration of the antidepressants tested, we will limit the discussion on the pharmacological relevance of the observed effects to a minimum, highlighting instead how brain circuitry may contribute to these observed effects.

1.1. Desipramine and Reboxetine Effect

The effect of desipramine on norepinephrine output appears to be complex since dose dependency can only be observed in the first hour after treatment, whereas afterwards, levels of norepinephrine remain elevated for over 3 hours, independently of dose. Desipramine is a rather selective NET blocker as its affinity for human NET is 0.8 nM, e.g., 22 and 4.000 times higher than it is for SERT and dopamine transporter (DAT), respectively (O'Donnell and Shelton, 2011). Desipramine's brain elimination half life in adult Sprague-Dawley rats is reported to be 3.3 hours, while brain concentration 2 hours after 10 mg/Kg i.p. administration (Kozisek *et al.*, 2007) is about 30 nM; this suggests that the enduring elevated norepinephrine output is likely

due to a slow clearance as further discussed below. It has been shown that desipramine increased basal and stimulated (70 mM K^+) norepinephrine output in the ventral BNST when administered locally (2-10 μM) through the microdialysis fiber, although the effect on basal output was weak and dose independent. Even though release in BNST was tetrodotoxin-sensitive and modulated by α2 drugs, these authors are suggesting that there is a minor role of NET blockade in BNST norepinephrine transmission (Forray et al., 1997). It is a proposition in apparent contrast with the prominent role of NET blockers in the therapy of mood disorders and with the relay role that has been attributed to BNST in anxiety related circuitry and in stress induced HPA activation (Koob et al., 1999; Forray and Gysling, 2004; Morilak et al., 2005; Choi et al., 2007; 2008). Although we observed that desipramine, at the higher doses tested, produced a strong increase (340 % of basal) in norepinephrine output, our data is only partly in contrast with that of Forray and colleaugues, suggesting that the effect of norepinephrine release in the BNST could also have a relevant extra-BNST component.

Reboxetine is the first selective norepinephrine inhibitor used in the treatment of depression. It has been claimed to have only minimal side effects (Hajós et al., 2004) although doubts have been recently raised on its efficacy (Eyding, 2010). Its affinity for human NET is 7 nM, e.g., 8 and 1.600 times higher than it is for SERT and DAT, respectively (O'Donnell and Shelton, 2011). Reboxetine is rapidly adsorbed (0.5-2 hours) and its half life is about 1-2 hours (Dostert et al., 1997). In this study, reboxetine increased norepinephrine output in a clearly dose-dependent manner and, as is the case with desipramine, the effect was long lasting. The voltammetry study of norepinephrine release in the ventral BNST (Park et al., 2009) showed that systemic desipramine (15 mg/Kg) increased stimulated norepinephrine release, whereas the observed slow reuptake was suggestive of volume transmission. On the strength of these observations, the long-lasting effect of desipramine and reboxetine on norepinephrine output observed here does appear to be compatible with volume transmission (Cragg and Rice, 2004).

Understanding depression and antidepressant action is complex because antidepressants act at different sites in different brain areas and, above all, long-term circuitry adaptation is crucial for both disorder development and for therapeutic effect. Interestingly, a relationship appears to exist between each of the three main brain monoamine neurotransmitters and specific symptoms of major depressive disorder

(e.g., serotonin - anxiety and obsessions; norepinephrine - alertness and energy; dopamine - motivation, pleasure, and reward); on the other hand, mood improvement is a common effect of various antidepressants (Della Pasqua, 2010). Some recent studies (Holmes *et al.*, 2003) have explored antidepressants that do not directly target monoamine neurotransmitters. The features of BNST in terms of location, inputs, and outputs suggest that it can have a crucial role in the circuitry that mediates the effects of antidepressants. Indeed, BNST is implicated in the modulation of behavioral and neuroendocrine responses to stress, a process whose alteration may well have repercussions in the etiology of depression (Morilak *et al.*, 2005; Itoi and Sugimoto, 2010). In particular, anterior BNST can modulate HPA axis through GABA and CRF innervations of the paraventricular nucleus (Radley and Sawchenko, 2009; 2011). Likewise, norepinephrine plays a role in this circuit by modulating glutamate and GABA transmission within BNST (Forray and Gysling, 2004; Park *et al.*, 2009). In fact, confirmation of the synaptic interaction between dopamine (e.g., tyrosine hydroxylase containing) and norepinephrine (e.g., dopamine-β-hydroxylase-containing) axons and CRF neurons in the BNST was demonstrated some years ago (Phelix *et al.*, 1994). These authors also showed that dopamine terminals form synapses with dendrites and soma of CRF neurons in the dorsolateral BNST, whereas norepinephrine terminals form synapses with dendrites of CRF neurons in the ventrolateral BNST. In addition, the role of CRF inputs and outputs of BNST in depression is supported by the fact that CRF_1 antagonists are tested clinically in depression (Kehne, 2010).

We also observed that desipramine or reboxetine increased dopamine output in the BNST in a time and dose dependent manner. In a previous study (Carboni *et al.*, 2006), we reported that besides reboxetine, GBR 12909 (selective DAT blocker) also increased dopamine output in the BNST, while a concurrent administration of the two drugs generated a cumulative effect, suggesting the likelihood that both carriers capture dopamine. The type of probe implanted in both studies collects the dialysate from dorsal and ventral anterior BNST (roughly in the proportion of two thirds and one third, respectively); thus, the increase in dopamine output can be attributed to the non-specific reuptake blockade only if dopamine and norepinephrine are released in the same sub-region. Conversely, through voltammetry studies, Park and colleagues (2009) suggested that catecholamine release in the ventral BNST should be exclusively norepinephrine, whereas Herr and colleagues (2012) suggested the presence of

norepinephrine innervation in the dorsomedial BNST. Therefore, although we cannot exclude that dopamine output increase may be partially due to non-specific reuptake of dopamine by NET in the dorsal BNST, it appears likely that most of the dopamine increase can be attributed to direct release stimulation, as a consequence of multiple synapse interaction activated by NET blockade. This hypothesis is supported by the fact that ventral tegmental area (VTA) and BNST are reciprocally innervated (Hasue and Shammah-Lagnado, 2002; Dong and Swanson, 2006) and by the report that reboxetine (i.v.) administration increased VTA burst firing and dopamine output in the prefrontal cortex (PFC) (Linner et al., 2001).

1.2. *Effects of Imipramine, Citalopram, and Fluoxetine*

A comparison of the effect of imipramine with that of desipramine or reboxetine shows that the dose of 20 mg/Kg produced a much stronger effect on norepinephrine and dopamine output. Imipramine's affinity for human NET is 37 nm, e.g., about 50 and 5 times less than desipramine and reboxetine, respectively, while its affinity for DAT is in the micromolar range. Conversely, imipramine's affinity for SERT is 1.4 nM, much higher than that of reboxetine and desipramine, 58.8 and 17.5, respectively (O'Donnell and Shelton, 2011). Thus, considering that desipramine is the major active liver metabolite of imipramine, we hypothesized that imipramine's effect on norepinephrine and dopamine output is the result of SERT and NET blockade succeeding. In view of the fact that selective serotonin reuptake inhibitors (SSRIs) are the most prescribed form of drugs for the treatment of anxiety disorders, and considering the substantial evidence suggesting that BNST mediates many forms of anxiety-like behavior in humans and animals (Walker *et al.*, 2003; Hammack *et al.*, 2009), we thought it would be of considerable interest to test the two selective SSRIs, fluoxetine and citalopram. In agreement with our hypothesis, citalopram and fluoxetine increased norepinephrine and dopamine output although they do not generate metabolites active on NET or DAT and do not directly interact with norepinephrine or dopamine reuptake. The affinity values of citalopram and fluoxetine for SERT are as high as 1.4 and 0.8 nM, respectively, whereas their affinity for NET and for DAT is in the micromolar range. Therefore, the increase of norepinephrine and dopamine could

be ascribed to a receptor-activated mechanism consequent to SSRIs induced serotonin synaptic increase either in the BNST or in other brain areas. Thus, SSRIs may produce catecholamine increase in the BNST by indirectly acting on serotonin receptors located on PFC glutamatergic neurons (Andrade, 2011). Conversely, the description of axo-somatic and axo-dendritic interactions that take place between serotonin and both the dorsolateral and ventrolateral sub-population of CRF neurons in the BNST (Phelix *et al.*, 1992) substantiates the direct role that BNST plays in the acute and pharmacological effects of SSRIs. In accordance with this view, Hammack and colleagues (2009) suggested that changes in the balance of the function of serotonin receptors in the BNST may be involved in the appearance of a pathological state of increased anxiety, whereas Oler and colleagues (2009) found that SERT availability in the BNST correlated positively with individual differences in anxious temperament and stress-induced metabolic activity.

1.3. *Effect of Bupropion*

Bupropion is a rather selective DAT and NET inhibitor and although it has a negligible SERT affinity (Stahl *et al.*, 2004), it has an antidepressant efficacy comparable to that of SSRIs (Fava *et al.*, 2005). Ki affinities for human DAT, NET and SERT are 0.52, 9.1, and 52 μM (O'Donnell and Shelton, 2011). Its effect on catecholamine output in the BNST is apparently predictable and while it is likely due to a local action, an involvement of extra BNST circuits cannot be excluded a "priori".

1.4. *Overall Considerations*

The complexity of neuronal innervations of BNST together with the fact that BNST and its major targets send projections to locus coeruleus (LC), VTA, and raphe nuclei, makes it difficult to characterize the mechanism of each drug tested. Interestingly, both LC and VTA neurons innervate each other in addition to projecting to the cortex and to the BNST (El Mansari *et al.*, 2010), suggesting that the release of each monoamine in the BNST can in turn influence other monoamine circuits. Moreover, VTA and LC

neurons send projections to serotonin neurons of dorsal raphe, which in turn project onto the cortex and the BNST, completing a circuit that is conceivably implicated in the mechanism of action of antidepressants. Hence, it seems reasonable to affirm that antidepressants may indeed produce comparable therapeutic effects even though they act specifically on different monoamine reuptake systems. In particular, the ability of SSRIs to increase both norepinephrine and dopamine in the BNST suggests that catecholamine transmission in the BNST might be part of a common downstream pathway that is involved in the therapeutic mechanism of action of various antidepressants. Thus, it can be hypothesized that a dysfunction of this transmission may well have a significant role in the etiology of affective disorders.

2. Effect of the Acute Systemic Administration of Ketamine on the Catecholamine Transmission in the BNST

The available antidepressant medications have substantial limitations and in particular the low percentage of responders and the two-three week time lag necessary to see the therapeutic effect are the most relevant (Trivedi *et al.*, 2006). However, recent clinical studies, suggested that the anesthetic ketamine, produces, at low doses, a rapid antidepressant response within hours (Berman *et al.*, 2000; Zarate *et al.*, 2006). More interestingly, the rapid action of ketamine was observed in patients who are resistant to several typical antidepressants. In fact, the intravenous infusion of a single sub-anesthetic dose evoked fast-acting and long-lasting antidepressant effects also in treatment resistant depressed patients (Berman *et al.*, 2000; Zarate *et al.*, 2006). Depressed patients reported alleviation of core symptoms within 2 hours of a single low-dose infusion, with effects lasting up to 2 weeks (Kavalali and Monteggia, 2015). Thus it was of interest to test the effects of ketamine on catecholamine transmission in the BNST, and compare its effect with that of typical antidepressants (Cadeddu *et al.*, 2014).

We observed in the present study, that acute systemic i.p. administration of ketamine increased in a time and dose dependent fashion dopamine and norepinephrine extracellular concentration in the rat brain bed nucleus of stria terminalis (BNST). The observed response was short-lasting, reaching a peak above basal level at 40 minutes after the treatment, yet dissipated within 2 hours. Microdialysis studies have shown that systemic administration of the same doses used in this study (10 to 40 mg/Kg), similarly increased extracellular dopamine concentrations in dialysate collected from the PFC (Verma and Moghaddam, 1996; Lorrain *et al.*, 2003).

The neurobiological mechanisms of the antidepressant action of ketamine are complex and ranges beyond the simple blockade of NMDA receptors. Cellular and molecular studies in rats revealed that ketamine remodels brain networks involved in the action of antidepressants through the activation of the mammalian target of rapamycin (mTOR) pathway (Li *et al.*, 2010), activating neurotrophic and synaptogenic processes, and thus producing antidepressant-like effects in several animal models of depression (Garcia *et al.*, 2009; Autry *et al.*, 2011; Li *et al.*, 2011). We do not know what could be the contribution of the increase in catecholamine transmission in the

BNST on the neurotrophic and synaptogenic effects of ketamine, but is significant the fact that very low doses of ketamine produce mild psychomimetic and dissociative effects 30-40 minutes after administration and completely dissipate by 80 minutes (Zarate *et al.*, 2006). The effect of ketamine on catecholamine transmission that we observed in the BNST was short-lasting too, and although we cannot exclude a role of BNST catecholamine transmission in the psycho-mimetic effects, we have to point out that this feature was common to the antidepressants we tested previously (Cadeddu *et al.*, 2014). Thus, considering that the acute effect of antidepressants cannot produce immediately the therapeutic antidepressant effect, understanding the meaning of the observed results appears difficult. Nonetheless, these results suggest that BNST can be a brain area that is directly involved in the antidepressant effect of ketamine. In fact, as far as regards ketamine interaction with the glutamatergic innervation in the BNST, it is relevant that the BNST plays a prominent role in brain integration of acute responses to stressful stimuli, and its ventral portion is one of the brain regions most innervated by norepinephrine (Palkovits *et al.*, 1979). The ventral BNST is interconnected with the dorsolateral sub-region receiving a consistent dopamine innervation, but having little norepinephrine content (Hasue and Shammah-Lagnado, 2002). Excitatory inputs arise from several prefrontal cortex (PFC) sub-regions, from the hippocampus via the ventral subiculum (vSub), and from the basolateral amygdala (Cullinan *et al.*, 1993; Mc Donald, 1998). Interestingly, glutamatergic projections from the vSub to the paraventricular nucleus of the hypothalamus (PVN) relay in different BNST nuclei (Cullinan and Herman, 1993). Several studies revealed the presence of glutamatergic neurons in the ventral BNST (Csaki *et al.*, 2000). The same neurons project to the PVN contributing to normalize the HPA axis activity perturbed by stressful conditions (Forray and Gysling, 2004). This suggest that the BNST is a site where the excitatory hippocampal output is converted into an inhibitory input to the PVN (Radley and Sawchenko, 2011), and provide anatomical support for a possible interaction between norepinephrine and glutamate neurotransmissions.

Additionally, the mRNA for the NR_1 subunit of the NMDA receptor is highly expressed onto noradrenergic cell bodies located in areas A1 and A2 innervating the ventral BNST (Forray *et al.*, 2000). A high density of presynaptic NR_1-containing NMDA receptors is also expressed in the BNST nerve terminals synapting with GABAergic interneurons (Forray *et al.*, 2000). It is also proved that norepinephrine extracellular

levels in the ventral BNST, are regulated by glutamate through NMDA receptors (Forray *et al.*, 1995), suggesting that the activation of subicular afferents may increase norepinephrine extracellular levels in this nucleus. Glu_{N2B} subunit-containing NMDA receptors are also largely expressed in the BNST (Regev *et al.*, 2011). Since their expression is increased after stressful situations (Ventura-Silva *et al.*, 2012), they have been implicated in the expression of anxiety-like behavior. A recent study proposed a specific role for the Glu_{N2B}-NMDA receptors expressed into the BNST in mediating the reduction of the latency for food observed after ketamine administration in the novelty-induced hypophagia test (Louderback *et al.*, 2012).

In addition to a direct effect of ketamine on catecholamine transmission in BNST, it can be considered an indirect effect that occurs through an effect on serotonin transmission in the PFC. In fact, we reported previously that the selective SERT blockers citalopram and fluoxetine could increase dopamine and norepinephrine output in the BNST via an action at PFC level. The interaction between serotonin and glutamate transmission is supported by the presence of cortical $5\text{-}HT_{1A}$ and $5\text{-}HT_{2A}$ receptors of layer V that might cooperate in regulating pyramidal neurons excitability and glutamate release (Andrade *et al.* 2011). Interestingly, ketamine does not produce antidepressant effects in serotonin deprived animals (Gigliucci *et al.*, 2013), suggesting that serotonin tone is required for ketamine's antidepressant activity (Dale *et al.*, 2015). An intriguing hypothesis takes into account a possible involvement of inhibitory systems, thus ketamine preferentially by targeting NMDA receptors expressed onto GABAergic interneurons might prevent GABAergic inhibition of glutamatergic neurons into the medial PFC (Moghaddam *et al.*, 1997). Particularly, the decrease of the inhibitory GABAergic tone may be directed over excitatory projection (Jodo *et al.*, 2005), leading to downstream changes of other neurotransmitter systems. The enhancement of the glutamate outflow from the medial PFC neurons to sub-cortical regions such as the ventral tegmental area or the raphe nuclei may thus increase the dopamine and the serotonin cell firing, and consequently the release of dopamine (Moghaddam *et al.*, 1997) and serotonin (Amargos-Bosh *et al.*, 2005) in the cortex or in other brain areas. Moreover, ketamine effect on dopamine transmission has been also reported by Belujon and Grace (2014).

In conclusion, although the multiple actions of ketamine on different systems make hard to appraise the role catecholamine transmission in BNST in ketamine

antidepressant effect, these results suggest that catecholamine transmission in BNST is likely involved in the antidepressant effect of ketamine.

3. Effect of Adolescent Stress on the Catecholamine Transmission in the BNST

It is widely accepted that chronic stress is strictly related to depression and that Major Depression and the stress response share many mediators, circuitries, and phenomenologies (Gold *et al.*, 2015). Stress precipitates major depression (Kessler *et al.*, 1994) and influences its severity, duration, and natural history (Kendler *et al.*, 1992; 1995; Frank and Thase, 1999; Gold, 2005). This relationship has been the basis for shaping animal models such as the chronic mild stress (CMS) (Willner, 1997). In fact, this model has been extensively used to shed light on the etiology of depression for its ability to induce behavioral, physiological, and neurochemical changes that parallel symptoms of the human disease (Willner, 2005). Moreover, the predictive validity of the model is confirmed by the fact that behavioral abnormalities are reversed by antidepressants of several classes (Willner *et al.*, 1992; Willner, 1997). Particularly, the CMS-induced anhedonia in rodents has been indicated as the equivalent core symptom of human depression. A plethora of studies demonstrated that rats subjected to CMS exhibited a decrease in the consumption of palatable sucrose solution, suggesting that these rats are vulnerable to stress (Willner *et al.*, 2013; Zurawek *et al.*, 2013). Additionally, features such as unpredictability and variability, avoid adaptation to the applied stressors (Bowman *et al.*, 2003; Herman, 2013), reproducing with greater accuracy real word stress situations.

We utilized in this study the procedure set by Willner and colleagues, and we refer to it as "unpredictable chronic mild stress" (UCMS). Negative consequences of stress appear to be exacerbated when they happen during periods of life crucial for the brain development and maturation (McEwen, 2008). In fact, the exposition to stressful events at pre-pubertal or adolescent age may be correlated with the appearance of depressive-related disorders in adolescence or adulthood (Kessler *et al.*, 2001). Thus, the possibility to apply this model at selected life time-points offers potential for identifying specific mechanisms induced by stress that may contribute to a major vulnerability to develop psychopathologies in adulthood.

Glucocorticoid stress hormone secretion represents the primary means by which humans, and other vertebrates, respond and adapt to stressful environmental stimuli throughout life. Within the central nervous system (CNS), the locus

coeruleus/noradrenergic system, the mesocorticolimbic system, the hippocampus and the extended amygdala (ExtA) can all be affected by elevated glucocorticoids, and in turn influence the activity of the stress system (Huizink *et al.*, 2004). The glucocorticoid receptor mediates the cellular effects of glucocorticoids in times of stress (Kitchener *et al.*, 2004), enabling adaptation of neurons and neural circuits, and additionally mediating negative feedback regulation of the HPA axis (Diorio *et al.*, 1993; Mizoguchi *et al.*, 2003; Weiser *et al.*, 2011).

It has been ascertained that stress hormones can also affect the development of both the dopamine and the norepinephrine system, although, because of the diverse experimental stress models used and the diverse parameters assessed, it is difficult to propose a convergent hypothesis that can mechanistically explain the effects of UCMS [see Willner, 2005; Willner et al., 2013 for reviews]. Considering the role of BNST in stress managing (Hammack *et al.*, 2010) and the role of catecholamine transmission in the mechanism of action of antidepressant action, we investigated the changes in catecholamine transmission in the BNST in adult rats exposed to UCMS at peri-adolescent age.

3.1. *Basal Levels*

We observed the following: (i) basal dopamine output in the BNST of adult rats was not affected by the exposition to peri-adolescent UCMS; (ii) basal norepinephrine output in the BNST of adult UCMS rats was higher than that of relative non-stressed controls.

The BNST is a forebrain nucleus in the ExtA positioned to relay between cortical, hippocampal, and amygdala inputs, and stress and reward centers (Drolet, 2009; Park *et al.*, 2009). BNST receives one of the major noradrenergic innervation in the brain (Forray and Gysling, 2004) and the highest norepinephrine levels are found in the anterolateral BNST (Palij and Stamford, 1993), a portion that comprises a high density of CRF-expressing neurons (Moga *et al.*, 1989; Phelix and Paull, 1990) and CRH_{1-2} receptors (Chalmers *et al.*, 1995). Moreover, noradrenergic terminals are mainly located in the ventral BNST (Feldman and Weidenfeld, 2004). It is of interest to consider that projections from the A1 noradrenergic nucleus to the BNST send collateral projections to the central nucleus of the amygdala and the paraventricular nucleus

(PVN) (Phelix *et al.*, 1992; Mulders *et al.*, 1997). Furthermore, anterolateral BNST can modulate HPA axis through GABA and CRF innervations of the PVN (Radley and Sawchenko, 2009; 2011). Norepinephrine signaling in the BNST has been implicated in mediating control over the HPA axis and in anxiety-like behavior (Cecchi *et al.*, 2002) and can release CRF within the BNST (McElligott *et al.*, 2010; Nobis *et al.*, 2011). Furthermore, prolonged norepinephrine signaling in the BNST induces synaptic plasticity that is modulated by stress (McElligott *et al.*, 2010; McElligott and Winder, 2008). Interestingly, we observed that UCMS exposition determined a long- term increase in norepinephrine basal extracellular concentration in the BNST. This result suggests that it might reflect an adaptive response of the catecholamine circuitry to the altered responsiveness of the HPA axis.

3.2. *Amphetamine Effect*

In this study, we observed that: (i) amphetamine-stimulated dopamine but not norepinephrine output (expressed as a percentage of basal level) in the BNST of adult UCMS-exposed rats was significantly higher than that observed in the relative controls; (ii) amphetamine-stimulated norepinephrine output (expressed as fmol/sample) in the BNST of adult UCMS rats was higher than that observed in the relative controls, although not significative.

Amphetamine interacts with brain circuitry on multiple levels acting as a dopamine, norepinephrine and serotonin indirect agonist (Carboni and Silvagni, 2004). In particular, amphetamine can increase dopamine (Carboni *et al.*, 2000a) and norepinephrine output by means of several mechanisms primarily involving dopamine reuptake and vesicular monoamine transport, but also synthesis and degradation (Sulzer, 2005). We investigated the effect of a low dose (0.5 mg/Kg) of amphetamine on dopamine and norepinephrine output in the BNST of UCMS and control rats, in order to ascertain whether UCMS could induce changes in catecholamine transmission that may be complementary to the effect on basal dopamine and norepinephrine output.

The main mechanism through which amphetamine enhances catecholamine output is by binding to the vesicular transporter VMAT2 (Sulzer *et al.*, 2005), thus increasing

catecholamines in the cytosol, and determining the reversed functioning of membrane transporter, evoking catecholamine diffusion in the extracellular space (Sulzer *et al.*, 2005). The stronger effect of amphetamine on dopamine output in adult UCMS rats might be due to the higher vesicle transmitter content available for amphetamine action. Amphetamine data suggest that UCMS effects may influence dopamine vesicle storing and releasing machinery. As far as regards, the percentage of norepinephrine increase was not different, but the absolute amount released was higher in UCMS exposed rats, suggesting the presence in these rats of a larger releasable pool available for amphetamine action. Interestingly, it was previously observed that amphetamine acute administration produced a higher dopamine and norepinephrine release in the nucleus accumbens (NAcc) shell of prenatally stressed rats (Silvagni *et al.*, 2008; Carboni *et al.*, 2010), suggesting a possible action of glucocorticoids on catecholamine transmission in the BNST.

On the other hand, the observed effects might be due to the indirect action of amphetamine on BNST dopamine and norepinephrine output through local or extra-BNST circuits. According, in a recent study, the learned helplesness (LH) model of depression altered the activity of dopaminergic neurons in the ventral tegmental area (VTA), and the normal processing information in the ventral subiculum (vSub)-NAcc shell pathway (Belujon and Grace, 2014). Abnormalities of the prefrontal cortex (PFC) and hippocampus function are moreover reported in a number of studies (Leuner and Shors, 2013; McEwen and Morrison, 2013). Thus, being the BNST an integrant part of these complex circuitries, it is feasible hypothesize an increased catecholamine release in this terminal region, as we observed in this study. The BNST is reciprocal connected with both inhibitory and excitatory VTA components, playing a crucial role as a relay station for the PFC excitation of the dopamine VTA neurons (Massi *et al.*, 2008). Other excitatory inputs include projections from the hippocampus via the vSub, and from the basolateral amygdala (Cullinan *et al.*, 1993; Mc Donald, 1998), a region involved in the expression of anxiety and fear-related behavior (Ledoux, 2000).

These results might also have importance for a major predisposition to develop drug abuse disorders in adulthood. It has been hypothesized that the progression to addiction requires the engagement of neuronal stress/anxiety systems (Koob and Wolkov, 2010), including the release of norepinephrine in the BNST (Delfs *et al.*, 2000; Koob, 2009). Consequently, UCMS may unbalance the delicate relationship between

dopamine and norepinephrine transmission in this critical area for the expression of emotional affective states, reward mechanisms, and stress response as well.

3.3. *Reboxetine and GBR 12909 Effects*

In this study, we observed that: (i) reboxetine-stimulated dopamine and norepinephrine output (expressed as a percentage of basal level or as fmol/sample) in the BNST of adult UCMS rats was not significantly different from that observed in the relative controls.

We also observed that: (i) GBR 12909-stimulated dopamine and norepinephrine output (expressed as a percentage of basal level) in the BNST of adult UCMS rats was not significantly different from that observed in the relative controls; (ii) GBR 12909-stimulated norepinephrine output (expressed as fmol/sample) was not significantly different between experimental groups, although in UCMS adult rats basal norepinephrine levels were significantly higher than that of relative controls.

The effect of systemic administration of reboxetine, a selective norepinephrine reuptake inhibitor, and GBR 12909, an inhibitor of the dopamine reuptake, has been evaluated in order to clarify a possible alteration of these mechanisms induced by the exposition to UCMS. Neurotransmitter levels in the extracellular space are determined by the balance between release and uptake (Wightman *et al.*, 1988). Release is a function of the rate of impulse flow (action potentials), the relative amount of neurotransmitter in the releasable pool, and regulation by auto-receptors. Reuptake is a fundamental mechanism by which transmitter monoamines are cleared from the extracellular space. Various drugs, by interfering with this mechanism, elicit an increase in monoamine concentrations around the terminals and on the receptors, thus resulting in a potentiation of monoaminergic transmission.

In particular, the fact that reboxetine effect was lower in UCMS exposed rats (although did not reach significance), as compared with controls, suggests that the catecholamine reuptake in BNST is reduced in UCMS rats although this effect has to be investigated further. The fact that a specific norepinephrine reuptake blocker increases dopamine output is not surprising because in BNST dopamine can be also captured also by norepinephrine transporter (NET) as previously observed (Carboni

et al., 2006). On the other hand surprisingly, we observed that the specific dopamine reuptake blocker GBR 12909 increased besides dopamine also norepinephrine in the BNST, and although this effect was not significantly different in UCMS exposed rats, we observed a tendency to lower effect in these rats. Of course although we observed an alteration of catecholamine transmission in the BNST of UCMS exposed rats, we cannot exclude that other changes may occur in other brain area and in turn produce an alteration of the firing dependent release. The local injection of reuptake blocker in UCMS rats will probably be able to verify this hypothesis.

As mentioned above, BNST receive a consistent norepinephrine, as well as dopamine innervation. Particularly, the anterior BNST receives dense inputs of both catecholamines. The dorsolateral BNST, receives dopaminergic innervation from the VTA, dorsal raphe nucleus and periacqueductal grey area, containing little norepinephrine (Hasue and Shammah-Lagnado, 2002; Meloni *et al.*, 2006). On the contrary, the ventral BNST has a dense noradrenergic innervation, but little dopamine content (Delfs *et al.*, 2000; Park *et al.*, 2009). In the present study, the probe implanted collects the dialysate from dorsal and ventral anterior BNST roughly in the proportion of two thirds and one third, respectively.

3.4. *Overall Considerations*

A number of evidence demonstrates a connection between the brain development and the mesocorticolimbic dopamine system maturation during adolescence. Dopamine systems undergo substantial reorganization (Spear, 2000), showing a considerable plasticity until late adolescence and early adulthood (Benes *et al.*, 2000). As in humans (Seeman *et al.*, 1987), dopamine forebrain innervation is still maturing during rodent adolescence (Spear, 2000). Structural and functional changes of mesolimbic dopamine system are found in several brain areas during this developmental stage (Spear, 2000; Robinson *et al.*, 2011). These modifications affect dopamine basal levels (Badanich *et al.*, 2006), dopamine synthesis, turnover (Teicher *et al.*, 1993; Andersen *et al.*, 1997), and reuptake (Wahlstrom *et al.*, 2010b). Nonetheless, dopamine receptors expression and binding are affected (Tarazi *et al.*, 1998b; 1999; Andersen *et al.*, 2000; Doremus-Fitzwater *et al.*, 2010).

Stress has complex interactions with development such that individuals may be especially vulnerable to stressors during specific developmental periods (Heim and Nemeroff, 2001; Casey *et al.*, 2010). Adolescence is a critical window in stress susceptibility as this is a time of substantial cerebral development and reorganization, as well as altered HPA function (Spear, 2000; Teicher *et al.*, 2003; Romeo and McEwen, 2006; Danese *et al.*, 2009; Casey *et al.*, 2010). The increasing prevalence of clinical depression in adolescence can be attributed to hormonal changes in brain (Angold *et al.*, 1999), an altered reaction of the post-pubertal body (Susman *et al.*, 1987), and excessive exposition to interpersonal stress (Leadbeater, 1999). Adverse life experiences occurring at this developmental stage, for instance inadequate emotional contact with parents (Slavich *et al.*, 2011), childhood abuse (Kendler *et al.*, 2002; 2006), bullying (Gladstone *et al.*, 2006), often are forerunner of adult depression (Kessler and Walters, 1998; McCormick *et al.*, 2008), as well as schizophrenia or drug abuse (Adriani and Laviola, 2004). The knowledge of neurobiological mechanisms and neuronal circuits involved in the effect of adolescence stress on depression and drug dependence, can be useful in understanding the mechanism of action of the drugs currently used as well as can contribute to the development of new therapeutic agents for depression.

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Acknowledgment

Table of Contents

ORIGINAL INVESTIGATION

Antidepressants share the ability to increase catecholamine output in the bed nucleus of stria terminalis: a possible role in antidepressant therapy?

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Abstract

Rationale Antidepressants include a relatively wide spectrum of drugs that increase the synaptic concentration of monoamines, mostly through neurotransmitter reuptake blockade. The bed nucleus of stria teminalis (BNST) is considered a relay station in mediating the activation of stress response but also in the acquisition and expression of emotions. BNST is richly innervated by monoamines and sends back projections to the nucleus of origin. We previously showed that the administration of selective blockers of norepinephrine transporter (NET) increases the extracellular concentration (output) of dopamine, suggesting that dopamine could be captured by NET in the BNST.
Objectives The aim of this study, carried out by means of in vivo microdialysis, was to ascertain the acute effects that antidepressants with varying mechanisms of action have on dopamine and norepinephrine output in the BNST.
Results We observed that all the antidepressants tested (5–20 mg/kg i.p.) increased the output of catecholamines, dose dependently. In particular, the maximum increases (as a percent of basal) for norepinephrine and dopamine respectively, were as follows: desipramine, 239 and 137; reboxetine, 185 and 128; imipramine, 512 and 359; citalopram, 95 and 122; fluoxetine, 122 and 68; bupropion, 255 and 164.

Conclusions These results suggest that catecholamine transmission in the BNST may be part of a common downstream pathway that is involved in the action mechanism of antidepressants. Consequently, it is hypothesized that a dysfunction of neuronal transmission in this brain area may have a role in the etiology of affective disorders.

Introduction

Among psychiatric disorders, depression has a high incidence and an elevated societal cost (Pincus and Pettit 2001). Although the etiology of depression is largely unknown, it is widely accepted that the concurrence of genetic and epigenetic factors determines the manifestation of this illness (Feder et al. 2009; Schroeder et al. 2010). There is growing evidence that among epigenetic factors, exposure to chronic stress has a prominent role in the etiology of depression (Belmaker and Agam 2008). When stress occurs, either in adolescence or adulthood, not only does it activate the hypothalamic–pituitary–adrenocortical axis (HPA) and the autonomic stress system (Ulrich-Lai and Herman 2009), but it also affects many brain areas innervated by monoamines. In particular, noradrenergic transmission plays a major role in the physiological response to environmental challenges and stress (Itoi and Sugimoto 2010). The homeostatic response to chronic stress may in turn lead to a malfunctioning of a broader brain neuronal circuitry, which may contribute to the manifestation of depression symptoms in individuals (Feder et al. 2009; Mann and Currier 2010).

One of the brain areas most innervated by norepinephrine neurons is the bed nucleus of stria terminalis (BNST; Brownstein and Palkovitzs 1984). In particular, the relationship of norepinephrine to *corticotrophin releasing factor* (CRF) neurons in the BNST suggests that BNST is involved in the

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adaptive response to stress (Morilak et al. 2005), and possibly in the etiology of depression (Koob 1999). In addition, it has been reported that immobilization stress significantly elevated plasma ACTH levels and as much as doubled norepinephrine levels in rats' lateral BNST (Pardon et al. 2003). Also, injection in the lateral BNST of the α1-adrenergic antagonist benoxathian attenuated the stress-induced increase in plasma ACTH (Pardon et al. 2003) and blocked anxiety-like behavioral responses (i.e., reduction of the open-arm exploration in the elevated plus maze; Cecchi et al. 2002). Further, removal of norepinephrine input by a bilateral BNST microinjection of an antibody against dopamine β-hydroxylase blunted a subsequent response of the HPA axis to a yohimbine challenge (Banihashemi and Rinaman 2006).

Oler et al. (2009) reported that availability of serotonin transporters in the BNST correlated positively with individual differences in anxiety behavior, so it appears that serotonin transmission is also involved in the role of BNST in anxiety and also most probably in depression. In addition, together with serotonin transmission, norepinephrine transmission is the main target of antidepressant therapy (Goddard et al. 2010; Haenisch and Bönisch 2011). Besides norepinephrine, norepinephrine transporter (NET) blocker antidepressants can also increase extracellular dopamine levels by preventing its reuptake by NET in brain areas such as the prefrontal cortex (PFC) and the BNST, being innervated by both norepinephrine and dopamine neurons (Carboni et al. 2006).

Conversely, antidepressants that selectively block the serotonin transporter (SERT) can influence norepinephrine and dopamine transmission in the PFC via mechanisms other than by reuptake blockade (Tanda et al. 1994; Pozzi et al. 1999; Bymaster et al. 2002). This evidence suggests that dopamine transmission in the PFC may play a role in the therapy of depression but also that other brain areas such as the BNST may also be involved. This is not surprising given that the BNST is an area that has been incorporated into the extended amygdala and is involved in the acquisition of emotions and in motivated behavior (Alheid et al. 1998). Because depression is considered to be a disease of diminished motivation (Nestler and Carlezon 2006), it appears reasonable to assume that BNST might be involved in depression etiology as well as in the mechanism of action of antidepressants. Furthermore, there is evidence to support the hypothesis that dopamine mesolimbic and mesocortical systems are involved in hedonia and motivation, two core symptoms of depression (Yadid and Friedman 2008). A dysfunctional dopamine system is, therefore, likely to be involved in the pathophysiology of depression (Martin-Soelch 2009).

Given this premise, we considered it expedient to study the effect of different antidepressants on dopamine and norepinephrine transmission in the BNST, through the assessment of their extracellular concentration (output) in freely moving rats, by means of the microdialysis technique. The results of this investigation are likely to shed light on the involvement of BNST catecholamine transmission in the therapeutic action of antidepressants.

Materials and methods

Animals Male Sprague–Dawley (S.D.) rats weighing 230–250 g [Harlan, S. Pietro Natisone, Italy] were group housed under standard conditions of temperature, humidity, and artificial light (light, 8 AM to 8 PM).

Probes Concentric dialysis probes were prepared with a 7 mm piece of AN 69 (sodium methallyl sulfate copolymer) dialysis fiber (310 and 220 μm, outer and inner diameter, respectively; Hospal, Dasco, Italy), sealed at one end with a drop of epoxy glue. Twenty-four hours later, the sealed end was sharpened to a bevel tip to reduce tissue damage during implanting. One of two 4-cm long pieces of fused silica (Composite Metal Services, Ilkley, UK) was sharpened to make a bevel tip and was then introduced into a 20 mm piece of stainless steel (obtained by cutting the end part of 24-gauge needle and perforating a side hole at 5 mm from the sharpened end using an abrasive disk), positioning the sharpened end of the silica to protrude from the sharpened end of the needle by 9 mm. The second 5-cm long piece of silica was introduced through the side hole of the 20-mm needle piece until it emerged from the sharpened end by a length of 6.5 mm. The two silica tubes were sealed to the sharpened end of the needle with epoxy glue and were pushed into the dialysis fiber, making sure that the longer of the two fused silica tubes reached the lower end of the dialyzing portion of the fiber (2.0 mm). The dialysis fiber was covered with a thin layer of epoxy glue, except for the dialyzing portion. The open end was then sealed to both the silica tubing and the stainless steel tubing. The segment protruding from the side hole of the 20-mm metal tubing was introduced into a 1.7-mm tubing (prepared in the same way as the 20 mm one), adapting the sharpened part to the side hole made in the 20 mm tubing. The two pieces of metal tubing were introduced into a 7-mm long piece of 200 μl micropipette tip, to which they were then glued. The fiber was covered with a thin layer of epoxy glue except for the dialyzing part. On completion of assembly, the probe was left to dry out for 24 h. In vitro probe recovery was 29.3±3.4 and 32.5±4.7 % (n=4) for dopamine and norepinephrine, respectively.

Surgery and experiments Rats, anesthetized with 100 mg/kg i.p. ketamine (Ketalar®, Farmaceutici Gellini, Milan, Italy) and 10 mg/kg i.p. xylazine (Sigma Milano, Italy) were placed in stereotaxic apparatus. A small hole was drilled on the side of the exposed skull. The probe was implanted vertically in the right BNST and then fixed to the skull with dental cement (Shofu CX-Plus, GmbH, Germany). The coordinates used

[expressed in millimeter from bregma, according to the atlas by Paxinos and Watson (2007)] were anterior, −0.4; lateral, 1.2; and vertical, −8.0. The rats were housed in a transparent (Plexiglas) hemisphere, covered with a top hemisphere, with food and water available.

Experiments were performed on freely moving rats 24 h after the probe implant. Ringer's solution (147 mM NaCl, 2.2 mM CaCl$_2$, 4 mM KCl) was pumped through the dialysis probe at a constant rate of 1 μL/min. Samples were taken every 20 min and analyzed.

When the basal output of norepinephrine and dopamine reached stable values, rats were given (i.p.) a challenge dose of the drug tested or saline. The norepinephrine and dopamine output was considered stable when the quantity evaluated through the last sample differed less than 10 % from the mean of the previous three samples. Stable levels of neurotransmitters were usually obtained after 2–3 h of dialysis. Basal values (as a mean±SE) of norepinephrine and dopamine were: 41.34 (±1.76; $n =119$) and 23.86 (±0.94; $n =119$) fmol/20 μl sample, respectively. Each implanted rat was challenged with a single dose of the test drug only once. Each drug was tested at doses of 5, 10, and 20 mg, respectively, as follows: desipramine (5, 7 and 8 times), reboxetine (5, 10, and 10 times), imipramine (6, 7, and 7 times), citalopram (5, 6, and 7 times), fluoxetine (5, 5, and 6 times), and bupropion (5, 5, and 6 times). Four rats were administered with saline.

All animal experimentation was conducted in accordance with the guidelines for care and use of experimental animals of the European Communities Council Directive of 24 November 1986 (86/609/EEC and Italian DL 116, dated 27/01/92) and approved by the "Ethics Committee" of the University of Cagliari.

Analytical procedure Dialysate samples (20 μL) were injected without any purification into the injector of a HPLC apparatus equipped with reverse-phase column (C-8 Simmetry, Waters) and a coulometric detector (first electrode, +125 mV; second electrode, −175 mV; ESA Coulochem II, Bedford, MA, USA). The mobile phase composition was 0.1 M sodium acetate, 0.3 mM Na$_2$EDTA, 1.8 mM octanesulfonic acid, 120 ml/L methanol, and pH 5.4. The flow rate was set at 0.6 ml/min while the sensitivity of the assay allowed for the detection of 5 fmole of norepinephrine and dopamine.

Histology Histological analysis was performed in order to locate the position of fiber. At the end of the experiment, rats were anesthetized with chloral hydrate (450 mg/kg i.p.) and killed. The brain was removed and stored in formaldehyde (10 %). Brains were cut on an oscillating microtome (Campden Instuments, Lafayette, IN, USA) in serial coronal slices oriented according to the atlas of Paxinos and Watson (2007). Results from rats implanted outside the BNST were discarded.

Drugs Desipramine HCl, imipramine HCl, bupropion HCl, and fluoxetine HCl from Sigma (Milan, Italy). Citalopram HCl was a gift from Innova Pharma (Milan, Italy) and reboxetine HCL was a gift from Pharmacia Upjohn (Milan, Italy). Drugs were dissolved in saline and injected immediately.

Statistics Statistical analysis was carried out by STATISTICA (Statsoft, Tulsa OK, USA). Two-way analysis of variance (ANOVA) for repeated measures was applied to the data expressed as a percentage of basal norepinephrine and dopamine concentration. Results from treatments showing significant overall changes were subjected to post hoc Tukey's tests with significance set at $p <0.05$. Basal value was the mean of three consecutive samples before treatment.

Results

Figure 1a, b shows that desipramine increased both norepinephrine (217, 255, and 339 %) and dopamine output (150, 206, and 237 %) when administered in doses of 5, 10, and 20 mg/kg, respectively. Two-way ANOVA of the results obtained showed a significant treatment effect ($F_{3,20}=5.44, p < 0.05$ and $F_{3,20}=3.83, p <0.05$), time effect ($F_{9,180}=13.71, p < 0.001$ and $F_{9,180}=16.75, p <0.001$), and time×dose interaction ($F_{27,180}=2.87, p <0.005$ and $F_{27,180}=3.12, p <0.001$) for norepinephrine and dopamine, respectively.

Figure 2a, b shows that reboxetine increased both norepinephrine (129, 204, and 274 %) and dopamine output (106, 206, and 228 %) when administered in doses of 5, 10, and 20 mg/kg, respectively. Two-way ANOVA of the results showed a significant treatment effect ($F_{3,25}=6.36, p <0.05$ and $F_{3,25}=5.35, p <0.01$), time effect ($F_{9,225}=8.99, p < 0.001$ and $F_{9,225}=6.46, p <0.001$), and time×dose interaction ($F_{27,225}=3.93, p <0.001$ and $F_{27,225}=1.86, p <0.01$) for norepinephrine and dopamine, respectively.

Figure 3a, b shows that imipramine increased both norepinephrine (202, 235, and 613 %) and dopamine output (169, 203, and 459 %) when administered in doses of 5, 10, and 20 mg/kg, respectively. Two-way ANOVA of the results obtained showed a significant treatment effect ($F_{3,20}=13.49, p < 0.001$ and $F_{3,20}=3.83, p <0.05$), time effect ($F_{9,180}=29.91, p <0.01$ and $F_{9,180}=16.75, p <0.001$), and time×dose interaction ($F_{27,180}=8.48, p <0.001$ and $F_{27,180}=8.97, p <0.001$) for norepinephrine and dopamine, respectively.

Figure 4a, b shows that citalopram increased both norepinephrine (148, 174, and 195 %) and dopamine output (129, 181, and 222 %) when administered in doses of 5, 10, and
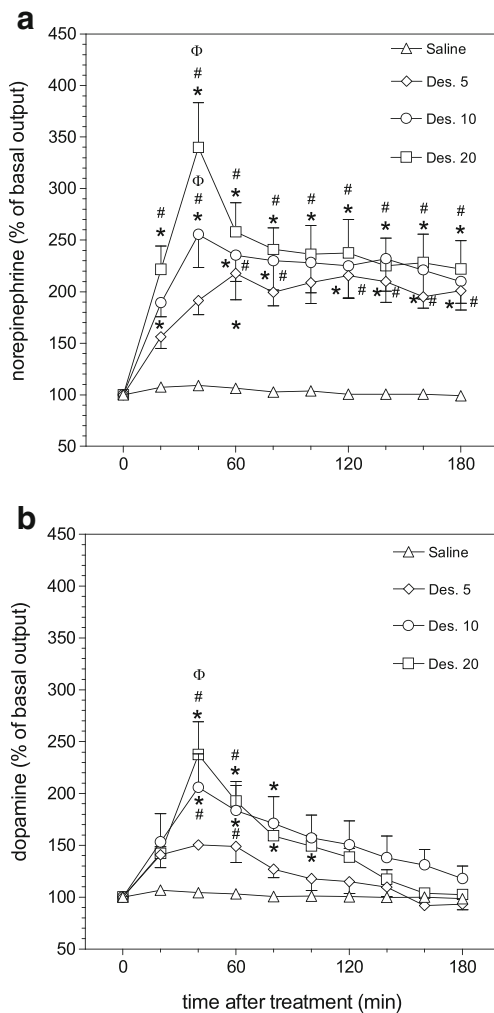
Fig. 1 Effect of desipramine (5, 10 and 20 mg/kg i.p.) on BNST dialysate norepinephrine (**a**) or dopamine (**b**), expressed as a percentage of basal output. Each point is the mean (±SE) of at least four determinations. *$p < 0.05$ from basal values; #$p < 0.05$ versus corresponding time point after saline; Φ$p < 0.05$ versus the corresponding time point of 5 mg/kg

Fig. 2 Effect of reboxetine (5, 10, and 20 mg/kg i.p.) on BNST dialysate norepinephrine (**a**) or dopamine (**b**), expressed as a percentage of basal output. Each point is the mean (±SE) of at least four determinations. *$p < 0.05$ from basal values; #$p < 0.05$ versus corresponding time point after saline; Φ$p < 0.05$ versus the corresponding time point of 5 mg/kg; Ψ$p < 0.05$ versus the corresponding time point of 10 mg/kg

20 mg/kg, respectively. Two-way ANOVA of the results obtained showed a significant treatment effect ($F_{3,20} = 7.26$, $p < 0.005$ and $F_{3,20} = 14.7$, $p < 0.001$), time effect ($F_{9,180} = 17.05$, $p < 0.001$ and $F_{9,180} = 28.88$, $p < 0.001$), and time×dose interaction ($F_{27,180} = 2.53$, $p < 0.001$ and $F_{27,180} = 2.86$, $p < 0.001$).

Figure 5a, b shows that fluoxetine increased norepinephrine (131, 154, and 222 %) when administered in doses of 5, 10, and 20 mg/kg, respectively, and dopamine output by 167 % when administered at 20 mg/kg. Two-way ANOVA of the results obtained showed a significant treatment effect ($F_{3,16} = 3.17$, $p < 0.001$ and $F_{3,16} = 6.48$, $p < 0.005$), time effect ($F_{9,144} = 11.30$, $p < 0.001$ and $F_{9,144} = 2.39$, $p < 0.05$), and time×dose interaction ($F_{27,144} = 2.41$, $p < 0.001$ and $F_{27,144} = 2.35$, $p < 0.001$) for norepinephrine and dopamine, respectively.

Figure 6a, b shows that bupropion increased norepinephrine (144, 266, and 355 %) and dopamine output (158, 250,
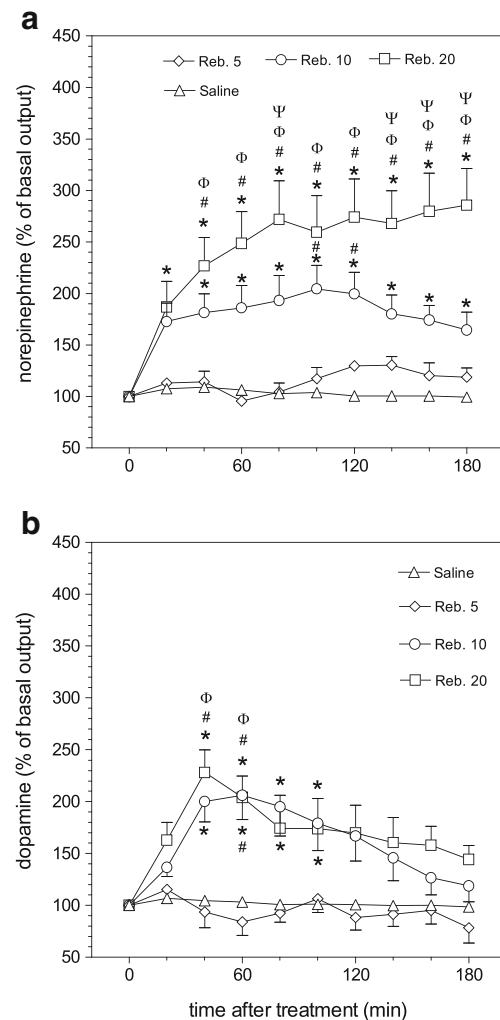
and 264 %) when administered in doses of 5, 10, and 20 mg/kg, respectively. Two-way ANOVA of the results showed a significant treatment effect ($F_{3,16} = 13.07$, $p < 0.001$ and $F_{3,16} = 20.55$, $p < 0.001$), time effect ($F_{9,144} = 43.14$, $p < 0.001$ and $F_{9,144} = 16.73$, $p < 0.001$), and time×dose interaction ($F_{27,144} = 9.58$, $p < 0.001$ and $F_{27,144} = 3.72$, $p < 0.001$) for norepinephrine and dopamine, respectively.

Discussion

What this study has shown is that various antidepressants share the property of increasing norepinephrine and dopamine output in the BNST. Because of the complexity of BNST monoamine innervations and projections (Dong and Swanson 2006), the results observed will be discussed separately.
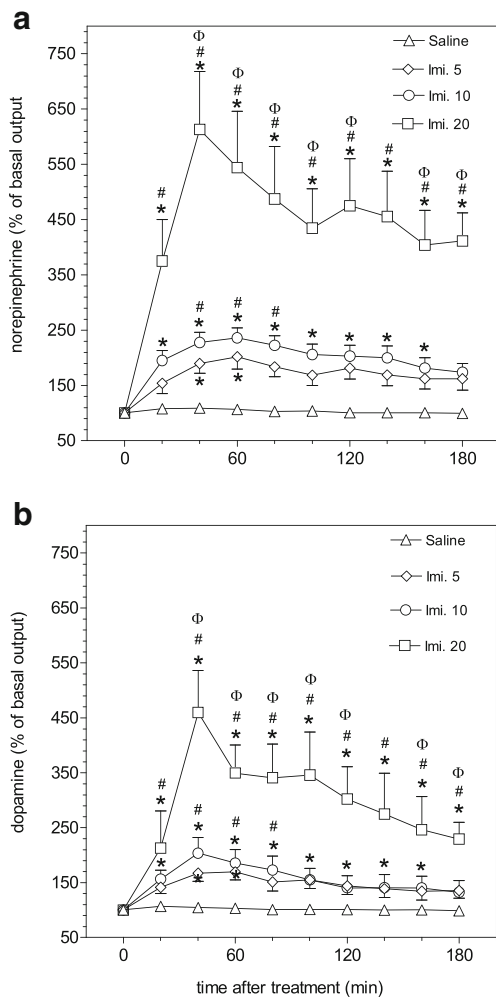
Fig. 3 Effect of imipramine (5, 10, and 20 mg/kg i.p.) on BNST dialysate norepinephrine (**a**) or dopamine (**b**), expressed as a percentage of basal output. Each point is the mean (±SE) of at least four determinations. $*p < 0.05$ from basal values; $^{\#}p < 0.05$ versus corresponding time point after saline; $^{\Phi}p < 0.05$ versus the corresponding time point of 5 mg/kg

Fig. 4 Effect of citalopram (5, 10, and 20 mg/kg i.p.) on BNST dialysate norepinephrine (**a**) or dopamine (**b**) expressed as a percentage of basal output. Each point is the mean (±SE) of at least four determinations. $*p < 0.05$ from basal values; $^{\#}p < 0.05$ versus corresponding time point after saline; $^{\Phi}p < 0.05$ versus the corresponding time point of 5 mg/kg

Moreover, given that the observed effects are produced by the acute administration of the antidepressants tested, we will limit the discussion on the pharmacological relevance of the observed effects to a minimum, highlighting instead how brain circuitry may contribute to these observed effects.

Desipramine and reboxetine effect

The effect of desipramine on norepinephrine output appears to be complex since dose dependency can only be observed in the first hour after treatment, whereas afterwards, levels of norepinephrine remain elevated for over 3 h, independently of dose. Desipramine is a rather selective NET blocker as its affinity for human NET is 0.8 nM, i.e., 22 and 4,000 times higher than it is for SERT and dopamine transporter (DAT), respectively (O'Donnell and Shelton 2011). Desipramine's brain elimination half life in adult S.D. rats is reported to be 3.3 h, while brain concentration 2 h after 10 mg/kg i.p.
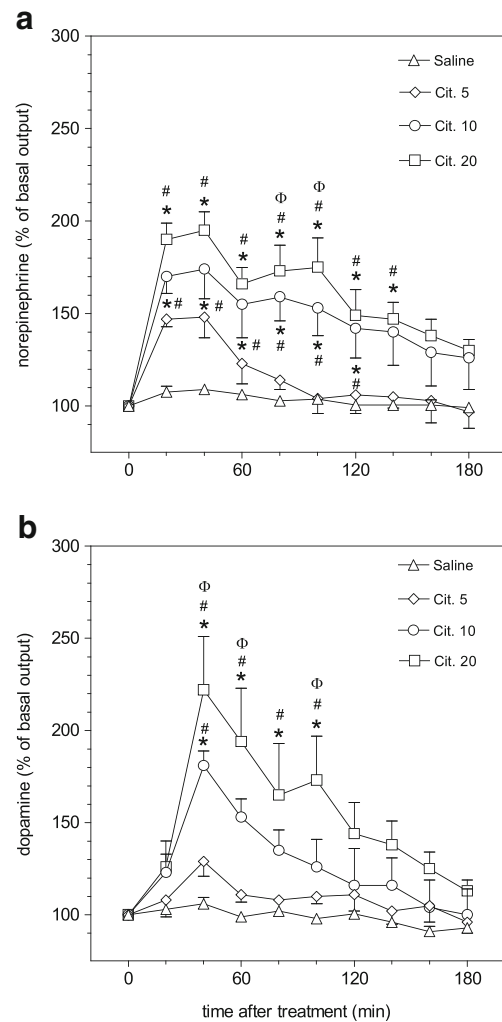
administration (Kozisek et al. 2007) is about 30 nM; this suggests that the enduring elevated norepinephrine output is likely due to a slow clearance as further discussed below. It has been shown that desipramine increased basal and stimulated (70 mM K$^+$) norepinephrine output in the vBNST when administered locally (2–10 μM) through the microdialysis fiber, although the effect on basal output was weak and dose independent. Even though release in BNST was TTX sensitive and modulated by α2 drugs, these authors are suggesting that there is a minor role of NET blockade in BNST norepinephrine transmission (Forray et al. 1997). It is a proposition in apparent contrast with the prominent role of NET blockers in the therapy of mood disorders and with the relay role that has been attributed to BNST in anxiety related circuitry and in stress induced HPA activation (Koob et al. 1999; Forray and Gysling 2004; Morilak et al. 2005; Choi et al. 2007, 2008). Although we observed that desipramine, at the higher doses
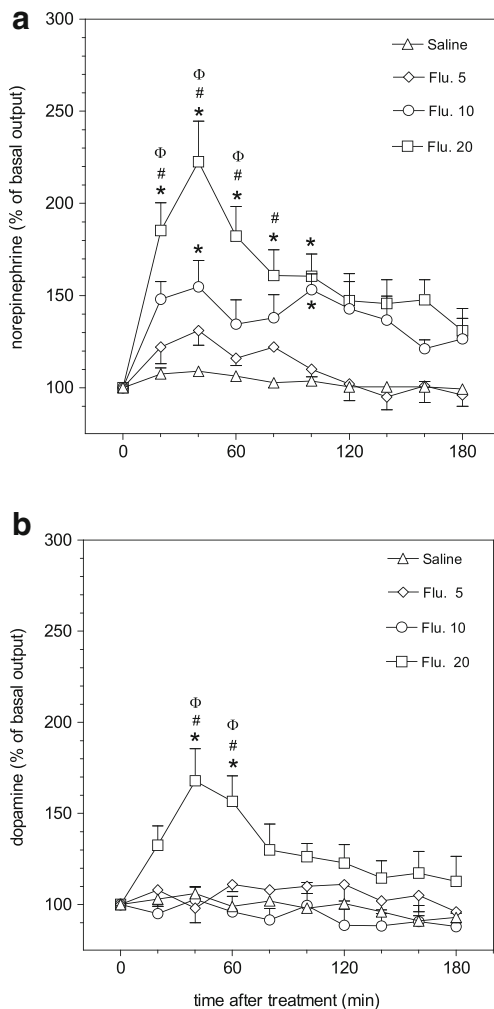
Fig. 5 Effect of fluoxetine (5, 10, and 20 mg/kg i.p.) on BNST dialysate norepinephrine (a) or dopamine (b), expressed as a percentage of basal output. Each point is the mean (±SE) of at least four determinations. *$p < 0.05$ from basal values; #$p < 0.05$ versus corresponding time point after saline; $\Phi$$p < 0.05$ versus the corresponding time point of 5 mg/kg
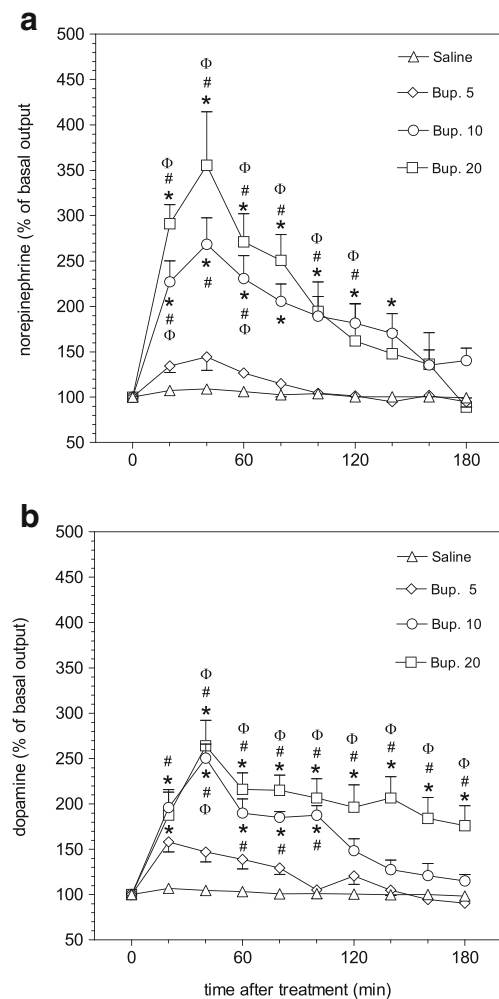

Fig. 6 Effect of bupropion (5, 10, and 20 mg/kg i.p.) on BNST dialysate norepinephrine (a) or dopamine (b) expressed as a percentage of basal output. Each point is the mean (±SE) of at least four determinations. *$p < 0.05$ from basal values; #$p < 0.05$ versus corresponding time point after saline; $\Phi$$p < 0.05$ versus the corresponding time point of 5 mg/kg

tested, produced a strong increase (340 % of basal) in norepinephrine output, our data is only partly in contrast with that of Forray et al. suggesting that the effect of norepinephrine release in the BNST could also have a relevant extra-BNST component. Reboxetine is the first selective norepinephrine inhibitor used in the treatment of depression. It has been claimed to have only minimal side effects (Hajós et al. 2004) although doubts have been recently raised on its efficacy (Eyding 2010). Its affinity for human NET is 7 nM, i.e., 8 and 1,600 times higher than it is for SERT and DAT, respectively (O'Donnell and Shelton 2011). Reboxetine is rapidly adsorbed (0.5–2 h) and its half life is about 1–2 h (Dostert et al. 1997). In this study, reboxetine increased norepinephrine output in a clearly dose-dependent manner and, as is the case with desipramine, the effect was long lasting.

The voltammetry study of norepinephrine release in the vBNST (Park et al. 2009) showed that systemic desipramine

(15 mg/kg) increased stimulated norepinephrine release, whereas the observed slow reuptake was suggestive of volume transmission. On the strength of these observations, the long-lasting effect of desipramine and reboxetine on norepinephrine output observed here does appear to be compatible with volume transmission (Cragg and Rice 2004).

Understanding depression and antidepressant action is complex because antidepressants act at different sites in different brain areas and, above all, long-term circuitry adaptation is crucial for both disorder development and for therapeutic effect. Interestingly, a relationship appears to exist between each of the three main brain monoamine neurotransmitters and specific symptoms of major depressive disorder (i.e., serotonin—anxiety and obsessions; norepinephrine—alertness and energy; dopamine—motivation, pleasure, and reward); on the other hand, mood improvement is a common effect of various antidepressants (Della Pasqua 2010). Some

recent studies (Holmes et al. 2003) have explored antidepressants that do not directly target monoamine neurotransmitters.

The features of BNST in terms of location, inputs, and outputs suggest that it can have a crucial role in the circuitry that mediates the effects of antidepressants. Indeed, BNST is implicated in the modulation of behavioral and neuroendocrine responses to stress, a process whose alteration may well have repercussions in the etiology of depression (Morilak et al. 2005; Itoi and Sugimoto 2010).

In particular, anterior BNST can modulate HPA axis through GABA and CRF innervations of the paraventricular nucleus (Radley and Sawchenko 2009, 2011). Likewise, norepinephrine plays a role in this circuit by modulating glutamate and GABA transmission within BNST (Forray and Gysling 2004; Park et al. 2009). In fact, confirmation of the synaptic interaction between dopamine (i.e., tyrosine hydroxylase containing) and norepinephrine (i.e., dopamine-beta-hydroxylase-containing) axons and CRF neurons in the BNST was demonstrated some years ago (Phelix et al. 1994). These authors also showed that dopamine terminals form synapses with dendrites and soma of CRF neurons in the dorsolateral BNST, whereas norepinephrine terminals form synapses with dendrites of CRF neurons in the ventrolateral BNST. In addition, the role of CRF inputs and outputs of BNST in depression is supported by the fact that CRF1 antagonists are tested clinically in depression (Kehne 2010).

We also observed that desipramine or reboxetine increased dopamine output in the BNST in a time and dose dependent manner. In a previous study (Carboni et al. 2006), we reported that besides reboxetine, GBR 12909 (selective DAT blocker) also increased dopamine output in the BNST, while a concurrent administration of the two drugs generated a cumulative effect, suggesting the likelihood that both carriers capture dopamine. The type of probe implanted in both studies collects the dialysate from dorsal and ventral anterior BNST (roughly in the proportion of two thirds and one third, respectively); thus, the increase in dopamine output can be attributed to the nonspecific reuptake blockade only if dopamine and norepinephrine are released in the same subregion.

Conversely, through voltammetry studies, Park et al. (2009) suggested that catecholamine release in the ventral BNST should be exclusively norepinephrine, whereas Herr et al. (2012) suggested the presence of norepinephrine innervation in the dorsomedial BNST. Therefore, although we cannot exclude that dopamine output increase may be partially due to nonspecific reuptake of dopamine by NET in the dorsal BNST, it appears likely that most of the dopamine increase can be attributed to direct release stimulation, as a consequence of multiple synapse interaction activated by NET blockade. This hypothesis is supported by the fact that ventral tegmental area (VTA) and BNST are reciprocally innervated (Hasue and Shammah-Lagnado 2002; Dong and Swanson 2006) and by the report that reboxetine (i.v.) administration increased VTA burst firing and DA output in the PFC (Linner et al. 2001).

Effects of imipramine, citalopram, and fluoxetine

A comparison of the effect of imipramine with that of desipramine or reboxetine shows that the dose of 20 mg/kg produced a much stronger effect on norepinephrine and dopamine output. Imipramine's affinity for human NET is 37 nm, i.e., about 50 and 5 times less than desipramine and reboxetine, respectively, while its affinity for DAT is in the micro molar range. Conversely, imipramine's affinity for SERT is 1.4 nM, much higher than that of reboxetine and desipramine, 58.8 and 17.5, respectively (O'Donnell and Shelton 2011). Thus, considering that desipramine is the major active liver metabolite of imipramine, we hypothesized that imipramine's effect on norepinephrine and dopamine output is the result of SERT and NET blockade succeeding. In view of the fact that selective serotonin reuptake inhibitors (SSRI) are the most prescribed form of drugs for the treatment of anxiety disorders, and considering the substantial evidence suggesting that BNST mediates many forms of anxiety-like behavior in humans and animals (Walker et al. 2003; Hammack et al. 2009), we thought it would be of considerable interest to test the two selective SSRI, fluoxetine and citalopram. In agreement with our hypothesis, citalopram and fluoxetine increased norepinephrine and dopamine output although they do not generate metabolites active on NET or DAT and do not directly interact with norepinephrine or dopamine reuptake. The affinity values of citalopram and fluoxetine for SERT are as high as 1.4 and 0.8 nM, respectively, whereas their affinity for NET and for DAT is in the micromolar range. Therefore, the increase of norepinephrine and dopamine could be ascribed to a receptor-activated mechanism consequent to SSRI induced 5-HT synaptic increase either in the BNST or in other brain areas. Thus, SSRI may produce catecholamine increase in the BNST by indirectly acting on 5-HT receptors located on PFC glutamatergic neurons (Andrade 2011). Conversely, the description of axosomatic and axodendritic interactions that take place between 5-HT and both the dorsolateral and ventrolateral subpopulation of CRF neurons in the BNST (Phelix et al. 1992) substantiates the direct role that BNST plays in the acute and pharmacological effects of SSRI. In accordance with this view, Hammack et al. (2009) suggested that changes in the balance of the function of 5-HT receptors in the BNST may be involved in the appearance of a pathological state of increased anxiety, whereas Oler et al. (2009) found that SERT availability in the BNST correlated positively with individual differences in anxious temperament and stress-induced metabolic activity.

Bupropion effect

Bupropion is a rather selective DAT and NET inhibitor and although it has a negligible SERT affinity (Stahl et al. 2004), it has an antidepressant efficacy comparable to that of SSRIs (Fava et al. 2005). Ki affinities for human DAT, NET and SERT are 0.52, 9.1, and 52 μM (O'Donnell and Shelton 2011). Its effect on catecholamine output in the BNST is apparently predictable and while it is likely due to a local action, an involvement of extra BNST circuits cannot be excluded a "priori".

Overall considerations

The complexity of neuronal innervations of BNST together with the fact that BNST and its major targets send projections to locus coeruleus (LC), VTA, and raphe nuclei, makes it difficult to characterize the mechanism of each drug tested. Interestingly, both LC and VTA neurons innervate each other in addition to projecting to the cortex and to the BNST (El Mansari et al. 2010) suggesting that the release of each monoamine in the BNST can in turn influence other monoamine circuits. Moreover, VTA and LC neurons send projections to serotonin neurons of dorsal raphe, which in turn project onto the cortex and the BNST, completing a circuit that is conceivably implicated in the mechanism of action of antidepressants. Hence, it seems reasonable to affirm that antidepressants may indeed produce comparable therapeutic effects even though they act specifically on different monoamine reuptake systems. In particular, the ability of SSRI to increase both norepinephrine and dopamine in the BNST suggests that catecholamine transmission in the BNST might be part of a common downstream pathway that is involved in the therapeutic mechanism of action of various antidepressants. Thus, it can be hypothesized that a dysfunction of this transmission may well have a significant role in the etiology of affective disorders.

Conflict of interest I certify that I have no financial interests to disclose.

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