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TITOLO TESI

Role of the Bed Nucleus of Stria Terminalis (BNST) in addiction and depression: a microdialysis study

Settore/i scientifico disciplinari di afferenza

BIO 14

Presentata da:	Dragana Jadžić
Coordinatore Dottorato	Prof. Antonio Argiolas
Tutor	Prof. Ezio Carboni

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Abstract

Introduction: The bed nucleus of stria terminalis (BNST) is a limbic brain area included in the extended amygdala. The BNST is innervated by norepinephrine, dopamine and serotonin projections, it sends and receives a robust corticotropin-realising factor innervation to/from the paraventricular nuclei of hypothalamus. These connections allow the BNST to play a crucial role not only in the control of the stress response [1,2], but also in a control of stressful state associated with drug-seeking that occurs after abstinence from chronic drug exposure [3,4]. Earlier we observed that stimulant and non stimulant drugs of abuse, dose-dependently, increased DA extracellular concentration (output) in the BNST [5], as well as traditional antidepressants [6], confirming a role of this nucleus in drug addiction and depression.

The non selective N-methyl-D-aspartate (NMDA) receptor antagonist ketamine has been suggested as a promising medication in a treatment of therapy-resistant patients, evoking long-lasting effect already after infusion of a single dose [7].

Aim: We investigated here by means of microdialysis the acute effect of stimulant and non stimulant drugs of abuse on norepinephrine transmission in the BNST, to better understand its role in drug abuse, addiction and relapse, as well as the effect of ketamine on NE and DA transmission in the BNST.

Methods: Each implanted rat received a single acute i.p. injection of each dose of tested drugs: nicotine (0.2-0.4 mg/kg s.c), morphine (1.0-3.0 mg/kg s.c.), cocaine (2.5-5.0 mg/Kg mg/kg i.p.), amphetamine (0.25-0.5 mg/kg s.c.) and ethanol (0.5-1.0 g/kg, i.p.); ketamine (10, 20 and 40 mg/kg i.p.).

Norepinephrine was assessed in dialysate samples by HPLC and coulometric detection (ESA). Samples were collected (and immediately analysed) every 20 min (flow: 1 mL/min) from freely moving rats implanted in the BNST (coordinates: ant. - 0.40; lat. 0.8; vert. 8).

Statistical analysis was carried out by STATISTICA (Statsotf, Tulsa, OK, USA). Two-way analysis of variance (ANOVA) for repeated measures was applied to the data expressed as a percentage of basal NE concentration. Results showing significant overall changes were subjected to post hoc Tukey´s tests with significance for $p < 0.05$.

Results: Two-way ANOVA showed that all above listed drugs significantly, dose and time-dependently increased norepinephrine output in the BNST (nicotine: $F_{2,11}$= 23.6, p <0.001, $F_{7,77}$= 18.05, p <0.001; cocaine: $F_{2,9}$ = 37.7, p <0.001, $F_{7,63}$ = 25.9, p <0.001; morphine: $F_{2,13}$ = 10.37, p <0.001 $F_{7,91}$ = 7.21, p <0.001; ethanol: $F_{2,13}$ = 4.85, p <0.05, $F_{7,91}$ = 8.0, p <0.001 and amphetamine: $F_{2,9}$= 41.7, p <0.001, $F_{7,63}$ = 16.85, p <0.001); Post-hoc analysis showed that the increase of NE output obtained in doses of 0.4 mg/Kg for nicotine, 5 mg/kg for cocaine, 3 mg/kg for morphine, 1 g/kg for ethanol and 0.5 mg/kg for amphetamine, was significantly higher than the output produced by lower doses of the same drug or saline.

When it comes to ketamine, it increased NE (127, 155, and 186 %) and DA output (114, 156, and 176 %) when administered in doses of 10, 20, and 40 mg/Kg, respectively. Two-way ANOVA of the results showed for doses of 20 and 40 mg/Kg a significant treatment effect ($F_{3,19}$ = 3.76, p < 0.02 and $F_{3,19}$ = 4.63, p < 0.01), time effect ($F_{9,171}$ = 31.41, p < 0.001 and $F_{9,171}$ = 12.72, p < 0.001), and time × dose interaction ($F_{27,171}$ = 6.29, p < 0.001 and $F_{27,171}$ = 3.04, p < 0.001) for NE and DA, respectively.

Conclusions: These results show that NE transmission in the BNST is involved in the acute effects of drugs of abuse. Taken together with previous findings, it seems that chronic exposure to drugs of abuse can produce an alteration of NE and DA transmission in the BNST, which further may modify the role that this nucleus plays in the stress response. So, we can support previous theories about the role of the BNST in addiction and state that these alterations of neurotransmission in the BNST may be crucial for a stress-induced drug relapse and for the anxiety/stress modulation of drug-seeking.

Results showed that ketamine produces a less increase in NE and DA levels compared with other antidepressants, pointing that the BNST may only be partly involved in its immediate antidepressant effect. Alternatively we may suggest that the modulation of the BNST catecholamine transmission is a condition necessary to produce an antidepressant effect but insufficient to determine the appearance of the effect. However, by influencing levels of DA and NE, ketamine also influences the complex circuit of BNST involved in a control of stress and reward response. Therefore, it is hard to define only one of its mechanisms of action.

***Key words**: ketamine, drugs, abuse, bed nucleus of stria terminalis, catecholamines*

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Preface

This dissertation is submitted for the degree of Doctor of Philosophy, at the University of Cagliari. The subject matter of this scientific report is based on the three year experimental research activity performed at the Department of Biomedical Sciences, Section Neuropsychopharmacology of the University of Cagliari, under the supervision of Prof. Ezio Carboni.

This work is to the best of my knowledge original, except where acknowledgments and references are made to previous work. I further state that no substantial part of my dissertation has already been submitted or is being concurrently submitted for any such degree, diploma or other qualification at the University of Cagliari or any other University or similar institution except as declared in the Preface and specified in the text.

One work presented here has been done in collaboration with colleagues from the same department, as may be seen on PubMed.

CONTENT

FIGURE CONTENT

Abbreviations

AADC- Aromatic L-amino acid decarboxylase

ACTH- Adrenocorticotropic Hormone

ADHD- Attention Deficite/Hyperactivity Disorder

AMPA- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPARs- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors

AT- Aminotransferase

AVP-Vasopresin

BDNF- Brain-derived Neurotrophic Factor

BNST- Bed Nucleus Stria Terminalis

BNSTal- anterolateral

BNSTm- medial

BLA- Basolateral Amygdala

CaMKII- Ca2+/calmodulin-dependent protein kinase II

CeA- Central Amygdala

CeM- Medial division of the Central nucleus of Amygdala

CNS- Central Nervous System

COMT- Catechol-O-methyltransferase

CRF- Corticotropin-releasing Factor

CRFR- Corticotropin-releasing Factor Receptor

CRH- Corticotropin-releasing Hormone

CSF- Cerebrospinal Fluid

DA- Dopamine

DAG- Diacylglycerol

DAT- Dopamine Transporter

DBH- Dopamine β-hydroxylase

DBS- Deep Brain Stimulation

DG- Dental Gyrus

DRD- dorsal raphe nucleus – dorsal division

DSM- Diagnostic and Statistical Manual of Mental Disorders

EATTs- excitatory amino acid transporters

ECF- Extracellular fluid

ED- Electrical Detection

ENK- Enkephalin

EPSCs- Excitatory postsynaptic potential

ExtA- Extended Amygdala

GABA- gamma-Aminobutyric acid

GCs- Glucocotricoids

GDH- Glutamate Decarboxylase

GLU- Glutamate

HNK- Hydroxynorketamine

HPA- hypothalamic–pituitary–adrenal axis

HPLC- High-performance liquid chromatography

HVA- Homovanillic acid

IGF-1- Insulin- like growth factor 1

IL- Infralimbic

IP-3- Inositol trisphosphate

LC- Locus Coeruleus

L-DOPA- L-3,4-dihydroxyphenylalanine

LH- Lateral Hipothalamus

LHb- Lateral Habenula

LTP- Long- Term Potentiation

MAO- Monoamino Oxidase

MAOIs- Monoamino Oxidase Inhibitors

MDD- Major Depressive Disorder

ME- Malic Enzyme

MeA- Medial Amygdala

MFB- Medial forebrain bundle

MHPG- 3-Methoxy-4-hydroxyphenylglycol

MPOA- Medial Preoptic Area

mPFC- medial Prefrontal Cortex

mRNA- Messenger RNA

MSN- Medium spiny neuron

NA- Noradrenaline

NAc- Nucleus Accumbens

NaSSAs- Noradrenergic and specific serotonergic antidepressants

NE- Norepinephrine

NET- Norepinephrine Transporter

NMDA- N-Methyl-D-aspartic acid or N-Methyl-D-aspartate

NMDAR- N-Methyl-D-aspartic acid or N-Methyl-D-aspartate Receptor

NPY- Neuropeptide Y

NTS- Nucleus of the solitary tract

PACAP- Pituitary adenylate cyclase-activating polypeptide

PAG- Phosphate activated glutaminase

PBP- parabrachial pigmented nucleus

PC- Pyruvate carboxylase

PDH- Piruvate dehydrogenase

PKS- Protein Kinase A

PI- Phosphatidyl Inositol

PL- Prelimbic Cortex

PFC- Prefrontal Cortex

PLC- Phospholipase C

PN- Pontine Nuclei

PNMT- Phenylethanolamine N-methyltransferase

POMT- Protein O-mannosyl-transferase

PVN- Paraventricular nucleus of the hypothalamus

RIMAs- reversible inhibitors of monoamine oxidase A

RMC- red nucleus, magnocellular part

S.D.- Sprague- Dawley rats

SERT- Serotonin Transporter

SOM- Somatostatin

SNC- Substantia Nigra pars Compacta

SNR- Substantia Nigra Reticulate

SNS- Sympathetic Nervous System

SNRIs- Serotonin–norepinephrine reuptake inhibitors

SSRIs- Selective serotonin reuptake inhibitors

SUD- Sunstance Use Disorder

TCA- Tricarboxylic acid

TCAs- Tricyclic antidepressant

TH- Tyrosine hydroxylase

TRH- Thyrotropin-releasing hormone

vBNST- Ventral Bed Nucleus of Stria Terminalis

VEGF- Vascular endothelial growth factor

VGluTs- Glutamate Transporters

VLPAG- Ventrolateral Periaqueductal Grey

VMAT2- Vesicular monoamine transporter 2

VTA- Ventral Tegmental Aera

WHO- World Health Orgaization

5-HIAA- 5-Hydroxyindoleacetic acid

5-HT- Serotonin

INTRODUCTION

1.Extended amygdala

The so called complex of the extended amygdala (ExtA) is a large basal forebrain macrostructure which contains highly interconnected and heterogeneous subnuclei, extending from the centromedial amygdala to the bed nucleus of stria terminalis (BNST) (Alheid, 2009). Extended amygdala includes two main structures: the BNST and the Central Nucleus of Amygdala (CeA) (Olmos and Heimer, 1999). Because of their complex interconnectivity and anatomical position, they are considered to be a central region for behavioral and physiological responses to a threat (Davis and Shi, 1999), and a large animal literature suggests that these structures are critical for phasic and sustained fear response to a threat (Davis et al., 2010). These findings, together with those of other researchers, have suggested that the extended amygdala plays a critical role in the development and maintenance of anxiety disorders, depression and substance abuse (Avery et al., 2016; Koob and Volkow, 2010; Shin and Liberzon, 2009).

Our main focus will be on the BNST, nucleus positioned to relay between cortical, hippocampal and amygdalar inputs and stress and reward centres (Drolet, 2009). The neurocircuitry of the BNST is maybe one of the most complex in the central nervous system (Dong et al, 2001b; Dong and Swanson, 2004; Larriva-Sahd, 2006; Bota et al, 2012) and understanding of its connections, of the effect of stress hormones and neurotransmitters on/from the BNST and its overall role in the stress-related disorders, will certainly help finding an efficient therapy for those disorders.

2.Bed Nucleus of Stria Terminalis (BNST)

2.1. Anatomy of the BNST in rodents

There is not only one nomenclature which is used to express anatomy of the BNST, but here it will be used a big number of papers that were published by Dong and Swanson in order to understand better the organization of this complex nucleus (Dong and Swanson, 2003, 2004, 2006a, 2006b, 2006c; Dong et al., 2000, 2001a, 2001b).

BNST is the site where all information correlated with the stress response and the control of emotional, cognitive, autonomic and behavioural functions converge. In particular, BNST plays an important role in the regulation of the hypothalamic–pituitary–adrenal axis (HPA) 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).

The Bed Nucleus of Stria Terminalis is placed between the lateral septal nucleus and the preoptic area of the hypothalamus, surrounding the crossing of the anterior commissure. It is surrounded by globus pallidus from all sides. The BNST has a complex structure which is still poorly understood and it is known now that it is made of different nuclei; initially the BNST was divided into medial and lateral divisions and this was based mostly on amygdalar inputs. That division was abandoned due to investigation of cytoarchitecture by Swanson and Lu which led to divide the BNST into anterior and posterior divisions with up to 12 subnuclei (Swanson and Lu, 1989). Recently, this number was updated and now BNST has up to 18 subnuclei (Bota and Swanson, 2010). By this new nomenclature, the anterior part is made of these accepted subregions: anterolateral, anteromedial, oval, fusiform, juxtacapsular, rhomboid, dorsomedial, ventral nucleus, magnocellular, and all these subregions are

interconnected and connected with regions outside of the BNST. On the other hand, the posterior division is less complex by its anatomy and it is divided in three nuclei: the principal, the interfascicular and the transverse nucleus (Dong and Swanson. 2004).

2.2 Anatomy of the BNST

The main anatomical division of the BNST on the anterior and posterior region was mentioned above. It seems that the posterior region of the BNST is involved in reproduction and social defensive behavior via modulation of the PVN (Lebow and Chen, 2016) and it will not be further discussed since our focus is on stress-related disorders and their mechanisms of development.

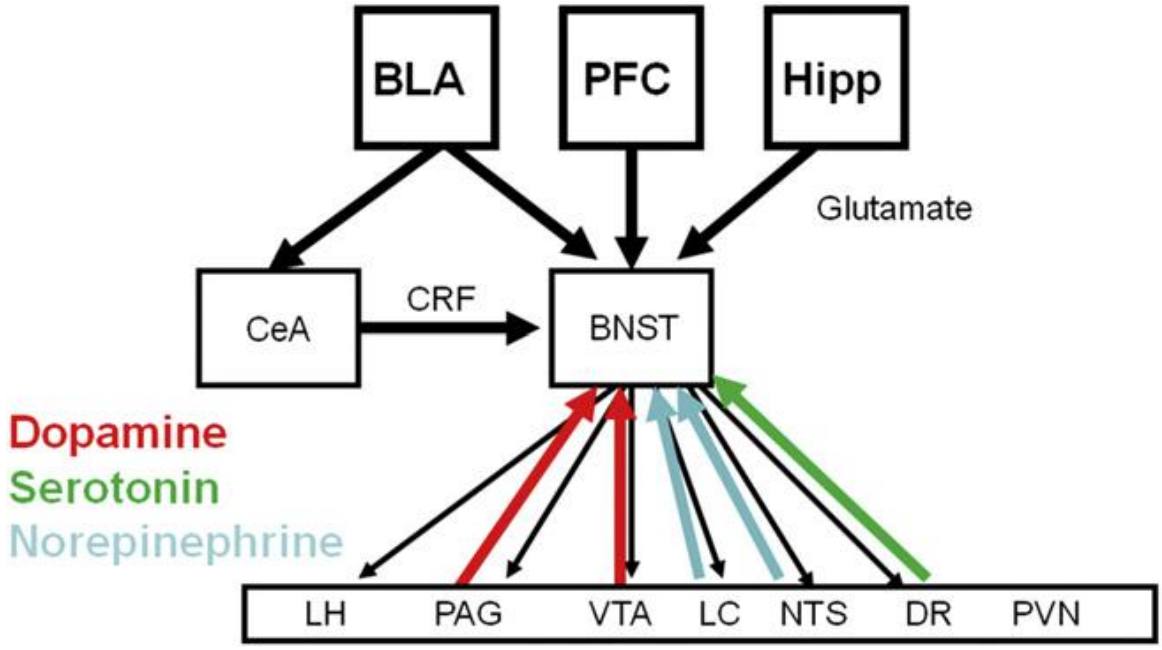

Figure 1- Connectivity of the BNST

(Kash et al., 2012)

On the other hand, the anterior part of the BNST is responsible for different behavioral responses to stress and noxious stimuli, as it will be explained below (Figure 1).

Division of the anterior region was changing during years, together with new discoveries of its anatomy and function. Dong and colleagues have divided the anterior part of the BNST into anterolateral (BNSTalg) and anteromedial (BNSTamg) cell groups. The oval, juxtacapsular, fusiform and rhomboid nuclei belong to the BNSTalg (Dong et al., 2000, 2001b; Dong and Swanson, 2003, 2004a), and this region sends dense projections to the autonomic-related parts of the hypothalamus and lower brainstem. Based on the work of Ju and colleagues (Ju and Swanson, 1989; Ju et al., 1989), Dong and Swanson (2001a) defined two divisions of the BSTamg: the anterodorsal (AD) area and the anteroventral area (AV)—with an undifferentiated region (also called the anteroventral area) and four embedded nuclei (dorsomedial, dorsolateral, ventral, and magnocellular). These areas are projecting to regions of the hypothalamus closely associated with the neuroendocrine system. Therefore, the anterior BNST has numerous connections with the hypothalamus, amygdala, midbrain, as well as with the lower brainstem regions associated with autonomic function, emotional processing, reward and pain (Dong et al, 2001b; Dong and Swanson, 2004). Our focus will remain on this group of nuclei.

2.2.1. *Physiological cell types and the transmitters they use*

There are five physiological classes of BNST neurons that have been described in the literature (Hammack et al., 2007; Francesconi et al., 2009; Szucs et al., 2010; Rodriguez-Sierra et al., 2013). Those are following: low-threshold bursting (LTB; Type II), regular spiking (RS, Type I), with a fast inward rectifying K^+ conductance (fIR; Type III), late-firing and spontaneously active neurons. Type I and II cells have a similar distribution in the anterior division of the BNST. On the other hand, the other three cell types are mostly found in one of the two regions: Type III cells are concentrated in the oval nucleus, spontaneously active cells in BNST-AV, and late-firing cells in BNSTalg (Rodriguez-Sierra et al., 2013).

Gamma-Aminobutyric acid (GABA) neurons, including projection cells, are predominant neurons in the anterior division of the BNST (Cullinan et al., 1993; Sun and Cassell, 1993; Polston et al., 2004; Poulin et al., 2009). Nevertheless, it seems that there is a distinct glutamatergic population of projection neurons, like some functional assays proves, as well as the presence of mRNA coding multiple vesicular glutamate transporters (Georges and Aston-Jones, 2002; Allen Institute for Brain Science, 2008). In addition, neurons of the BNST can express a variety of peptides in multiple combinations (Gray and Magnuson, 1987; Ju et al., 1989; Moga et al., 1989). Among these are corticotropin-releasing factor (CRF), enkephalin (ENK), neuropeptide Y (NPY), neurotensin, and somatostatin (SOM) (Walter et al, 1991). This gives a functional diversity to the BNST, since it has been shown that different subregions within the same nucleus are found to exert opposite effects on the anxious state (Kim et al., 2013), which will be explain later.

2.2.2. BNST regions

As previously said, the anterior region of the BNST is divided in two main cell groups: lateral group, also known as anterolateral group (BNSTalg; Dong et al., 2000, 2001a; Dong and Swanson 2003, 2004a) and the medial group of the anterior division (Dong and Swanson 2006b).

BNSTalg. The BNST anterolateral region is involved in a mechanism of negative feedback of the HPA axis (Lebow et al., 2012), sending GABAergic projections to neuroendocrine centers of the paraventicular area of the hypothalamus (Dong and Swanson, 2004) and receiving inputs from the ventral subiculum's glutamatergic projections (Forray et al., 2014). Therefore, these inputs are of importance for labelling one experience as a positive, or as anxiety- like experience. Moreover, anterolateral region has many sensory and motor related projections towards the vagus nerve. In that way, BNST controls digestive

homeostasis (Dong and Swanson, 2006), especially in response to noxious stimuli ("learned anorexia associated with noxious stimuli").

The oval nucleus is a place for integration of mood and negative valence information, using GABA, CRF, pituitary adenylate cyclase-activating polypeptide (PACAP), dopamine (DA) and enkephalin (Kim et al., 2013). Thus, this nucleus promotes anxiety-like behaviour. GABAergic neurons project to the CeA, ventral tegmental area (VTA) and lateral hypothalamus; some of these projections co-express CRF (Forray and Gysling, 2004). Levels of the CRF in the dorsal BNST, where the oval nucleus is positioned, are increased after chronic stress and foot shock (Daniel and Rainnie, 2015). Dopaminergic receptors in the oval nucleus receive VTA and dorsal raphe inputs (Park et al., 2013). Optogenetic stimulation of these DA neurons in the oval nucleus led to the expression of anxiogenic behaviour (Forray and Gysling, 2004).

The fusiform nucleus sends projections to the CeA, PVN, nucleus accumbens (NAc), peri-aqueductal gray and reticular nuclei (Lebow and Chen, 2016). On the other hand, the juxtacapsular nucleus projects to the CeA, basolateral amygdala, substantia nigra and dorsal raphe (Dong et al., 2001; Dong and Swanson, 2004). Therefore, their outputs go to a similar circuitry, but to different levels. CRF mRNA is highly expressed in the fusiform nuclei and oval nuclei (Choi et al., 2007).

The rhomboid nucleus projects to the CeA and to important brain centers, from mesolimbic reward centers (e.g. VTA) to the substantia nigra, NAc and globus pallidus (Dong and Swanson, 2004). Moreover, it sends GABAergic and glutamatergic projection to the hypothalamus (Li et al., 2012).

BNSTamg. The anteromedial cells group of the BNST, also called the BNST anteromedial area (BNSTam; Dong and Swanson 2006a), along with the dorsomedial nucleus

of the BNSTamg (Dong and Swanson, 2005a), is generating the most prominent BNST inputs directly to the region of hypothalamic neuroendocrine secretomotoneuron pools. This region has the highest density of direct projections to the PVN of any brain region and also projects directly to the CeA (Dong and Swanson, 2006a).

The anteromedial area has a role in stress-regulated autonomic control; it receives inputs from medial preoptic area (MPOA) and other hypothalamic areas, from the medial amygdala (MeA) and posterior BNST (Dong and Swanson, 2006; Gomez and Newman, 1992). In addition, this region has the highest density of direct projections to the PVN of any brain region and also projects directly to the CeA (Dong and Swanson, 2006). Thanks to these connections, HPA axis peripheral changes can be either activate or attenuate by BNST, depending on the olfactory information received. Moreover, this BNST region projects to Barrington's nucleus, which is involved in drinking and fluid homeostasis. BNST projections to the PVN descend to the brainstem. In this way BNST is regulating pelvic functions via the Barrington's nucleus (Dong and Swanson, 2006a). This involvement of the BNST in a connection between the PVN and digestive homeostasis is most likely critical for HPA axis response and 'fight or flight' implementation (Lebow and Chen, 2016; Allgulander, 2009).

The dorsomedial BNST has the most extensive direct projections to the hypothalamus; it sends mostly GABAergic outputs to the parvocellular PVN neurons that produce thyrotropin-releasing hormone (TRH), CRF, somatostatin and gonadotropin-releasing hormone (Dong and Swanson, 2006b). This region receives input from the basomedial amygdala (BLA), MeA and the CeA, as well as the ventral subiculum of the hippocampus, the insula and infralimbic prefrontal regions; moreover, DA inputs from the VTA, serotonergic inputs from the raphe nuclei and NA inputs from the locus coeruleus and nucleus of the solitary tract (Dong and Swanson, 2006b). Overall, the dorsomedial region of the BNST might be a place where social information integrates, and based on processing of

these information, it might lead to activation of the HPA axis to initiate fight or flight behaviors and adjustment of mood and reward circuitry (Lebow and Chen, 2016).

The largest density of noradrenergic fibers in the brain is located in the AV BNST, which gives it a role of the center of arousal (Forray et al., 2014); these fibers originate from A1 (caudal ventrolateral medulla) and A2 (nucleus of the solitary tract) cell bodies in the brainstem, and the small number originates from the locus coeruleus (Forray et al., 2014; Park et al., 2013). The anteroventral area of the BNST medial cell groups is also contributing to the regulation of the HPA axis (Herman et al., 2005), regulating in this way anxiety-like responses. Indeed, the most part of the PVN BNST projections originates from BNST-AV (Sawchenko and Swanson, 1983; Moga and Saper, 1994). These projections are mostly GABAergic (Radley et al., 2009; Radley and Sawchenko, 2011), although some glutamatergic (Csa´ki et al., 2000) and CRF-expressing (Moga and Saper, 1994) cells also project to PVN. In this region, excitatory inputs from medial prefrontal cortex (mPFC) and subiculum are transformed into inhibitory output onto PVN (Radley et al., 2009; Radley and Sawchenko, 2011). On contrary, there are evidences suggesting that although glutamatergic projections to PVN count for minority, their influence, excitation of the PVN, appear to be dominant (Choi et al., 2007; Crane et al., 2003).

2.3. Projections of the BNST

2.3.1. Noradrenergic and CRFergic Inputs to the BNST

The BNST is the place with the highest concentration of noradrenalin in the brain (Brownstein and Palkovits, 1984; Kilt and Anderson, 1986). As it was previously said, these noradrenergic nerve terminals in the vBNST originate mainly from cell bodies located in the brainstem noradrenergic cell groups A1 and A2 (Forray et al., 2014, Park et al., 2013) and in a lesser degree, from the LC. The neurons of these regions are the same group of cells that innervate the PVN, CeA and, in a lesser extent, the MeA (Riche et al., 1990; Roder and Ciriello, 1994; Wong et al., 2000; Woufle et al., 1990). It has been shown that the A1 group of cells send collateral axonal projections to the CeA and the BNST (Roder and Ciriello, 1994). Although the distribution of NA nerve terminals in the vBNST is heterogeneous, the highest concentration is found in the rostral region (Fuentealba et al., 2000), and it seems that there is a tonic NA activity in this BNST division (Forray et al., 1997; Palij and Stamford, 199240, 90). As a result, there is a constant noradrenergic tone over the most of the output neurons in the vBNST.

It has been shown that the oval and fusiform nucleus of the BNST contains a high density of corticotropin-releasing factor expressive neurons (Dong et al., 2001a; Dong and Swanson, 2006a). These neurons are predominantly GABAergic (Dabrowska et al., 2013). Furthermore, anterolaterale BNST receives strong CRF inputs from the lateral sector of the CeA (Sakanaka et al., 1986). Norepinephrine neurons make synaptic contacts with dendrites of the CRF neurons in the ventrolateral BNST; dopamine neurons innervate soma and dendrites of the CRF neurons in the dorsal part (Phelix et al., 1994); also serotonin neurons innervate CRF neurons in both areas (Phelix et al., 1992).

2.3.2. Dopaminergic Inputs to the BNST

The BNST takes place in the centre of dopamine-rich areas, positioned laterally (caudate putamen/globus pallidus) and dorsally (nucleus accumbens shell). In particular, the dorsal-lateral BNST is heavily interconnected with the VTA (Dong and Swanson, 2004). Furthermore, BNST receives dopaminergic inputs from periacqueductal grey area (Li et al., 2016). 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. More about the role of the BNST in a reward circle will be discussed in the Addiction chapter.

2.3.3. Glutamate in the BNST

The BNST is densely innervated by ventral subiculum of the Hippocampus, as its efferents for the PVN relay in different BNST and in other hypothalamic nuclei (Cullinan et al., 1993). These subicular projections arrive to the BNST through two main output pathways of hippocampus and amygdala: the fimbria–fornix and the stria terminalis, in this order (Cullinan et al., 1993; Kohler, 1990; Krettek and Price, 1978). Furthermore, the nature of these projections, according to existed biochemical evidence, is mainly glutamatergic/aspartergic (Fonnum et al., 1981; Walaas et al., 1980). Overall, it can be concluded that the BNST is a place where the excitatory hippocampal output is converted into an inhibitory input to the PVN. To confirm this statement we should look at work of Herman et al. that have proposed that the hippocampus, through previously described subicular projections, enhances the GABAergic tone at the PVN and therefore exerts an inhibitory action over the activation of the HPA axis (Herman et al., 1997; 1998). Moreover, the same group has showed that by damaging the subiculum or transecting the fornix upon

restraint-stress and open field exposure, the expression of CRH mRNA and its peptide in the PVN is increasing, as well as the corticosterone secretion. This is supporting the hippocampal inhibitory role upon the HPA axis activity (Herman et al., 1992; 1995; 1998).

As it was mentioned before, although the most of the vBNST neurons are GABA-ergic, there are evidences proving that there is a presence of glutamatergic (GLU) neurons (Csaki et al., 2000). In addition, the mRNA encoding the NR1 subunit of the N-methyl-daspartate (NMDA) glutamate receptor is expressed on NA bodies in A1 and A2 innervating the vBNST (Forray et al., 2000), and this subunit is distributed both in dendrites and in nerve terminals (Gracy and Pickel, 1995). Another study (Forray et al. 1995) showed that NMDA, a specific agonist of NMDA receptors, stimulates in vitro NA release in the ventral BNST minislices; moreover, it was demonstrated that in the presence of d-serine, an agonist of the glycine site associated with the NMDA receptor, this effect was significantly higher. Overall, it can be concluded that the extracellular levels of NA in the ventral BNST are regulated by glutamate through NMDA receptors, suggesting that the activation of subicular afferents may increase NA in the extracellular space of this region of the BNST.

On the other hand, another study of Forray et al. (1999) showed that the BNST NA, acting trough α_2- and α_1-adrenergic receptors, exerts an inhibitory effect upon GLU release. Moreover, NA also has an inhibitory effect upon extracellular GLU, though through activation of α_2-adrenergic receptors. Thus, vBNST NA could regulate the conversion of excitatory subicular outputs into an inhibitory input to the PVN.

2.3.4. Serotoninergic inputs to the BNST

The main source of the BNST serotoninergic inputs is the dorsal raphe nucleus (Phelix et al., 1992). Some subclasses of this nucleus, which are targeting the BNST, can be activated by stress events and anxiogenic drugs (Hammack et al., 2009). Furthermore, several studies have suggested the function of serotonin receptors located in the BNST. For example, inhibition of 5-HT_{1a} receptor in the BNST is producing an anxiolytic effect (Levita et al., 2004). On the other hand, activation of excitatory receptors 5-$HT_{2a,2c,7}$, would have the opposite effect (Guo et al., 2009). In accordance with this is also a study on 5-HT_{2c} receptor knockout mice, which shows that there is reduced stress-induced activation of CRF neurons in the BNST. Immunohistochemical studies suggest that this receptor might regulate CRF neurons in the BNST (Heisler et al., 2007).

2.3.5. BNST and its efferents towards Ventral Tegmental Area

The ventral BNST sends numerous efferents of a different nature to the VTA (Geisler and Zaham, 2005; Dong and Swanson, 2004; Dumont and Williams, 2005; Jalabert et al., 2009; Kudo et al., 2012). First it was reported that the influence of the BNST on DA cell firing within VTA is mostly excitatory, through glutamatergic efferents (Georges and Aston- Jones, 2002). Later studies have showed that the most of the vBNST efferents are GABAergic (Jennings et al., 2013; Kudo et al., 2012); since there is a small population of GLU neurons in the vBNST, the effect of these projections to VTA might have an additional role in the regulation of the VTA. It is known now that VTA GABAergic neurons are sending inhibitory projections to local DAergic neurons, controlling in that way reward-related and aversive behaviours (Johnson and North, 1992; Omelchenko and Sesack, 2009; Tan et al., 2012; van Zessen et al., 2012). Taking this into account, the conclusion is that BNST GABAergic neurons mainly exert disynaptic disinhibition of VTA DAergic neurons via VTA

GABAergic neurons. It was also found that some of BNST GABAergic neurons send projections directly to DA VTA neurons, but in lesser extent. The role of this BNST- VTA connection will be further discussed in the Addiction chapter.

3. Stress

3.1. Definition of Stress

When our organism is confronted with endogenous or exogenous unpleasant events, it responds by serious of behavioural, endocrinal, immune and neuronal events called stress response. Those unpleasant events are called stressors and stress is body's reaction to a challenge, which is causing a disbalance in the homeostasis of the body. Stress response, and therefore the adaptation of the body to disruption of homeostasis, is individual and depends on the continuous physiological interaction between three basic systems involved in the stress response: hormonal, immune and nervous systems. These systems are closely interconnected and regulate each other by means of feedback mechanisms. Stress response is meant to protect us from stressors, to move our organism to the homeostatic point which is our optimal condition for living. Therefore, once the threat is gone and the stress response occurred, the system reaches basal conditions again and this stress episode does not have any long term negative impact on our health. However, some stressful situations are continuous, like every-day life challenges, and the stress response becomes more or less uninterrupted. In this case, stress is causing long-term consequences and may render stress systems hyperresponsive or even hyporesponsive, putting the organism at risk for stress-related diseases such as depression.

This complex mechanism of physiological and pathological stress-response of the body's systems, as well as the possible role of stress in a development of stress-related diseases, will be further discussed in details.

3.2. Physiology of stress response- The HPA axis

Physiological stress responses have a fast onset and the goal to optimize the re-establishment of homeostasis through mobilization of resources (Munch et al., 1984). The main system of stress response is the HPA axis that consists of three major structures: the paraventricular nucleus of the hypothalamus, the anterior lobe of the pituitary gland and the adrenal gland (Smith and Vale, 2006). In addition to this, some other systems are also included in the regulation of stress response: the noradrenergic system (locus coeruleus), the sympathetic/adrenomedullary system and the parasympathetic systems (Habib et al., 2001; Chrousos, 1992; Whitnall 1993).

The HPA axis is the main regulatory system of adaptive response of organism to stress. The principle regulator of this axis is the corticotropin-releasing factor, produced by hypophysiotropic neurons localized in the medial parvocellular subdivision of the PVN (Vale et al., 1981; Rivier and Vale, 1983). Activation of CRF receptors on pituitary corticotropes results in release of adrenocorticotropic hormone (ACTH) into the systemic circulation. ACTH then is transported by bloodstream to the adrenal gland, where it binds to the receptor in the adrenal cortex. In this way ACTH is stimulating glucocorticoid synthesis and secretion from the zona fasciculata. Therefore, glucocorticoids present the downstream effectors of the HPA axis (Figure 2). Moreover, after acute exposure to stressors, elevated levels of circulating glucocorticoids inhibit HPA activity via negative feedback at the level of the hypothalamus and pituitary gland (Keller-Wood and Dallman, 1984). In addition, parvocellular neurons of the PVN synthesize and release vasopresin (AVP) into the portal circulation, where this peptide potentiates the effects of CRF on ACTH release from the anterior pituitary gland (Whitnall, 1993; Rivier and Vale, 1983; Antoni, 1993).

Beside this endocrine regulation of the HPA axis there is also neuronal regulation, which relays on many afferent projections to the hypophysiotropic neurons of the PVN of the hypothalamus. These afferents include those from brainstem, other hypothalamic nuclei and forebrain limbic structures (Figure 2).

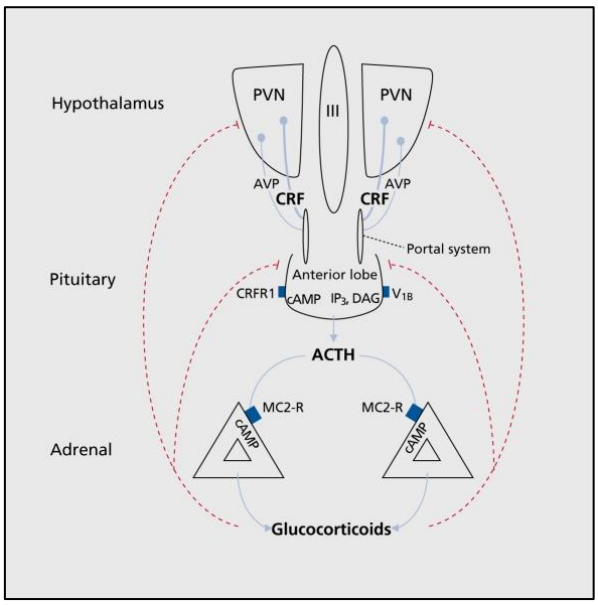

Figure 2- Anatomy of the stress response

(Smith and Vale, 2006)

3.2.1. Neuronal regulation of the HPA axis

One of the most important systems of the brainstem is the catecholaminergic system and it includes the Nucleus of the solitary tract (NTS). This nucleus receives inputs from periphery through cranial nerves and central inputs from limbic structures that regulate behavioral responses to stress, including the medial prefrontal cortex (mPFC) and the central nucleus of the amygdala. Catecholaminergic input to the HPA axis represents a major excitatory drive and this input, through α_1 adrenergic receptor-dependent mechanism, induces

CRF expression and protein release (Plotsky 1987; Widmaier et al., 1988; Plotsky et al., 1989).

PVN receives also inputs from hypothalamic nuclei and these connections are using mostly GABA as a neurotransmitter (Roland and Sawchenko 1993). Hypophysiotropic neurons of the PVN express GABA-A receptor subunits (Cullinan, 2000), and through these receptors, GABA exerts inhibitory effect on glutaminergic transmission in the PVN upon exposure to stress (Cullinan et al. 1996; Cullinan and Wolfe, 2000).

Limbic structures included in the regulation of stress response, hippocampus, mPFC and amygdala, are implicated in cognitive/affective responses to stress and in endocrine adjustments (Cullinan et al., 1995, Dayas et al., 2001, Li and Sawchenko, 1998). The hippocampus and mPFC have excitatory outputs, using glutamate as a transmitter (Swanson and Cowan, 1977, Walaas and Fonnum, 1980), but having GABAergic relays (Cullinan et al., 1993; Herman et al., 2003).

Hippocampus plays an important role in terminating HPA axis responses to stress (Jacobson and Sapolsky, 1991; Herman et al., 2005). Activation of hippocampal neurons results in a decrease of neuronal activity in the parvocellular division of the PVN and therefore in the inhibition of glucocorticoid secretion (Rubin et al. 1966; Sapolsky at al., 1984; Saphier and Feldman, 1987). The study of Cullinan and colleagues (1993) showed that these inhibitory influences of the hippocampus on the PVN might be relayed through specific portions of the BNST.

While localization of stress-induced inhibitory sources of hypothalamus has been successful, doing the same with the mPFC is more elusive. mPFC has a stress-inhibitory influence on HPA axis (Dario et al., 1993; Figuerido et al., 2003) and neurons of the mPFC release catecholamines when activated by stress (Cullinan et al., 1996; Finlay et al., 1995;

Jedema et al., 1999). There is a topographic organization of mPFC: prelimbic (PL) and infralimbic (IL) regions have an opposite role in a regulation of HPA axis. IL region lesions lead to inhibition of the HPA axis in response to acute emotional stress, which means that IL region normally has an excitatory influence on the activation of the HPA axis (Radley et al., 2006). On the other hand, lesions to PL region enhance HPA axis activation in response to acute stressful experiences; therefore, this region has capacity to restrain HPA axis activation (Radley et al., 2006). It seems that PL region exhibits this influence over acute stress-induced HPA output through the anterior subdivision of the BNST; the BNST has a discrete population of GABAergic, PVN-projecting neurons (Dong et al., 2001) that show a diminished activation following PL lesions. In addition, selective ablation of these neurons results in an improvement of stress-induced HPA axis activation, as in a case of PL lesions (Radley et al., 2009). To date, there is only one work that suggests that the same region of the BNST is also involved in IL modulation of stress response (Spencer et al., 2005), probably through a contiguous or interposed group of PVN-projecting excitatory neurons (Choi et al., 2007).

The last but not the least key to the limbic control of stress response is the amygdala. Opposite to the hippocampus and the prefrontal cortex, the amygdala is thought to activate the HPA axis since some studies have shown that the stimulation of amygdalar neurons promotes glucocorticoids synthesis and their release into the systemic circulation (Matheson et al., 1971; Van de Kar and Blair, 1999). The medial and central nuclei of the amygdala regulate the HPA axis by contributing to the majority of afferent projections from the amygdala to cortical, midbrain and brain stem regions that regulate adaptive responses to stress. The CeA sends numerous projections to the NTS (Schwaber et al., 1982), while the MeA sends a limited number of direct projections to the parvocellular division of the PVN (Canteras et al., 1995). In contrast to the effects on hippocampal and cortical neurons,

glucocorticoids increase expression of CRF in the CeA, potentiating autonomic responses to chronic stressors (Herman et al., 2005; Akana et al., 2001).

3.2.2. Sympathetic circuits and stress response

Activation of the brain stem noradrenergic neurons and sympathoadrenomedullary system occurs within seconds of perceived stress. Sympathetic nervous system (SNS) excitation promotes norepinephrine-induced changes in numerous bodily systems.

The locus coeruleus is the part of the brain with the largest cluster of noradrenergic neurons and it has been involved in a wide range of physiological and behavioral functions including emotion, vigilance, memory and adaptive responses to stress (Aston-Jones et al., 1986 and 1991; Valentino et al., 1998). Stressful events activate LC neurons and this induces NE release (Abercrombie and Jacobs, 1987; Passerin et al., 2000; Dayas et al., 2001) which in turn activates HPA-axis.

There is one pronounced interaction of CRF and norepinephrine systems central nervous systems. This interaction works as a feedforward system at multiple levels: CRF activates norepinephrine and norepinephrine in turn activates CRF (Koob, 1999). There are many evidences supporting an important role of CRF-NE interaction in the LC region in a response to stressors (Valentino et al., 1991, 1993; Van Bockstaele et al., 1998). This will be discussed in details in the next chapter.

3.2.3. CRF and stress response

Discovery of the CRF by Vale et al. (1981) led to the series of discoveries in a field of stress physiology and stress-related pathologies. CRF is synthesised by the hypophyseotropic neurons in the medial dorsal parvocellular subdivision of the PVN (Figure 3). But that is not

the only source of CRF since it is widely distributed in the CNS (Merchenthaler, 1984; Palkovits et al., 1985; Swanson et al., 1983).

Upon the exposition to stress, there is a robust and immediate release of corticotropin-releasing hormone (CRH) from the PVN neurons into the hypophyseal portal circulation that depletes neuropeptide stores at the axon terminals (Plotsky, 1985). As it was previously explained, the PVN receives stress-evoked impulses from the brainstem, the hippocampus and the limbic structures. Connections with the brain stem are direct and noradrenergic and the stress-induced increase of CRH mRNA in PVN depends on the integrity of this connection (Pacak et al., 1996). On the other hand, limited factor for basal and stress-induced CRF activity in the PVN are glucocorticoids.

A lot of studies show the capacity of stress-induced corticosterone to stimulate CRH expression in several brain areas. The most prominent CORT effect was found in the CeA and the BNST (Makino et al., 1994; Schulkin et al., 1998; Watts and Sanchez-Watts, 1995), which might mean that these regions are of foremost importance for the role of the CRF in adaptive stress response. Moreover, the levels of mRNA for CRF in the PFC and hippocampus increase after exposure to the stressful stimuli (Givalois et al., 2000; Chen et al., 2004).

CRH is involved in many aspects of the stress response (Figure 2). Hypophyseotropic CRH starts the neuroendocrine stress cascade, whereas the central CRH initiates a series of physiological and behavioral changes in order to prepare the organism for “fight or flight” reaction, and at the same time it inhibits vegetative and reproductive functions and alters immunity. As it was mentioned above, upon the stressful event the central CRH stimulates LC neurons which results in elevated NA levels (Berridge and Waterhouse, 2003; Valentino et al., 1991). In return, NA stimulates further CRF release. However, norepinephrine also

stimulates CRF release in the PVN of the hypothalamus (Alonso et al., 1986), BNST and CeA. Moreover, it has been shown that stress-induces increase of CRH in the BNST and amygdala, which results in anxiety in rats (Shepard et al., 2000, 2009). In addition, infusion of CRH into the BNST and amygdala provokes number of fear-related behavioral responses (Lee and Davis, 1997; Shepard et al., 2000).

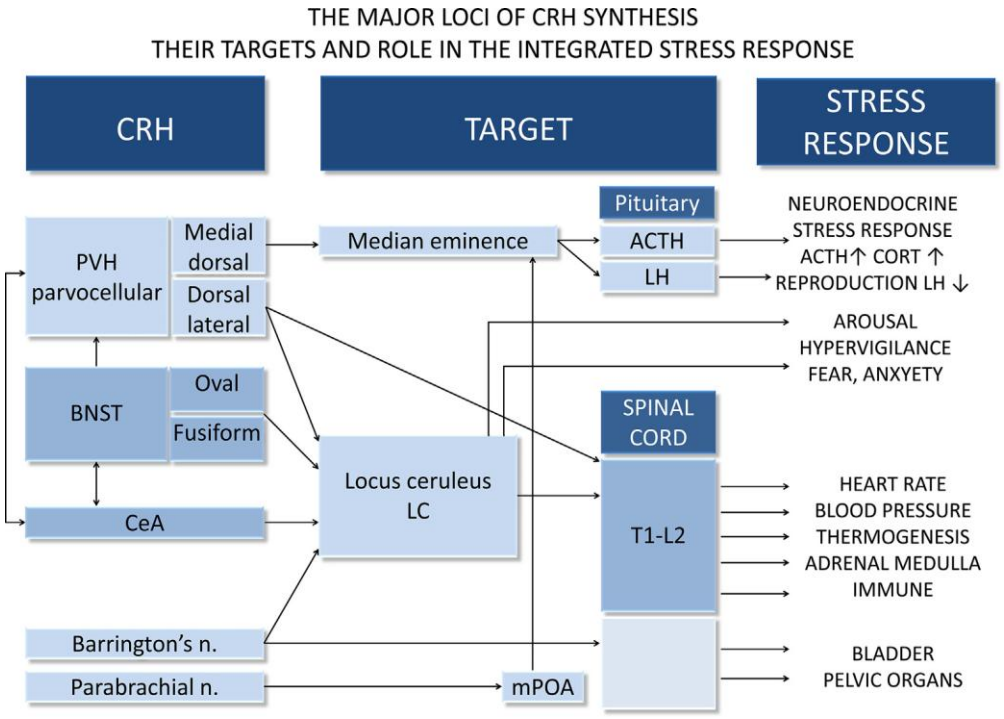

Figure 3- CRF and adaptive stress response

(Kovacs, 2013)

3.3. Metabolic changes during stress adaptation

Acute or chronic, stress is modifying eating patterns and metabolism and it seems that it makes the base of the metabolic syndrome (Charmandari et al. 2005, Maniam and Morris, 2012). Glucocorticoids (GCs) excess or lack leads to several metabolic problems because they act on multiple organs (Rose and Herzig, 2013). Among many effects, chronic glucocorticoid administration leads to an increase in food intake, leptin levels and to the development of insulin resistance and visceral fat accumulation (Dallman et al. 2007, Maniam and Morris 2012).

One of the most interesting metabolic changes provoked by stress and GCs is the metabolism of thyroid gland. Increased levels of GCs decrease the expression of Thyrotropin-releasing hormone (TRH) in the PVN and decrease of levels of Thyroid-stimulating hormone (TSH) in serum, in rat and in human (Kakucska et al. 1995, Alkemade et al. 2005). It was noted that patients with Cushing's disease present hypothyroidism, and by analysing post-mortem brains of patients that received GCs treatment or suffered major depression, it was discovered that there was a reduction of TRH expression in the PVN (Alkemade et al. 2005). Moreover, GCs rapidly suppress glutamatergic excitatory inputs onto various parvocellular neurons of the PVN, including those expressing TRH (Di et al., 2003). Therefore, TRH mRNA levels in the PVN are negatively correlated with corticosteron serum concentrations.

These observations are important because disturbances in thyroid function may significantly affect mental status including emotion and cognition and, on the other hand, depression can be accompanied by subtle thyroid dysfunction (Wolkowitz and Rpthschild, 2003).

3.4. Stress and Neurogenesis

As it was previously described, glucocorticoids represent the messenger of a stress response and under normal circumstances they serve many beneficial homeostatic functions. Nevertheless, GCs dysregulation is correlated with cognitive impairments and depression (Holsboer and Ising, 2010; McEwen, 2007). Neurogenesis in adult brain is based on functional granules which are produced in neurons of hippocampus, specifically in dental gyrus (DG) of hippocampus (Kempermann, 2008). These neurons dynamically regulate stress reactivity at both the endocrine and behavioral level (Seri et al., 2001). It has been shown that stress, together with GCs, strongly inhibits adult neurogenesis in the hippocampus (Mirescu and Gould, 2006), and it seems that one possible mechanism is through corticosteroid inhibition of granule cell precursor proliferation (Tanapat et al., 2001; Cameron et al., 1998). But, this connection between stress and neurogenesis is probably much complex than simple inhibitory role. For example, physical activity in mice is boosting adult neurogenesis and promotes neuronal differentiation (van Praag et al., 1999 and 2002; Snyder et al., 2009). On the other hand, it has been shown that physical activity is a strong activator of the HPA axis; therefore, it leads to an increase in GCs in a circulation (Droste et al., 2003; Stranahan et al., 2006). But, it has to be said that this relationship depends also on the type of stressor, where control or no control over stress may have opposite effect on neuronal plasticity, including adult neurogenesis (Lehmann et al., 2013). When stressor is unpredictable and its nature severe, stress generally reduces neurogenesis.

Besides reduce in neurogenesis, stress and the resulting rise in GCs also slow down neuronal differentiation and reduce the survival of existed neurons; in a case of prolonged action of stress (chronic stress), there are notable physiological and morphological changes at the level of hippocampus: reduction in hippocampal excitability, long-term potentiation

(LTP) and memory, as well as reduction in volume and atrophy of dendritic spines (Joels et al., 2007, 2012; Sapolsky et al., 1985, 1990; Lucassen et al., 2014). Although the underlying mechanism is largely unknown, this might be due to inhibition of neurotrophic factor like brain-derived neurotrophic factor (BDNF) (Schmidt and Duman 2007). All these changes, reduction of hippocampal volume and excitability, consequently damaged control over HPA axis, as well as decreased levels of the BDNF and other neutrophic factors, might have an important role in etiology of depression (see Depression/Neurotrophic hypothesis).

4. Depression

4.1. Definition of depression

Major depressive disorder (MDD) is a mental illness defined in the *Diagnostic and Statistical Manual of Mental Disorders* (DSM). Depression is, unfortunately, a common illness worldwide, and by data of World Health Organization (WHO) more than 300 million people are affected. Depression is a more severe state of mind than usual mood fluctuations and emotional responses of a short nature to challenges in everyday life. Moreover, if depression is persistent, with moderate or severe intensity, it may become a serious health condition. It is affecting all aspects of the ill person: family, social and relationships at work, etc. And there is a high risk of committing suicide, as the most severe outcome. Suicide is the second leading cause of death in 15-29-year-olds (WHO).

The diagnostic criteria for MDD by new DSM-V require the occurrence of one or more major depressive episodes. Symptoms of a major depressive episode include the following: depressed mood, anhedonia (decreased feeling of pleasure in almost all activities), weight, appetite and sleep disturbance, loss of energy, low self-esteem and diminished cognitive abilities etc. These symptoms must last for at least two weeks per episode and cause significant distress or severely impact on social, occupational or other important life areas. Major depressive disorder can be rated mild, moderate or severe. MDD can be diagnosed with psychotic symptoms. If MDD continues for more than two years, the DSM labels it as chronic depression or dysthymia.

The exact cause or trigger of depression is still unknown, but it is currently accepted that the combination of genetic and environmental factors can determine the appearance of the disease (Agam and Belmaker, 2008). Among environmental factors, it seems that chronic stress plays a decisive role in development of depression.

4.2. Biological and Pathophysiological Bases of Depression- Hypotheses

As it was said above, the exact mechanism of depression development and, in some cases persistence, are unknown. But, over the past decade scientists have been working tireless to reveal the cause and therefore an efficient therapy for MDD. Here, some neurobiological and molecular mechanisms will be described in details.

4.2.1. Monoamine Hypothesis

The monoamine hypothesis of depression is among the first hypothesis and it predicts that the underlying pathophysiologic basis of depression is a decrease in the levels of serotonin (5-HT), norepinephrine and, showed by recent studies, dopamine in the central nervous system (CNS).

This hypothesis, that depression represented the outcome of a functional disorder of the diffuse modulatory systems was developed after one observation concerned the natural alkaloid reserpine, made in the 1950s. In that time, reserpine was used for the treatment of hypertension and schizophrenia. But then clinicians have noted that, in some patients, reserpine caused depressive-like symptoms. Accordingly, animals given reserpine also developed a depression-like syndrome. Subsequently, it was proved that reserpine caused the depletion of a synaptic monoamine availability of NE, 5-HT and DA by inhibiting their vesicular storage. Overall, this drug was a key to development of psychopharmacology and studies on neurochemistry (Barchas and Altemus, 1999).

After the notion on reserpin, the next evidence of involvement of monoamines in the development of depression was found. Iproniazid was synthesized during 1950s and it was used for the treatment of tuberculosis. But as for reserpin, other symptoms were observed, with the difference in a nature of those symptoms: in some patient iproniazid produced

euphoria and hyperactive behaviour. Later it was discovered that it acts through monoamine oxidase (MAO) inhibition, therefore it increases brain concentrations of NE and 5-HT.

The third class of drugs, tricyclic antidepressants (such as amitriptyline) proved to be effective in a treatment of depression, supporting the monoamine hypothesis of mood disorders. They act by blocking the reuptake by presynaptic terminals of monoamine transmitters, increasing the synaptic concentration of monoamines.

Overall, based on the actions of reserpine, MAO inhibitors and tricyclics, researchers developed the hypothesis that the regulatory mechanisms of the mood tone are closely associated with the monoamine levels in the brain.

Nevertheless, soon after some inconsistencies came out. Other antidepressants which pharmacological activities were not in accordance with monoamine hypothesis were discovered. Moreover, lithium, the antimanic agent which doesn't increase monoamine transmission in a chronic matter, can also be used to treat depression. And the best example of this inconsistence is cocaine, a potent inhibitor of monoamine reuptake that has no antidepressant activity. In addition, studies on reserpin, MAO inhibitors and tricyclics showed dissimilarity among them. Even though reserpin caused depressive-like symptoms in patients, it has been shown that only in about 6% of patients it will produce depression. Moreover, since MAO inhibitors and tricyclic antidepressants are acting directly on the catecholaminergic neurotransmission, one would expect immediate results. However, their clinical antidepressant effects develop quite slowly, generally over 2 to 6 weeks.

On the other hand, efficacy of serotonin-reuptake inhibitors (SSRIs) has been taken as a proof that some mood disorders may be due to serotonin deficiency (Bloom and Kupfer, 1995). It has been shown that chronic administration of SSRIs increases the efficiency of serotonergic neurotransmission. In addition, one important discovery has been made in a field

of biological research related to mental disorders. It has been shown that patients with low cerebrospinal fluid (CSF) levels of 5-hydroxyindoleacetic acid (5-HIAA) are prone to commit suicide (Mann et al., 1996). [26].

Overall, the monoamine hypothesis might have some background, mostly if we look the previous example. But, it is hard to explain such a disease as depression by only one theory, especially if there is a certain degree of inconsistence with it, as it was mentioned in this chapter.

4.2.2. A Neurotrophic Hypothesis and Neurogenesis

A neurotrophic hypothesis proposes that decreased neurotrophic support leads towards the development of depression through mechanism such as neuronal atrophy, diminished hippocampal neurogenesis and loss of glia, and that the mechanism of action of antidepressants is that of blocking or reversing this neurotrophic factor deficit, thereby reversing the atrophy and cell loss (Duman et al., 2006; Duman et al., 1997). This theory is based on many preclinical, clinical and imaging studies, and some of those will be named here. One specific paper, work of Duman and Li, was used as a reference for numerous studies that confirm this theory.

In the past years more attention has been given to discovering of pathophisiology of mood disorders, providing us with evidences such as a reduction of the brain volume, especially of limbic areas connected to depression (Dervets et al., 2008; Macqueen et al., 2008). Furthermore, post-mortem biopsy of depressed patients discovered neurons of diminished size and reduced number of glia cells (Drevets et al., 2008; Miguel-Hidalgo et al. 2002). In addition, it seems that repeated exposure to stress can cause atrophy of neurons in the hippocampus and PFC, as well as loss of glia (Duman et al. 2006; Krishnan and Nestler, 2008).

Neurotrophic theory is based on studies of many neurotrophic factors in the brain and one of the most studied is brain-derived neurotrophic factor. It has been shown that stress, either social or physical, can cause reduction in BDNF levels in the hippocampus and PFC in rodent models; in addition, treatment with antidepressants increases the expression of BDNF in these two regions (Duman et al., 2008; Krishnan and Nestler, 2008 Castrén and Rantamäki, 2010). As it was said before, there is the reduction of brain volume in patents who suffered depression but also a reduction of BDNF in the same regions was discovered (Duman et al., 2008). To support this theory, many genetic studies have been conducted on BDNF knockdown or BDNF over-expression rodents. What these behavioural studies showed was that local BDNF infusion has an antidepressant effect, providing us with the strong evidence that the BDNF is necessary for a response to antidepressant treatments (Duman et al., 2006; Castrén and Rantamäki, 2010). Nevertheless, the big number of studies based on BDNF-deletion mutant mice report normal behaviour in models of depression.

These genetic studies have a lack of environmental factor, as some recent studies have provided evidence for a BDNF gene × environment interaction (Ibarguen-Vargas et al., 2009), showing that environmental factor is important for expression of depressive phenotype. Stress has a great impact on the process of neurogenesis in the hippocampus; this process also involves changes in BDNF levels in hippocampus. So it seems that there is a potential interaction between stress, neurogenesis and depression. Stress leads to changes in hippocampus functioning and volume and these changes can contribute to the etiology of depression; moreover, stress causes reduction in BDNF expression in hippocampus (Rasmusson et al., 2002; Roceri et al., 2004), and reduced BDNF levels results in neuronal atrophy and cell death in the hippocampus. Therefore, changes in hippocampus correlated to stress lead to the changes in hippocampal BDNF levels which results in downstream

signalling pathways that may play an essential role in a regulation of depression-related behaviors.

Contrary to the effects of stress, different classes of antidepressants significantly increase the expression of BDNF in the major subfields of the hippocampus (Russo–Neustadt et al., 2004). Chronic pretreatment with antidepressants blocks the stress-induced decrease in BDNF mRNA expression in the hippocampus (Nibuya et al., 1995). In addition, other studies show that antidepressants are increasing the number of adult-born neurons (Boldrini et al, 2009; Malberg et al, 2000), which takes about 4 weeks to form synaptic connections (Toni et al, 2007) and contribute to behavior in rodents (Denny et al, 2012; Kee et al, 2007). This all led to the hypothesis that BDNF is necessary for the effect of antidepressants and that this combination might affect mood by increasing adult hippocampal neurogenesis (Duman et al, 2001). Later studies proved that chronic antidepressant treatment, specifically with SSRIs and norepinephrine-selective reuptake inhibitors (NSRIs), increased neurogenesis (Duman et al., 2006; Sahay and Hen, 2007).

Studies on other neurotrophic/growth factors have been conducted and it has been showed that these factors are also implicated in a development of depression: vascular endothelial growth factor (VEGF), fibroblast growth factor 2 and insulin-like growth factor 1 (IGF-1) (Duman and Monteggia, 2006; Akil et al., 2008; Fournier and Duman, 2012). As well as for BDNF, stress and antidepressant treatments have opposing effects on the expression of these factors.

4.2.3. Diathesis–stress model

Psychology is trying to explain a model of stress-related disorders as the outcome of the interaction between a stress caused by life experience and individual (predispositional) vulnerability. This hypothesis is called the diathesis–stress model, where the term diathesis

has Greek origins: διάθεσις, which means a predisposition, or vulnerability. A wide range of factors make diathesis: genetic, psychological, biological, or situational factors (Ingram and Luxton, 2005).

In this model, the diathesis/predisposition interacts with exposition to strongly negative aversive, stressful event. Once this reaction exceeds the threshold, the person will develop a disorder (Lazarus, 1993). The diathesis model is used to explain a wide range of mental disorders, where every pathology has a different diathesis. So called "windows of vulnerability" of evolvent of a specific disorder, are believed to exist at different points of the lifespan. For example, children who have a family history of depression are generally more vulnerable to development of a depressive disorder themselves. According to this, the diathesis can help with prediction who will develop a disorder and who will not, or how is that when two people are exposed to the same stressor, one person may develop a disease and the second one may not (Oatley et al., 2006; Sigelman and Rider, 2009). For example, if one child has a family history of a depression, and if it will be exposed to a particular stressor, such as rejection by his or her peers, she/he would be more likely to develop depression than a child with the same family history that has an otherwise positive social network of peers (Gazelle and Ladd, 2003).

Therefore, this model might be used to investigate how the interaction between biological and genetic traits (diathesis), and environmental events (stress exposure) can increase the probability to develop stress-related mental disorders such as anxiety or depression (Belsky and Pluess, 2009; Willner et al., 2013).

4.3. Therapy of Depression

Antidepressants are the group of drugs used in the therapy of MDD. Among the most frequently prescribed drugs are those which are increasing monoamine concentration in a synaptic cleft, usually those of norepinephrine and serotonin. These antidepressants are SSRIs, NSRIs and selective serotonin and norepinephrine reuptake inhibitors (SNRIs). Other antidepressants are: tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), reversible inhibitors of monoamine oxidase A (RIMAs), tetracyclic antidepressants (TeCAs), and noradrenergic and specific serotonergic antidepressant (NaSSAs). All these antidepressants are called "traditional" and they have something in common: many antidepressants change monoamine levels within hours but effective changes in mood are not seen for 3–4 weeks and not all patients are responsive to existed therapy (Nestler e tal., 2002).references).

One chapter of this thesis will be dedicated to Ketamine, psychotomimetic at low doses and a dissociative anaesthetic at high doses, which antidepressant properties were discovered recently.

4.3.1. Selective Serotonin Reuptake Inhibitors (SSRIs)

SSRIs specifically inhibit the serotonin reuptake through the selective Serotonin reuptake transporters (SERT) blockade. They act primarily at the 5-HT transporter protein and have low affinity for other neurotransmitter systems. By blocking the reuptake of serotonin, more of it remains in the synaptic cleft; therefore more serotonin is free to bind to more distant receptors as well as to continue bonding with nearby receptors. It is unclear whether SSRIs bind to the same SERT domain as serotonin or operate through more indirect mechanisms. Recent evidence suggests that binding of SSRIs to SERTs occurs at the same site as 5-HT binding, but it has not been determined conclusively. After a chronic use of these

drugs, development of downregulation of synaptic serotonin receptors is inevitably (Goodman et al., 2009).

Since there is more serotonin in a synaptic cleft, the main side effects of SSRIs are a result of its binding to the 5-HT_2 receptor. Some of main adverse effects are sexual dysfunction, loss of libido in both, males and females, insomnia and/or sedation. Moreover, nausea and vomiting, as well as intestinal problems may occur since serotonin acts on peripheral 5-HT_3 receptors. Compounds like fluoxetine, citalopram, its enantiomer escitalopram, fluvoxamine, paroxetine and sertraline belong to this category.

4.3.2. Selective Norepinephrine Reuptake Inhibitors (NERIs)

Representative of this group is Reboxetine. There were many studies conducted in order to understand if Reboxetine is more efficacious than placebo in the treatment of depression. In Europe it is still used as antidepressant, especially for the cases of severe depression (Drug Safety Uptade). It has high selectivity for norepinephrine transporter (NET), it is less sensitive for SERT and has no affinity for dopamine transporter DAT (DAT; Rossi, 2013).

4.3.3. Triciclic Antidepressants (TCAs)

These were among the first antidepressants that were used in a clinical practice. The exact mechanism of action was never found, but we know that the majority of the TCAs act by blocking of SERT and NET. This leads to an increase of concentration of these neurotransmitters in a synaptic cleft and therefore enhances neurotransmission (Tatsumi et al., 1997; Gillman, 2007). TCAs also antagonise serotoninergic, α1 adrenergic, H_1 histaminergic, NMDA and muscarinic M_1 cholinergic receptors (Sanches and Hyttel, 1999;

Silli et Lu, 1989; Cusack et al., 1994). Action on some of these receptors can contribute to their efficacy in a treatment of depression, as well as their side effects.

Although TCAs are no longer the first line of therapy for depression after SSRIs were introduced, some of drugs from this group are still in use. Imipramine is a representative TCA drug that has high affinity for SERT, less affinity to NET but still strong and it is anticholinergic. Its major active metabolite is desipramine. Desipramine is a rather selective NET blocker (O´Donnell and Shelton, 2011). Other drugs belonging to the TCAs category are chlorimipramine (serotonin–norepinephrine reuptake inhibitor) and amitriptyline (it has strong actions on SERT and moderate on NET).

4.3.4. Selective Serotonin and Norepinephrine Reuptake Inhibitors (SNRIs)

SNRIs, along with SSRIs and NRIs, are second-generation antidepressants. They are blocking NET and SERT, but in a comparison with TCAs they don't produce the same adverse effects. Representative of this group is an antidepressant called Venlafaxine, which in low doses of preferentially blocks the serotonin reuptake, while higher doses are required to block the norepinephrine reuptake.

4.3.5. Atypical Antidepressants

There are drugs that have antidepressant effect, but cannot be categorized in other groups because they act in an atypical manner.

Mirtazapine (Specific Noradrenergic Antidepressants, NASSAs) is an antagonist of α2 adrenergic receptors, serotonin 5-HT_{2A}, 5-HT_{2C} and 5-HT_3 receptors, and histaminic H_1 receptor (Anttila and Leinonen, 2001; Frazer, 1997). It doesn't inhibit MAO and neither the transporters for monoamines. Acting through α_2-adrenergic inhibitory autoreceptors mirtazapine enhances adrenergic and serotonergic neurotransmission, and since it acts trough

central 5-HT_{1A} receptor, it mediates transmission in the dorsal raphe nucleus and hippocampus; hence, mirtazapine is classified as a NaSSA.

4.3.6. Antidepressants Interacting with Dopaminergic Transmission

Bupropion is a rather selective DAT and NET inhibitor, with insignificant SERT affinity (Shelton and Stahl, 2004). Its effect as antidepressant is mild and usually the addition to prescribed SSRIs is a common strategy when people do not respond to the SSRI. This combination, although possible interactions are unknown, may result in an improvement in some people who showed a resistance to the antidepressants of first choice (Zisook et al., 2006).

4.3.7. Monoamine Oxidase Inhibitors (MAOIs)

These antidepressants, together with TACs, make the first generation of antidepressants. This class of drugs inhibits the activity of MAO-A and MAO-B enzymes, increasing the levels of monoamines in a synaptic cleft. They are particularly used in cases of atypical depression (Cristancho, 2013). The first MAOIs are binding to the enzyme irreversibly thus, only synthesis of a new enzyme could overcome this inhibition. But, a few newer drugs as moclobemid have reversible effect. When it comes to selectivity, mentioned moclobemid acts more selective than the first ones, inhibiting only MAO-A. This inhibition reduces the breakdown of primarily serotonin, norepinephrine and dopamine. The most severe side effect of MAO-A inhibitors is hypertensive crisis, which can be fatal (Grady et al., 2012). This happens because if food high in tyramine is ingested after the inhibition of MAO-A, tyramine can displace stored monoamines, such as dopamine, norepinephrine and epinephrine from pre-synaptic vesicles.

4.3.8. Other antidepressants and methods

Besides drugs that act on monoaminergic neurotransmission, other substances 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 were suggested as modulators of monoaminergic neurotransmission, as well as factors influencing intracellular signal transmission pathways and cytokines (Berton and Nestler, 2006). Moreover, aglomelatine, an agonist of melatonin receptor agomelatine, was introduced in the drug therapy of depression. But, aglomelatine didn't differ from the traditional antidepressants by its limits in effectiveness and speed of action (Kennedy and Emsley, 2006).

Deep brain stimulation (DBS) showed to be effective in a treatment of drug-resistance Parkinsonian patients and for some mental diseases, including MDD (Laxton et al., 2013). Over the past 20 years, the anterior cingulate and subcaudate areas and their combination were chosen for DBS in a case of MDD. A recently proposed target of DBS intervention in depression is the superolateral branch of the medial forebrain bundle (slMFB), since the stimulation of this region leads to surprisingly rapid antidepressant effects in patient resistant to the traditional treatment (Schlaepfer et al., 2013). It has been proposed that DA system contributes to this effect and it has been proved that DBS of the MFB activates the DA system: this results in an increase in overall concentration of DA (Klanker et al., 2017).

Many other studies are still being done in order to establish the DBS antidepressant effect and we still don't have a clear picture about its efficacy (Lévêque, 2014).

4.4. Ketamine

What previous described drugs have in common is the slow onset of clinical response and possible resistance to therapy. Therefore, researchers are working on discovering antidepressants that might be more efficient, with a faster action and better response of patients, especially of those with a resistant type of depression or in danger of suicide.

In the past few years, particular attention was given to glutamate, as one of the most important factors in a mechanism of dysfunction of systems related to stress. In particular, the modulation of the ionotropic N-methyl-D-aspartate receptor (NMDAR) has been proposed as a promising target for the treatment of depression (Hashimoto, 2010). This is understandable since glutamate is considered as one of the main factors correlated with stress-related mental illnesses (Musazzi et al., 2013). In addition, in some animal models of depression an altered glutamate function has been found (Sanacora et al., 2012) and could be related to smaller hippocampal volume (Drevets et al., 2008). Among NMDA acting drugs, the special interest has been given to ketamine because the single intravenous infusion of a sub-anesthetic dose of ketamine resulted in rapid and long-lasting antidepressant effect; moreover, this effect was also present in a treatment of therapy-resistant patients (Berman et al., 2000; Zarate et al., 2006). Depressed patients reported alleviation of core symptoms within 2 hours after a single low-dose infusion, with effects lasting up to 2 weeks (Kavalali and Monteggia, 2015). Interesting fact about ketamine effect is that it exerted an enhanced antidepressant effect in cases of patients with a relatively smaller hippocampus (Abdallah et al., 2014).

The exact mechanism of action of ketamine is still a puzzle. At first, it was accepted that this effect comes from ketamine blockade of NMDA receptors, which prevents GLU from activating NMDAR and so inhibiting signalling processes triggered by the receptor. But, for many researchers this mechanism didn’t make sense. NMDA receptor is important

for a process of LTP, which occurs widely in the brain (Collingridge et al., 1983); LTP is enhancing signalling and therefore it is important for forming of associative memories (Morris et al., 1986; Nabavi, 2014). All this means that, by blocking NMDAR, ketamine would cause a brief block in the formation of memories and this mechanism cannot explain its antidepressant effects. Recent work of Zanos and colleagues (2016) presents one interesting result: it is a metabolite of ketamine, called hydroxynorketamine (HNK) that has antidepressant activity. They showed that HNK increases the levels of postsynaptic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, although through unknown intermediates. This is enhancing neural activity but how it produces an antidepressant effect still remains unclear. In addition, it has been proved that ketamine regulates synaptic transmission/neuronal plasticity. Li et al. (2011) have showed that after a single dose of ketamine, the number of spines on the apical dendrites of layer V pyramidal neurons increased, as well as a spine function. In accordance with this finding they examined a hypothesis that ketamine can reverse the atrophy of dendrites caused by chronic stress. The result was positive: a single dose of ketamine caused fast and complete reversal of the deficit in spine number and function caused by three weeks of chronic unpredictable stress exposure (Li et al., 2011). Ketamine has proved antidepressant effect in behavioural tests also (Li et al., 2010).

In this work we will try to explain the networks and neurotransmitters involved in ketamine antidepressant effect by using a method of microdialysis.

5. Addiction

5.1. Basic Concepts of Substance Abuse and Dependence

In order to define the term substance abuse and dependence, it is necessary to define the term reward. Reward is a pleasurable feeling that occurs after certain behaviour (in this case after use of substances); the pleasurable feelings provide positive reinforcement so that the behaviour is repeated. There are two types of reward: natural (sex, food, etc.) and artificial reward, as drugs.

Addiction is a state of compulsive engagement in rewarding stimuli, even when faced with negative consequences. Addiction should be distinguished from dependence, because someone can be dependent on prescription medication but not addicted. Substance dependence, also known as drug dependence, is an adaptive state that develops from repeated drug administration and it results in withdrawal upon cessation of drug use.

The next term that has to be defined is Substance use disorder (SUD), also known as drug use disorder. Substance use disorder in DSM-V combines the DSM-IV categories of substance abuse and substance dependence into a single disorder measured on a continuum from mild to severe. This is a condition in which the use of one or more substances leads to a clinically significant impairment or distress. SUD recognizes the use of ten separate classes of drugs: alcohol, caffeine, cannabis, hallucinogens, other hallucinogens such as LSD, inhalants, opioids, sedatives, hypnotics or anxiolytics, stimulants (including amphetamine-type substances, cocaine, and other stimulants), tobacco, and other or unknown substances.

5.2. The Main Pathological Features of Substance Use Disorder

Substance use disorders for all above mentioned substances have some features in common: compulsive drug seeking, loss of self-control and propensity to relapse. This implies a common neuronal mechanism, which has been seen in the addictive brain.

Koob and LeMoal (Koob and LeMoal, 1997) defined three stages of addiction: binge/intoxication, withdrawal/negative affect, and preoccupation/ anticipation- that worsens over time and involves allostatic changes in the brain reward and stress systems and this division will be used in a further text. Reinforcement plays an important role in this process. There are two primary sources of reinforcement: positive and negative. Positive reinforcement is defined as: "the process by which presentation of a stimulus increases the probability of a response; negative reinforcement is defined as the process by which removal of an aversive stimulus (or negative emotional state of withdrawal in the case of addiction) increases the probability of a response". The main brain regions which are sources of reinforcement and that play a key role in allostatic neuroadaptations are two main motivational systems: the brain reward and stress system (Koob, 2013).

5.2.1. Brain Reward System and Addiction

Brain reward system involves widespread neurocircuitry throughout the brain, but the most sensitive sites consist of the VTA, the NAc and PFC (Olds and Milner, 1954, Koob et al., 1977; Simon et al., 1979). These brain systems are involved in the acute reinforcing actions of drugs of abuse: in a binge/intoxication phase.

It has been seen that all drugs of abuse acutely decrease brain stimulation reward thresholds [i.e., increase or facilitate reward; (Volkow et al., 2010)]. But after chronic drug use, this threshold increases by withdrawal from drugs of abuse (decrease reward). Among

the first observed changes in the brain immediately following drug exposure was an elevation of dopamine levels, particularly in the NAc (Di Chiara and Imperato, 1988). This was a reason for establishment of the dopaminergic theory of addiction and researches have devoted much attention to triggers and consequences of dopamine release, especially in relationship to drug-dependent behaviours (Willuhn et al., 2010, 2012; Volkow et al., 2011; Tritsch and Sabatini, 2012; Carboni et al., 2000). Initial work on DA's role in drug reward focused on the mesolimbic DA pathway—DA neurons in the VTA projecting onto the NAc. It has been proven that all drugs of abuse activate this pathway (Di Chiara and Imperato, 1988), but as it will be shown later, dopamine-independent reinforcement occurs at the level of the nucleus accumbens, suggesting multiple inputs to the activation of critical reinforcement circuitry in these brain regions (Koob, 1992; Nestler, 2005).

The Ventral Tegmental Area has a central and very complex role in motivated behaviours (Salamone and Correa, 2012). It is a heterogeneous structure, containing DA, GABA, and glutamate projection neurons (Hnasko et al., 2012; Li et al., 2012). As it was mentioned before, it has long been appreciated that DA plays a major role in reinforcement learning and now optogenetic studies have shown that local activation of VTA dopaminergic neurons (Tsai et al. 2009; Witten et al., 2011; Ilango et al., 2014) or their terminals within the nucleus accumbens (Steinberg et al., 2014) is rewarding. In contrast, activation of VTA GABAergic neurons is aversive (Tan et al., 2012), whereas their inhibition is rewarding (Jennings et al., 2013). Moreover, the VTA contains glutamatergic neurons expressing vesicular glutamate transporter-2 (VGluT2; Yamaguchi et al., 2011), which are creating both local connections (Dobi et al., 2010) and long-range connections within the PFC, NAc, lateral habenula (LHb), amygdala, and basal forebrain (Yamaguchi et al., 2011; Hnasko et al., 2012; Root et al., 2014a; Taylor et al., 2014; Zhang et al., 2015). Activation of GLU terminals within the VTA activates VTA dopaminergic and nondopaminergic neurons (Dobi et al.,

2010). Immediately after the first exposure to an addictive drug, some changes in synaptic transmission occur: a single *in vivo* exposure to an addictive drug results in an increase in synaptic strength at glutamatergic synapses onto DA neurons of the VTA. Furthermore, it has been showed that a single injection of cocaine, administrated *in vivo* to mice and rats, provoked an excitatory postsynaptic currents (EPSCs) in DA neurons in midbrain slices, only after 24h (Ungless et al., 2001).

The NAc integrates reward-related information from several areas throughout the brain, including the VTA (Haber and Knutson, 2010). The main cell type in the NAc is a GABAergic projection neuron known as a medium spiny neuron (MSN). On the other hand, it seems that glutamatergic transmission in the NAc plays an important role in drug seeking. For example, some studies have shown that administration of AMPA into the NAc elicits significant reinstatement of cocaine-seeking behaviour; on the other hand, administration of an AMPAR antagonist prevents reinstatement of cocaine-seeking behaviour (Cornish and Kalivas, 2000; Ping et al., 2008). Overall conclusion is that glutamatergic signaling in the NAc plays an important role in addictive behavior therefore the synaptic plasticity at glutamatergic synapses onto NAc MSNs is likely to be involved in addiction.

5.2.2. Brain Stress Systems and Addiction

Modulation of brain stress systems is thought to be the main cause of the second phase of addiction, withdrawal/negative affect stage, as an attempt of brain to overcome the chronic presence of the drug (Koob and Volkow, 2010). The HPA axis and extrahypothalamic CRF pathways are activated during withdrawal from chronic use of all major addictive drugs, leading to a common response of elevated ACTH, corticosterone, and amygdala CRF during acute withdrawal (Koob, 2008; Koob and Kreek, 2007). During this phase, an aversive or anxiety-like state is present, in which CRF and other stress related systems (including

noradrenergic pathways) have the key roles. But stress system is not important for this stage of addiction only. The preoccupation/anticipation or craving stage of the addiction cycle might be a key element of relapse in humans and this stage is what defines addiction as a chronic relapsing disorder. This is when the individual reinstates drug-seeking behavior after abstinence. In animal studies there are three typical triggers of reinstatement: (1) re-exposure to the same or related drug previously administered (drug-induced reinstatement); (2) exposure of animals to the drug-associated stimuli or cues (cue-induced reinstatement); or (3) exposure to a variety of stressors (stress-induced reinstatement) (Silberman et al., 2013). For the purposes of this work, the main focus will stay on stress systems and their involvement in addiction and stress-induced reinstatement.

5.3. Stress, BNST, Noradrenaline and Addiction

Stress-induced reinstatement is maybe one of the most important targets for therapy of addiction. Everyday situation like family issues, finding and maintaining work, and even traffic is causing stress response in any person but it becomes more important in addicts who are recovering. Thus, it is not surprising the fact that stress is a major trigger for relapse in addicted patients (Sinha, 2007).

A great number of studies have examined the role of stress in addiction and they revealed the key neurobiological mechanism of withdrawal and stress-induced reinstatement of drug-seeking. The particular focus was on the effects of two stress-related neuromodulatory systems, norepinephrine and corticotropin releasing factor, in two related brain regions of the extended amygdala, the CeA and the BNST (Shaham et al., 2003; Epstein et al., 2006; Sofuoglu and Sewell, 2009; Erb, 2010; Haass-Koffler and Bartlett, 2012).

It seems that withdrawal from chronic drug abuse can lead to NE dysfunction and this is associated with increased vulnerability to anxiety (McDougle et al., 1994). More precisely, intracerebroventricular injection of the NE leads to an increase of fos in the BNST (Brown et al., 2011), and stressed-induced reinstatement can be blocked by microinjection of β antagonist into the extended amygdala (Leri et al., 2002). All this suggests that the key factor in enhanced drug-seeking following stress might be NE dysfunction in the extended amygdala.

It has been discovered that also CRF plays an important role in stress- induced reinstatement of drug seeking during addiction cycle. It seems that alcohol and other drugs of abuse can modulate CRF activity in the BNST. Furthermore, protracted withdrawal from cocaine, heroin, and alcohol can result in a dysregulation of the intrinsic excitability of some BNST neurons via a CRF-mediated mechanism (Francesconi et al., 2009). Therefore, the

development of symptoms during drug-withdrawal relays on repeated activation of CRF receptors in the BNST. Moreover, stress-induced reinstatement of drug seeking can be blocked by microinjections of $CRFR_1$ antagonists into the BNST (Erb and Stewart, 1999; Erb et al., 2001); on the other hand, microinjections of CRF into the BNST can drive reinstatement for drug-seeking (Erb and Stewart, 1999).

Overall, it seems that both NE and CRF in the extended amygdala are the key components of both acute drug-withdrawal syndromes and reinstatement, but the mechanism of sensitization of stress pathways upon chronic exposure to drugs is still unknown. Nobis et al. (2011) examined the theory of this interaction. What they have found is that during the acute withdrawal phase from chronic cocaine exposure, β agonist and CRF increased the frequency of spontaneous GLU neurotransmission in the BNST. These effects were blocked by previous treatment with CRF_1 antagonist. This means that serial NE-CRF signalling in the BNST is engaged *in vivo* during drug exposures (Nobis et al., 2011).

5.3.1. Role of CRF BNST Neurons in Addicition

There are two main sources of the CRF in the BNST: local CRF neurons and CRF projections from the CeA (Erb et al., 2001). Silberman and colleagues (2013) have examined the source of elevated CRF in the BNST during stress response. They used β- adrenergic agonist isoproterenol in the BNST and this resulted in a depolarization of CRF neurons. Therefore, stress-induced NA firing in the BNST leads to enhanced local CRF neuron activity, leading to enhanced CRF release. This CRF release might be further regulated by CeA CRF afferents (Erb et al., 2001). Enhanced extracellular CRF levels in the BNST results in enhanced glutamatergic activity and thus increased BNST excitation.

5.4.2. BNST-VTA Circuitry

All above mentioned studies describe how NA-CRF interaction is enhancing the BNST excitability but it is still not clear how this excitability is influencing neurociruitry of addiction. Since drug-seeking behavior in all types of reinstatement models is due to modulation of mesolimbic pathway, the connection between BNST and VTA should be examined in order to discover the mechanism of reinstatement of drug-seeking behaviour upon stress exposure.

The most of VTA-projecting neurons in the BNST are GABAergic, and the rest of the projections are glutamatergic or contain a mixture of transmitters (Figure 4; Kudo et al., 2012). Furthermore, it seems that at least some of the BNST VTA-projecting neurons contain CRF (Rodaros et al., 2007). Therefore, BNST sends numerous projections to the VTA with different types of neurotransmitters, and some studies tried to discover to which VTA neurons these projections are connected. Recent studies have showed that after optogenetic activation of VTA GABAergic neurons there is a disruption in reward consumption (van Zessen et al., 2012). Moreover, recent immunoelectron microscopy work of Kudo and colleagues (2012) indicates that BNST GLU projection neurons may selective target VTA DA neurons, while GABAergic BNST projection neurons may specifically target GABAergic neurons in the VTA (Kudo et al., 2012).

Although the nature of VTA-BNST connection is not completely clear, we know that disruption of this pathway reduces cocaine preference (Sartor and Aston-Jones, 2012), and that VTA projecting BNST neurons become activated during reinstatement to cocaine seeking (Mahler and Aston-Jones, 2012). Moreover, antagonism of glutamatergic receptors in the VTA can block BNST stimulation mediated enhancement of VTA DA neuron firing

while having minimal effects on putative VTA-GABA neuron firing (Georges and Aston-Jones, 2002).

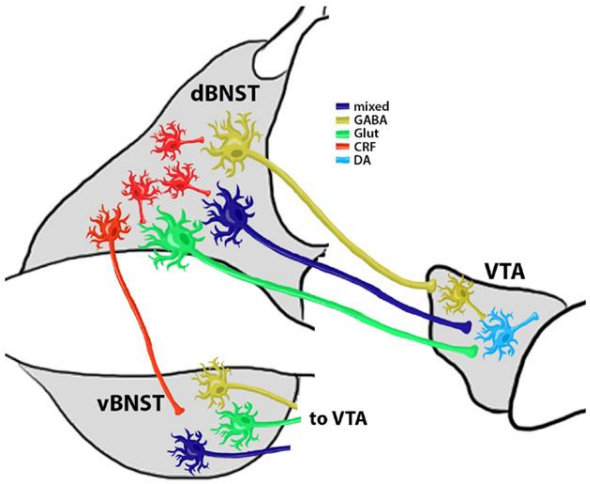

Figure 4- Model of Reinstatement

Summary Model of Reinstatement Related BNST and VTA Connectivity (Silberman et al., 2013).

Overall, it seems that the BNST, thanks to its role in the regulation of stress-responses, its NA-CRF interaction and connectivity is the key to discovering mechanisms that lead to the development of addiction. This thesis is based on this theory and substances used in the experiment of our lab will be described next.

5.4. Drugs of interest

5.4.1. Cocaine

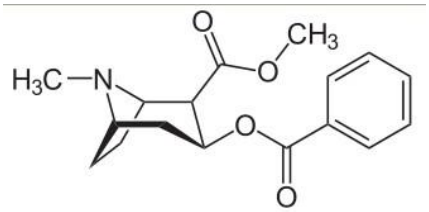

Figure 5- Cocaine chemical structure

Cocaine, an alkaloid that is produced from coca leaves (Erythroxylum coca), acts by blocking the recapture of catecholamines; thus, enhancing the transmission of dopamine, noradrenaline and adrenaline, as demonstrated by in vitro and animal studies. The mechanism of action involves blocking the transporter responsible for dopamine reuptake, acting on the binding site for dopamine (Howland and Mycek, 2007).

The rewarding action of cocaine occurs through the activation of dopaminergic neurons in the limbic system. It acts in the nucleus accumbens, one of the limbic areas on which the axons of endogenous dopaminergic neurons terminate (Carboni et al., 1989). Moreover, cocaine has an inhibitory action on the reuptake of NA and serotonin and has a local anesthetic effect, as it blocks the conduction of the nervous signal in Na^+ sensitive channels. Cocaine induces considerable psychological dependence by evoking the production of dopamine in the centers for perception of pleasure (ventro-medial nucleus of the mesencephalus; Childress et al., 2002). The exact mechanism of development of cocaine addiction is not well known and since the specific therapy doesn't exist, it is important to discover neurobiological mechanisms of its action on the brain.

5.4.2. Nicotine

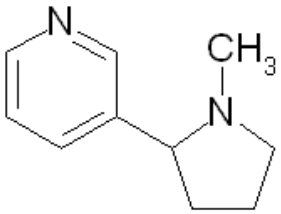

Figure 6- Nicotine chemical structure

Nicotine is an alkaloid of vegetable origin, particularly concentrated in tobacco leaves (*Nicotiana tabacum*). It binds to the cholinergic nicotinic receptor, a channel receptor consisting of the assembly of five polypeptide subunits delimiting a cavity through which the ions pass from one to the other side of the cell membrane. At least ten subunits (α; β) have been identified, by their genes, that may be a part of the receptor structure. They can be organized to form homomeric receptors consisting of repetition of the same subunit (α7) or heteromeric receptors, constituted by the association of a different subunits (α and β) (Di Chiara, 2010). The receptors are present in different areas of the brain: the most are in the prefrontal cortex, thalamus, interpendicular nucleus and slightly less in the amygdala, septum, medulla oblongata and locus ceruleus. Their activation is able to influence cognitive functions, neuronal development, neuronal degeneration and the transmission of impulses from the central nervous system to peripheral organs.

The nicotine bond with the receptor determines the transient opening of the channel, allowing the positively charged ions (Ca^{2+}) to enter the cell. Calcium is involved in the release mechanism of several neurotransmitters such as acetylcholine, dopamine, noradrenaline, serotonin, β-endorphine, growth hormone, prolactin and ACTH. Positive reward and rewarding action is determined by the release of dopamine at the level of pleasure circuits or limbic areas (Jones and Benowitz, 2002).

5.4.3. Morphine

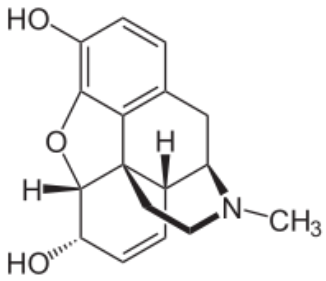

Figure 7- Morphine chemical structure

Morphine is extracted from opium, obtained from latex processing from *Papaver sonniferum*. It is an alkaline that acts on the central nervous system interacting with opioid receptors μ, δ and ƙ. In low doses, morphine is selective for μ receptors, while in high doses it is also able to bind to receptors δ and ƙ. Morphine acts on these receptors by imitating the action of endogenous agonists: encephalins, dinorphins and endorphins (Negri et al., 2004). Endogenous peptides are located in the areas of the Central Nervous System associated with pain perception such as lamina I and II of spinal cord, the nucleus of trigeminal nerve and periacqueduttal gray matter. They intervene in adjustment of the mood (globus pallidus, stria terminals, locus coeruleus), and like neuromodulators in the endocrine glands of the gut, they modulate synaptic responses by binding to receptors and limiting their actions. Morphine acts on the μ receptor of GABAergic neurons, inhibiting the neurons of the VTA, which are a part of dopaminergic pathways with enhanced dopamine release on the NAc of the limbic system (Regoli and Calò, 2010).

Morphine is used to treat moderate to severe pain. Short-acting formulations are taken as-needed for pain. The extended-release form of morphine is for around-the-clock treatment of pain.

5.4.4. Amphetamine

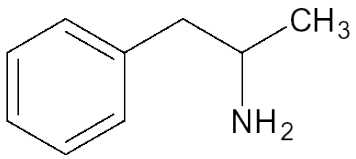

Figure 8- Amphetamine chemical structure

Amphetamine has been synthesized for the purpose of obtaining a compound with effects similar to the ephedrine (bronchodilator); only later its psychotropic action and the effects of physical and psychological dependence were discovered. This substance acts centrally, increasing the release of catecholamines from the nerve endings by various mechanisms: it inhibits the vesicular carrier, monoamino-oxidase, enzymes involved in the degradation of monoamines and inverts the direction of membrane carrier transport (Howland and Mycek, 2007). Therefore, the block of vesicle carrier prevents noradrenaline and dopamine from being stored in the vesicles; these neurotransmitters accumulate in the neuron cytoplasm also because the MAOs are blocked. The membrane conveyor, normally carrying "DA / NA" from the synaptic space to the cytoplasm (reuptake mechanism), when the concentration of "DA / NA" in the cytoplasm is lower than that in the synaptic space, inverts the transport direction: "DA / NA "are transported from the cytoplasm to the synaptic space. This results in increased synaptic concentration of catecholamines, similar to that occurring with cocaine but by a different mechanism (Di Chiara G., 2010); in both cases it occurs preferentially at the level of the limbic system and especially of the nucleus accumbens (Carboni et al., 1989).

5.4.5. Ethanol

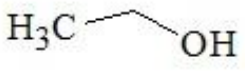

Figure 9- Ethanol chemical structure

Ethanol or Ethyl alcohol is a free chain alcohol that represents the basic component of all alcoholic beverages. In the past it was believed that alcohol acts on cellular systems through a chemical-physical mechanism, solubilizing in the membranes and altering its fluidity, permeability and organization. However, thanks to numerous studies, today it is known that ethanol has well-defined targets in the brain. In fact, it acts on different types of channels: NMDA of glutamate, GABA receptor of GABA, serotonin $5HT_3$ ionotropic receptor, nicotinic acetylcholine receptor and strychnine-sensitive glycine receptor (Bagetta and Amantea, 2010). At the central level, ethanol has a dual action: initially stimulative and subsequently depressive. The depressive action is due to the activation of mechanisms that have an inhibitory action on cellular activity; for example, the opening of the GABA-A channel determines the passage of chlorine ions (Cl^-) inside the cell with the effect of hyperpolarization. The stimulative action, responsible for the gratification of alcohol, is due to two mechanisms: inhibition of the inhibitory tone on the dopaminergic neuron, which is thus free to release dopamine in the gratification circuits; and formation of Salsolinol that is responsible for dopaminergic stimulation in limbic areas. Salmolinol is a metabolite of ethanol that results from condensation of dopamine with acetaldehyde in the brain, the dopamine present in the synaptic spaces of areas rich in dopaminergic neurons such as the VTA and substantia nigra (Quintanilla et al ., 2015).

6. Neurotransmitter Systems of the BNST

6.1. Catecholaminergic neurotransmission

Catecholamine is a term that groups together substances of low molecular weight that are sharing a mutual chemical structure. This structure consist of a benzene ring with two hydroxyl side groups in the position 1 and 2, and a side-chain of ethylamine.

There are three forms of catecholamines: norepinephrine (also known as noradrenaline/NA), dopamine (DA) and epinephrine (adrenalin). First two acts primarily as neurotransmitters in the central (NA and DA) and peripheral nervous system (NA), while epinephrine is highly localized in the adrenal medulla, released in a blood as a reaction to stress and fear.

6.1.1. Biosynthesis

Catecholamines are produced mainly by the chromaffin cells of the adrenal medulla and the postganglionic fibers of the sympathetic nervous system.

There are two amino acids which are essential for the synthesis of cateholamines. Those are phenylalanine and tyrosine. As essential amino acids, they cannot be produced *de novo* in humans and animals, but have to be introduced by food. But phenylalanine can be converted to tyrosine by the enzyme phenylalanine hydroxylase. After this reaction, tyrosine has to be transported to the cytosol of the cells by a specific transporter, where biosynthesis takes place. This first step of synthesis in a cytosol of nerve terminals and chromaffin cells consists of conversion of tyrosine to 3,4- dihydroxyphenylalanine (l-DOPA), catalyzed by the enzyme tyrosine hydroxylase (TH). The speed of this reaction, however rather slow, represents the limiting factor of catecholamine synthesis and is under tight control of neuronal activity. Therefore, the hydroxylation of l-tyrosine by TH results in the formation of

the DA precursor l-DOPA, which is then metabolized by non-specific cytosolic DOPA decarboxylase, also called l-aromatic amino acid decarboxylase (AADC; see Cooper et al., 2002) to the transmitter dopamine. This step occurs so rapidly that it is difficult to measure l-DOPA in the brain without first inhibiting AADC. Dopamine is then transported into the synaptic vesicle by the specific vesicular transporters for monoamines. This reaction is the last step in neurons that are using DA as a transmitter. But in those neurons using noradrenalin or adrenalin, the enzyme dopamine b-hydroxylase (DBH) is also present. This enzyme converts dopamine to noradrenalin. In still other neurons in which epinephrine is transmitter, the third enzyme phenylethanolamine N-methyltransferase (PNMT) converts NE into epinephrine. So, the cells using DA as a transmitter contain only two enzymes (TH and AADC), those using NA three (TH, AADC and DBH) and finally, chromaffine cells contain four enzymes (TH, AADC, DBH and PMNT; Figure 10).

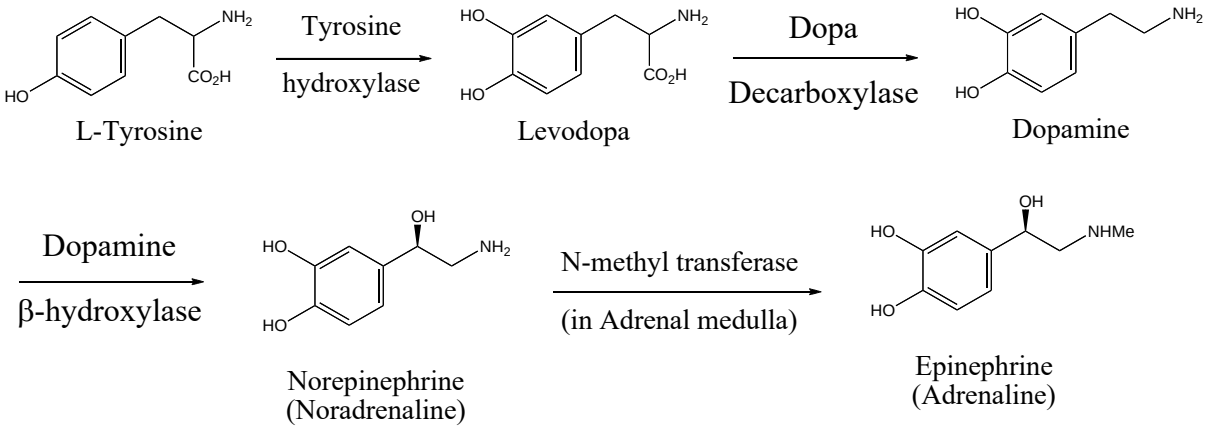

Figure 10- Synthesis of Catecholamines

6.1.2. Regulation of Catecholamine Synthesis

Despite the fluctuation in activity of catecholaminergic neurons, concentration of catecholamines is controlled through regulation of the synthesis (Alousi and Weiner, 1966). There is a short-term and long-term regulation of a biosynthesis.

When it comes to the short-term regulation, there are two main mechanisms. TH is a limiting enzyme in a biosynthesis of catecholamines and it is inhibited by the end-product (Alousi and Weiner, 1966). Therefore, free catecholamines inside of a nerve terminal are inhibiting TH. When there is a depolarization of a nerve terminal, release of neurotransmitter leads to a decrease of its cytoplasmic concentration and therefore to disinhibition of enzyme. The other mechanism is modulation of TH by depolarization. After depolarization, there is a change in the kinetic of the enzyme: it has a higher affinity for the pterin cofactor and is less sensitive to end-product inhibition. Activation of the enzyme is associated with reversible phosphorylation of the enzyme (Zigmond et al., 1989).

Long-term regulation of catecholaminergic biosynthesis involves modification of TH and DBH concentration in nerve (Molinoff and Axelrod, 1971). When there is a prolonged activity of sympathetic neurons, the amount of mRNA coding for TH and DBH increases in the neuronal perikarya. The newly synthesized enzyme molecules are then transported down the axon to the nerve terminals.

6.1.3. Vesicular Storage and Release

After DA has been synthesized from L-DOPA, the reaction which happens in cytosol, it is transported to the storage vesicles. In NA-containing neurons the final reaction of β hydroxylation occurs within the vesicles. On the other hand, the final reaction of N-methylation of NE by Protein O-mannosyl-transferase (POMT) occurs in the cytoplasm and

then epinephrine is transported back into chromaffin granules for storage. Catecholamines are transported into vesicles thanks to membrane protein, referred to as vesicular membrane transporter 2 (VMAT2). This protein has a high affinity for reserpine, which blocks vesicular uptake in vivo (Liu et al., 1992). Thanks to an ATP-dependent process linked to a proton pump, catecholamines are storaged in a high concentration. The concentration of catecholamines is approximately 0.5M and they are in a complex with ATP and acidic proteins known as chromogranins. The vesicles play a double role: they are storing catecholamines, which are ready to be released and they also mediate the process of release. When an action potential reaches the nerve terminal (when depolarization happens), Ca^{2+} channels open, letting the cation enter into the terminal; increased intracellular Ca^{2+} promotes the fusion of vesicles with the neuronal membrane and release of catecholamines. Then neurotransmitters are reaching postsynaptic receptors, producing the biological response.

6.1.4. Interruption of a Synaptic Signal

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 of Catecholamines

As said before, one of the mechanisms of signal interruption is action of enzymes MAO and COMT (Figure 11).

MAO is a flavin-containing enzyme which is distributed all over our body. It can be found only inside of a cell (outer membrane of mitochondria); therefore, it can metabolise only free catecholamines since those in vesicles are protected by its wall. This enzyme oxidatively deaminates catecholamines to their corresponding aldehydes. Two MAO

isozymes have been identified, characterised by a different affinity towards substrate: MAO-A, that favourites NE and serotonin, and MAO-B that acts on a broad spectrum of phenylethylamines, including β-phenylethylamine. MAO is found also in gastrointestinal system where it plays a protective role, since food can contain a large amount of tyramine, which can produce hypertensive crises.

COMT is found in almost all cells, including erythrocytes (Nikodejevic et al., 1970). Therefore, enzyme can act on catecholamines outside of the nervous system. This enzyme has a great capacity for supstrate (e.g. it is not substrate specific as MAO). Its mechanism of action consists of transfering a methyl group from the cosubstrate S-adenosylmethionine to the 3-hydroxy group on the catecholamine ring. For this action, COMT requires Mg^{2+}.

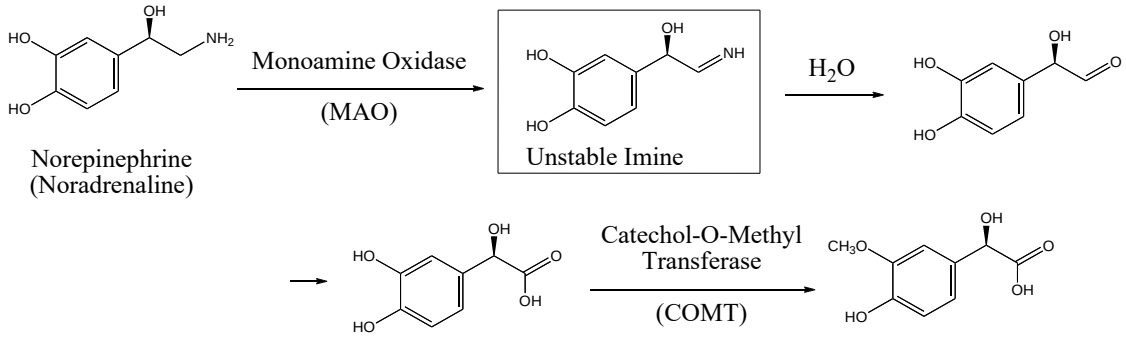

Figure 11- Catabolism of Catecholamines

Measurement of metabolites of catecholamines can give us information about their production and turnover. This measurement is assisted in a CSF since there is a large amount of catecholamines produced in the peripheral nervous system. Homovanillic acid (HVA) is a major metabolite of DA and it can be measured in a spinal fluid. A metabolite of NE formed relatively selectively in the brain is 3-methoxy-4-hydroxyphenylglycol (MHPG). But it requires measurement in both urine and CSF.

6.1.6. Reuptake

Reuptake makes the most efficient way of catecholamines removal from the synaptic cleft. The uptake process by presynaptic nerve terminal is mediated by a carrier or transporter located on the outer membrane of the catecholaminergic neurons. It is saturable and obeys Michaelis-Menten kinetics. A transport for NA is found only in noradrenergic neuronal cells, whereas a transporter with different specificity is found in DA-containing neurons. Work of our lab in early nineties showed that NET in the prefrontal cortex of rats has an affinity for dopamine four times higher than that for norepinephrine, thus increasing dopamine extracellular concentration after administration of drugs that selectively block the NET (Carboni et al., 1990).

Reuptake is energy-dependent process since it can be inhibited by incubation at a low temperature or by metabolic inhibitors. The energy requirements reflect a coupling of the uptake process with the Na^+ gradient across the neuronal membrane, dependent on the presence of Cl^-. Transport of catecholamines can be inhibited by opening of Na^+ channels (veratridine), which are selectively inhibited by such drugs as tricyclic antidepressants and cocaine. Furthermore, a variety of phenylethylamines, such as amphetamine, can compete with catecholamines for the transporter.

6.1.7. Catecholaminergic Receptors

After the release of catecholamines from the nerve terminal or adrenal gland, they are free to bind to the specific receptor on the effectors' cells. The reaction that they cause inside of a cell is responsible for expressing of their function.

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 one intracellular C-terminal. The crucial step for recognition of specific G-proteins is the third cytoplasmatic loop and the COOH terminal portion. As it is well know, G-coupled protein receptors mediate slow responses and often involve second messengers and amplification of the signal. Catecholamine receptors are classified into adrenergic (for epinephrine and norepinephrine) and dopaminergic receptors.

- *Noradrenergic receptors*

The actions of noradrenalin and adrenaline are exerted through two main groups of receptors, of which there are nine distinct forms. First group is named α (alpha) and the second β (beta). Moreover, there are two functionally distinct classes of α adrenergic receptor, classified as the α_1 and α_2 forms, while β adrenergic receptors are divided into three subtypes, β_1, β_2 and β_3.

The α_1 adrenergic receptors are coupled to Gq/11 proteins, thus responsible for mobilization of cytosolic Ca^{2+} and stimulation of phosphatidyl inositol (PI) turnover. α_1 adrenergic receptors are distributed widely in the CNS (Nicholas et al., 1996), but dominantly located on postsynaptic neurons (Santana et al., 2013). These receptors mediate slow depolarization and facilitation of neuronal excitation and are found in rat's cerebral cortex, thalamic nuclei, and dorsal raphe. On the other hand, presynaptic α_1 adrenergic receptors have been found in the nucleus accumbens and it seems that they may regulate dopamine release (Mitrano et al., 2012).

The α_2 receptors consists the α_{2A}, α_{2B}, and α_{2C} receptors. They are expressed mostly on the presynaptic nerve terminals (autoreceptors). They can also be located postsynaptically, regulating serotonin and dopamine transmission (Gobert et al., 1998; Devoto et al., 2001). Activation of α_2 autoreceptors leads to inhibition of cAMP production through Gi-type G-

proteins. Therefore, receptor activation results in decreased levels of cAMP and consequently reduced levels of active protein kinase A (PKA), hyperpolarization and neuronal inhibition. They are predominantly expressed 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.

As it was mentioned before, the β adrenergic receptors are divided in three groups: β_1, β_2 and β_3. Each of these three types is coupled with Gs-type G-proteins, leading to activation of adenylate cyclase and increase in cAMP. But only to types, β_1 and β_2 receptors are expressed in the CNS (Nicholas et al., 1996).

The β_1 adrenergic receptors are heterogeneously localized with high expression peaks in the cerebral cortex and the caudate. The β_2 adrenergic receptors are located on neuronal and glial elements, with the highest density in the cerebellum. Epinephrine and norepinephrine are equipotent at β1, whereas epinephrine is considerably more potent than norepinephrine at β_2.

- *Dopaminergic Receptors*

Dopamine binds to its specific receptors called D-type receptors and they are divided into 5 subtypes: D_1, D_2, D_3, D_4 and D_5 receptors. Moreover, D_1 and D_5 receptors belong to so called D_1-like family and D_2, D_3 and D_4 to D_2-like family of receptors. They are all coupled with G proteins but have to opposite effects: activation of D_1-like family receptors leads to the activation of of Gs-type G-proteins and, therefore, receptor activation results in activation of adenylate cyclise, whereas activation of D_2-like family coupled to Gi-type G-proteins results in the inhibition of adenylate cyclase.

After the activation of AC in the D_1-like family receptors, protein kinase A (PKA) activation happens and leads to 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). This inhibitory interaction regulates neural plasticity through extracellular signal regulated kinase (ERK) pathways (Valjent et al., 2005).

The phospholipase C (PLC) pathway can be induced by activation of both D_1 and D_2 receptors, resulting in a production of inositol 1,4,5-triphosphate (IP-3) and diacylglycerol (DAG). These two induce the increase of Ca^{2+} levels, leading to the 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 (Valjent et al., 2005).

In the CNS dopamine receptors are widely expressed because they are involved in the control of locomotion, cognition, emotion, affection and in the neuroendocrine secretion (Missale et al., 1998). Commonly, the most expressed subtypes of dopaminergic receptors are the D_1 and the D_2 receptors.

Based upon Northern blot and in situ hybridization, D_1 mRNA expression in the central nervous system is highest in the dorsal striatum (caudate and putamen) and ventral striatum (nucleus accumbens and olfactory tubercle). (Schetz and Sibley, 2007)

D_2 receptors are expressed presynaptically, as well as postsynaptically. Presynaptic D_2 receptors are located on the soma and dendrites of dopaminergic cells and the act as autoreceptors, decreasing firing frequency, while D_2 receptors located on the nerve terminals reduce dopamine synthesis and release. The expression of these receptors in subcortical areas is approximately equal, whereas in the cortex the D_1 type expression is prevalent. D_3

receptors are predominantly localized in limbic regions, while D_4 and D_5 receptors are expressed in both cortical regions and limbic areas (Tritsch and Sabatini, 2012).

6.2. Catecholamine Systems

6.2.1. Noradrenergic Projections

There are two main groups of noradrenergic neurons in the CNS and their cell bodies are located 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 (Malenka et al., 2009). Neurons of the lateral tegmental nuclei project to other regions such as the hypothalamus, part of the amygdala and the spinal cord.

Noradrenergic transmission has many different functions. Some of its main functions are correlated with stress response (Arnsten and Li, 2005; Kvetnanski et al., 2009; Ma and Morilak, 2004), arousal (Aston-Jones et al., 2001), decision making (Aston-Jones and Cohen, 2005), enhancing signal to noise ratio in target areas (Berridge and Waterhouse, 2003), and depression (Karolewicz et al., 2005; West et al., 2009); this system 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 (Attention-deficit/hyperactivity disorder).

6.2.2. Dopaminergic Pathways

There are four main Dopaminergic pathways in the central nervous system. They are grouped based on innervate brain areas: (i) nigrostriatal, (ii) mesocortical, (iii) mesolimbic, and (iv) tuberoinfundibular pathway (Dahlström and Fuxe, 1964).

The nigrostriatal pathway is characterized by neurons with long axons, projecting from the substantia nigra pars compacta (A9) predominantly to the dorsal striatum (particularly to the caudate putamen). The extrapyramidal system is involved in the motor coordination, learning of complex movements and in the regulation of muscle tone. Neuronal degeneration of substantia nigra leads to the development of Parkinson's disease and all related symptoms.

The "meso" prefix in the word "mesolimbic" refers to the midbrain, or "middle brain", since "meso" means "middle" in Greek. Dopaminergic neurons from the VTA, which is located in the midbrain, are sending projections to the ventral striatum, which includes both the nucleus accumbens and olfactory tubercle (Ikemoto, 2010; Malenka et al., 2009). This pathway is involved in primordial functions such as emotional responses, behavior, motivation, stimuli processing and reward mechanisms

The other group of dopamine cells located in the VTA (A10) is innervating prefrontal cortex (mesocortical pathway). This system is 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.

"Infundibular" in the word "tuberoinfundibular" refers to the cup or infundibulum, out of which the pituitary gland develops. Short projecting dopaminergic neurons, which cell bodies are located in the arcuate and periarcuate nucleus of the hypothalamus (A12) send axons to the median eminence. The median eminence is closely connected with capillaries of the hypothalamic-pituitary portal circulation where dopamine, released into the portal circulation, is reaching the anterior pituitary gland where it inhibits prolactin release via activation of D_2 receptors.

6.3. Glutamatergic Neurotransmission

In neuroscience, glutamate is the anion of glutamic acid which has a role of neurotransmitter. It is the most abundant neurotransmitter in the vertebrate nervous system (Meldrum, 2000). Glutamate is an excitatory neurotransmitter, used by every major excitatory function in the vertebrate brain and being present in total for well over 90% of the synaptic connections in the human brain. In addition, it also serves as the primary neurotransmitter for some well localized brain regions, such as cerebellum granule cells.

6.3.1. Synthesis and Metabolism

Glutamate is among the most abundant amino acids in a human body and consequently it is a constituent of a wide variety of proteins (Meldrum, 2000). Although glutamate is presented to our body through diet in big quantities so it doesn't have to be synthesized, it is formally classified as a non-essential amino acid because it can be synthesized from alpha-ketoglutaric acid, which is produced as part of the citric acid cycle. Since glutamate cannot cross the blood-brain barrier, it is actively transported into the bran by a high affinity transport system, which maintains its concentration in brain fluids at a fairly constant level (Smith, 2000).

It should be noted that *de novo* synthesis of glutamine takes place only in astrocytes. After its interaction with the receptors in a synaptic cleft, glutamate is manly taken up by surrounding astrocytes. There it is either converted to glutamine, catalyzed by glutamine synthetase (GS) as part of the glutamate-glutamine cycle, or metabolized in the tricarboxylic acid (TCA) cycle. After the first reaction, glutamine is transported to the glutamatergic neuron where it will be used for synthesis of glutamate, catalyzed by phosphate activated glutaminase (PAG). In the presynaptic cell glutamate is synthesized from glutamine as part of the glutamate-glutamine cycle by the enzyme glutaminase (Rothman et al., 2003). Glutamate

itself serves as metabolic precursor for the neurotransmitter GABA, via the action of the enzyme glutamate decarboxylase.

In the second case, glutamate enters the TCA cycle by the activity of glutamate dehydrogenase (GDH) or an aminotransferase (AT), and the carbon skeleton may either be completely oxidatively metabolized via pyruvate recycling including malic enzyme (ME) activity, or phosphoenolpyruvate carboxykinase and pyruvate kinase. Alternative, the carbon skeleton supports the pool of TCA cycle intermediates and in that way potentially increases the oxidation of acetyl CoA in the TCA cycle. *De novo* synthesis of glutamate and glutamine from glucose occurs via the concerted action of glycolytic enzymes, pyruvate dehydrogenase (PDH) and pyruvate carboxylase (PC), making a net synthesis of TCA cycle intermediates.

6.3.2. Regulation of Glutamatergic Release

A number of presynaptic receptors are involved in the regulation of glutamate release and not only glutamatergic, but also cholinergic (nicotinic and muscarinic, adenosine (A1), kappa opioid, g-aminobutyric acid (GABA) B, cholecystokinin and neuropeptide Y (Y2) receptors (see Meldrum, 1998).

Glutamate extracellular concentrations are low and tightly regulated in order to prevent excitotoxicity. Glutamate plasma membrane transporters regulate glutamate concentrations and are situated on both pre- and post-synaptic neurons, as well as on surrounding astrocytes (Kanai et al., 1994). There are five cloned plasma membrane transporters, called excitatory amino acid transporters (EAATs): EAAT-1 to EAAT-5. Those are expressed predominantly on cells in brain regions rich in glutamate (Eulenburg and Gomeza, 2010). Glial cells are accepted as cells responsible for maintaining extracellular glutamate concentrations. Nevertheless, the presence of EAATs on multiple cell types suggests a high level of cooperation (Eulenburg and Gomeza, 2010; Foran and Trotti, 2009; Tanaka, 2000). Under

normal conditions, glutamate is recycled continuously between neurons and glia in what is known as the glutamate–glutamine cycle. Excess glutamate in the synapse is taken up by glial cells via EAAT transporters, where it is converted to glutamine. The destiny of glutamine is described above. However, glial cells, under certain conditions, may also release glutamate by at least six mechanisms, one of which is reversal of uptake by glutamate transporters (Malarkey and Parpura, 2008). This kind of reverse transport may be involved in the mechanism of brain damage and stroke (Grewer et al., 2008).

6.3.3. Vesicular Storage and Release

After its production in cytoplasm of the neuron cell, glutamate is transported into vesicles by the group of glutamate vesicular transporters (VGLUTs). These transporters are ATP dependent and when protons enter the vesicle, they make it more acid and therefore pH gradient is generated across vesicle membrane. The second result of this proton influx is creation of membrane potential on the surface of vesicular membrane.

Glutamate is released from vesicles in presynaptic terminals by a Ca^{++} - dependent mechanism that involves N- and P/Q-type voltage-dependent Ca^{++} channels (Birnbaumer et al. 1994), that appear to be closely linked to vesicle docking sites. Once AMPA receptors are activated, an excitatory postsynaptic potential causes a release of a single vesicle.

6.3.4. Interruption of the Signal

After depolarization of neuronal cell, glutamate released in the synaptic cleft must be removed. This is the main role of astrocytes- maintenance of the low resting glutamate concentration of 1–10 μM and its conversion to glutamine (Bergles et al. 1999; Matsui et al. 2005).

6.3.5. Catabolism

There are two main pathways of glutamate catabolism inside of astrocyte. It can be either converted to glutamine, reaction catalyzed by GS, as part of the glutamate-glutamine cycle or oxidatively metabolized in the TCA cycle (Look 6.3.1.)

6.3.6. Glutamatergic Receptors

The family of glutamate receptors is numerous and highly complex; there have been discovered more than 20 glutamate receptors in the mammalian CNS. They fall into two main categories, ionotropic (voltage sensitive) and metabotropic (ligand sensitive). In these two categories there are three types of receptors, divided by binding specificity, ion permeability, conductance properties and other factors. Although their properties differ somewhat, as do their anatomical distribution, glutamate receptors are best known for mediating glutamate's role in learning and memory through neuronal plasticity, enhanced glutamate neurotransmission and gene expression (Barco et al., 2006).

- *Ionotropic receptor channels*

Ionotropic receptor channels are formed by assemblies of heterotetrameric or homotetrameric protein subunits. The three types of ionotropic receptors are named after the ligand that expressly binds to one, but not to the other two: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainic acid. After discovering of these agonists, many others whether agonists or antagonists were subsequently found (Lesage and Steckler, 2010).

Upon binding of glutamate to NMDA receptors (NMDARs), a cation non-selective ion channel opens. This phase of opening, as well as phase of closing, is primarily gated by ligand binding but, it is also voltage-dependent. Extracellular magnesium (Mg^{2+}) and zinc

(Zn^{2+}) ions can bind to specific sites on the receptor, blocking in this way the passage of other cations through the open ion channel. But depolarization of the neuronal cell dislodges and repels these ions from the pore, therefore allowing a voltage-dependent flow of sodium (Na^{+}) and small amounts of calcium (Ca^{2+}) ions into the cell and potassium (K^{+}) out of the cell (Dingledine et al., 1999; Liu and Zhang, 2000; Cull-Candy et al., 2001; Paoletti and Neyton, 2007). The NMDA receptor is primarily a ligand-gated channel, but it does display weaker voltage-dependence modulation of the ligand-dependent gating. NMDA requires co-activation by two ligands: glutamate and either D-serine or glycine (Kleckner and Dingledine, 1988). On the other hand, the voltage-dependence of current through the channel is mainly due to binding of Mg^{2+} or Zn^{2+} ions to the protein as described above.

Furthermore, NMDA receptors are divided in subtypes depending on intracellular protein structure: NR1, NR2 and NR3. NR1 has eight different subunits generated by alternative splicing from a single gene. There are four different NR2 subunits (A-D) and late in the 20 century NR3A and NR3B subunits have been reported.

NMDA receptors are highly expressed on neurons, but not only. They are also expressed on astrocytes (Lee et al., 2010). NMDA signalling is important for human brain capacity for plasticity, learning, memory and recovery from injury, especially in the hippocampus and other regions (Barco et al., 2006). In pathological circumstances, overactivation of NMDA receptors can lead to excitotoxicity. This is implied to be involved in some neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and Huntington's disease (Chen et al., 2006; Lipton, 2006; Koch et al., 2004).

AMPA receptors are composed of four types of subunits, designated as GluA1, GluA2, GluA3, and GluA4 (Shi et al., 1999; Song and Huganir, 2002); these receptors are mostly heterotetrameric, consisting of symmetric 'dimer of dimers' of GluA2 and either GluA1,

GluA3 or GluA4 (Mayer, 2005; Greger et al., 2007). On each AMPAR there are four binding sites to which an agonist (such as glutamate) can bind, one for each subunit (Mayer, 2005). Channel only opens if two binding sites are occupied, with increase in its current as more binding sites are occupied (Rosenmund et al., 1998). Once opened, the channel may undergo rapid desensitization, stopping the current. Since AMPARs open and close quickly (1ms), they are responsible for most of the fast excitatory synaptic transmission in the CNS (Platt, 2007). GluA2 subunit is governing the AMPAR's permeability to calcium and other cations, such as sodium and potassium. Thus, if GluA2 subunit is lacking, AMPAR's will be permeable to sodium, potassium and calcium. The presence of a GluA2 subunit will almost always render the channel impermeable to calcium.

Both of ion channels, NMDA and AMPA, are important for plasticity and synaptic transmission at many postsynaptic membranes. The most studied form of neuronal plasticity is long-term potentiation, or LTP. Two main components of LTP are: depolarization of a presynaptic nerve terminal, with release of glutamate, and postsynaptic depolarization. It seems that AMPARs play an integral role in LTP process since both GluR1 and GluR2 are important for the synaptic plasticity. It is now known that the underlying physiological correlate for the increase in EPSP size is upregulation of AMPARs on a posynaptic membrane (Maren et al., 1993).

Glutamate released to the synaptic cleft binds to postsynaptic AMPARs and NMDARs, thus causing the AMPARs to open and Na^+ flows into the postsynaptic cell, resulting in a depolarization. NMDARs, on the other hand, do not open directly but are depended on a depolarization from the AMPAR activation. After GLU binding to NMDARs, calcium enters the cell through it and this is resulting in activation of Ca^{2+}/calmodulin-dependent protein kinase (CaMKII; Fukunaga, 1993). Blocking either this influx or the

activation of CaMKII prevents LTP; therefore, these receptors are one of the key elements for LTP (Lisman et al., 2002).

- *Glutamate metabotropic receptors (mGluR)*

Metabotropic glutamate receptors are contrast to ion channel receptors; they are slower acting and exert their effects indirectly, typically through gene expression and protein synthesis. These receptors have a common structure with other G protein–linked metabotropic receptors, i.e., they have seven trans-membrane domains with an extracellular N-terminal and intracellular COOH terminal. When glutamate binds with a metabotropic receptor, it activates a post-synaptic intracellular G-protein, which, in turn, triggers a second messenger system that opens a membrane channel for signal transmission. Furthermore, the activation of the G protein also triggers functional changes in the cytoplasm, resulting in gene expression and consequently protein synthesis. There are three groups of glutamate metabotropic receptors.

Group I receptors are coupled with phospholipase C, producing diacylglycerol and inositol triphosphate as second messengers. They are mostly expressed on the postsynaptic membrane. It has been shown that they have been involved in problems with learning and memory, addiction, motor regulation, and Fragile X syndrome (Niswender and Conn, 2010).

Groups II and III are negatively coupled to adenylyl cyclase. Group II metabotropic receptors are located on both, presynaptic and postsynaptic membrane, possibly to suppress glutamate transmission (Swanson et al., 2005). It is possible also that this dual location give them ability to exert a greater degree of modulation of glutamate signaling (Lesage and Steckler, 2010). Impaired functioning of group II metabotropic receptors has been implicated in anxiety, schizophrenia and Alzheimer's disease. Group III metabotropic receptors are positioned presynapticaly and like the II group, they inhibit neurotransmitter release. They

are found within the hippocampus and hypothalamus and may play a role in Parkinson's disease and anxiety disorders (Swanson et al., 2005).

6.4. Glutamatergic system

Glutamate is the most pervasive neurotransmitter in the central nervous system (CNS). Its dysfunction is thought to be correlated with a wide range of nervous system diseases and disorders. These glutamate-related disorders include neuropsychiatric disorders such as schizophrenia, neurodegenerative diseases (such as Alzheimer's), substance abuse, pain disorders, and traumatic brain and spinal cord injuries.

Five glutamatergic pathways have been indentified (Stahl, 2008; Figure 12): a) The cortical brainstem glutamate projection starts from cortical pyramidal neurons in the PFC and goes down to brainstorm neurotransmitter centers (raphe, locus coeruleus, ventral tegmental area, substantia nigra) and regulates neurotransmitter release. (b) One more descending glutamatergic pathway projects from the PFC to the striatum (corticostriatal glutamate pathway) and to the NAc (cortico-accumbens glutamate pathway), and constitutes the "corticostriatal" portion of cortico-striatal-thalamic loop. (c) Thalamocortical glutamate pathways start in the thalamus and project to pyramidal neurons in the cortex. (d) Corticothalamic glutamate pathways descend from the prefrontal cortex to the thalamus. (e) Intracortical pyramidal neurons can communicate with each other via the neurotransmitter glutamate. These pathways are known as cortico-cortical glutamatergic pathways. Three of the five pathways project from the frontal cortex and penetrate into deeper brain areas where they exert control over the neuroanatomic structures residing there.

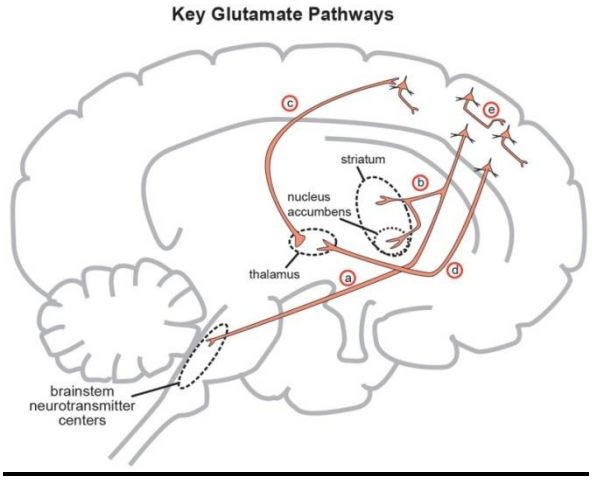

Figure 12- Glutamatergic pathways in the brain

(Stahl, 2008)

6.4.1. BNST and Glutamate

The BNST receives glutamatergic inputs from different brain structures such as hippocampus, amygdala and medial prefrontal cortex (Vertes, 2006; Choi et al., 2007; Canteras et al., 2010), as well as mixed dopaminergic/ glutamatergic inputs from the periaqueductal gray matter (Li et al., 2016). Moreover, glutamate receptors, such as NMDA and AMPA receptors, are expressed throughout the BNST (Walker and Davis, 1997; Weitlauf et al., 2004).

7. Microdialysis Coupled with Electrochemical Detection

7.1. Introduction

Brain microdialysis technique coupled with electrochemical detection (ED) is a method that permits us monitoring *in vivo* of levels of substances, such as neurotransmitters and neuromodulators, at the specific brain areas. Since there is an opportunity to measure direct changes in the brain after administration of one substance or handling an animal, microdialysis is a valuable tool for the study of the mechanisms of action and preclinical profile of centrally-acting drugs and effects of environmental changes on the brain. This method consists of a surgery with implantation of special dialysis fibers (probes) in a specific brain area, followed by perfusion of the fiber with artificial cerebrospinal fluid (Ringer´s solution). Thanks to the concentration gradient, molecules that are small enough to pass through dialysis membrane are passing from an area of high concentration (the extracellular compartment) to an area of low concentration (the inner space of the dialysis membrane). This collection of liquid and substances is called dialysate. Dialysate is then assayed by high pressure liquid chromatography (HPLC) for evaluation of substances concentration in it, which is closely related to their extracellular concentration in the investigated area. Overall, using this method allows us to assay the concentration of wanted substances in freely moving animals, after their treatment with drugs; therefore, it gives us the opportunity to compare the observed concentration variations with the related behaviour.

7.2. Basis of Monoamine Electrochemical Detection (ED)

The possibility of detecting biogenic amines, such as dopamine, norepinephrine, serotonin and their metabolites, is due to their capacity to be oxidized. Although the reaction of oxidation of monoamines 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. During oxidation reaction, every molecule gives up two electrons to the electrode. Because the result of applied potential to the electrode immersed in an electrical conducting solution (mobile phase) is 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 electrical current produces a chromatogram in which one of the detected peaks corresponds to a substance of interest, and its height or area is proportional to its sample concentration. By use of a standard curve, the absolute amount of substance can be assessed.

7.3. Dialysis Probes: Design and Time After Implantation

On the basis of their geometry, microdialysis probes are classified into three categories: (i) transcerebral, (ii) U-shaped and (iii) concentric (Di Chiara 1990).

Transcerebral probe was developed initially as a probe for anesthetized rats (Di Chiara and Imperato 1984), then adjusted to the form that can be used in freely- moving rats (Di Chiara 1985). The advantage of this probe over other two types is simplicity of construction, less tissue damage at a place of implantation, closer contact with a tissue and given a straightforward geometry, the flow of solution injected and dialysate is constant. Moreover, transcerebral probe has an elastic connection with a skull, which means that there is less damage along their track, due to the constant brain oscillations in the fluids. The main disadvantage of transcerebral probes is that they cannot reach deep brain tissues as midbrain structures, or dialyze symmetrical brain areas, simultaneously. In addition, due to elasticity of this probe, a rather complex surgery is needed in order to place it in wanted region of the brain.

7.4. Membrane properties

The diffusion of dialysate, called recovery of the fluid, and its content depend on physico-chemical properties of the membrane.

7.4.1. Membrane "cut-off"

During microdialysis, dialysate passes through a semipermeable membrane from the extracellular fluid (ECF) into a perfusate that is collected over a predetermined time and volume. Because of the semipermeable nature of membrane, only some solutes, namely low-molecular-weight solutes, will be recovered. The "cut-off" is an expression of this specificity of permeability of the membrane constituting the probe. In the case of commonly used

probes, it is allowed the diffusion of solutes of low molecular weight (400-600 Dalton), thus crossing of proteins is prevented and relatively clean samples are obtained. Furthermore, probe membrane material may also affect dialysate recovery (Ungerstedt, 1984; Kendrick 1989, 1990; Hsiao et al., 1990; Mason and Romano, 1995). The membrane materials currently used in microdialysis probes include regenerated cellulose (Cuprophan from Gambro AB), polyacrylonitrile (PAN), and polycarbonate-ether (proprietary to CMA/Microdialysis). Kendrick (1989, 1990) showed that in vitro recovery, using probes representative of each type of material, there is a great degree of variation in recovery among probe types. However, in vivo recovery this variation may be more due to the diffusional characteristics of the analyte in a tissue, so those differences in recovery among membrane types may not occur in vivo.

7.5. Neurotransmitter Recovery

Recovery of neurotransmitter from extracellular space depends on many factors and some of those are mentioned above. Taking into consideration these factors during the design phase of a microdialysis experiment and understanding the factors that contribute to changes in analyte recovery will greatly enhance the success of experiment.

7.6.1. Relative and Absolute Recovery

By comparing the concentration of the substance in the dialysate with the concentration of the medium, it is possible to calculate the recovery of the substance. Distinguishing the relative recovery and the absolute recovery is important.

In the case of relative recovery, the concentration of a specific substance in the perfusate, when leaving the probe, is expressed as a percentage of the concentration of the surrounding medium (Larson, 1991); Relative recovery will approach 100% as the flow rate approaches zero, and consequently decrease as the flow rate increases.

Absolute recovery is represented by the total amount of the recovered substance in a given period of time and it is expressed in moles /litre. It reflects the actual mass of a substance that is dialysed from the probe during a specific time period (mol/unit time). Absolute recovery is calculated as the product of the concentration in the dialysate, perfusion flow rate and the relative recovery (Lonnroth et al., 1987; Ungerstedt, 1991; Chaurasia, 1999; Hansen et al., 1999). Unlike relative recovery, absolute recovery is zero when the flow rate is zero, and will reach a maximum at higher flow rates.

Expression of microdialysis can be presented in % or in absolute amount of neurotransmitter detected. It has been debated about which expression should be used. But, there is a high variability of the basal neurotransmitter recovery, even under unchanged

working conditions; it is difficult to obtain statistical significance using the raw data when the change of output induced by drug treatment is minimal. On the other hand, if the change of the output induced by drug treatment is expressed as % of basal output (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.

7.6.2. Flow Rate

As it was mentioned above, relative recovery of an analyte (concentration of analyte per sample) is inversely proportional to the perfusate flow rate. Furthermore, for analytes with low extracellular concentration or low diffusivity, reducing the flow rate will increase the relative recovery and, therefore, increase the probability of obtaining a detectable concentration of analyte in each sample. When flow rates are extremely low (<0.1 μl/min), it is possible to reach near 100% recovery of an analyte, which means that the dialysate concentration of that analyte is equal to the ECF analyte concentration (Van Wylen et al., 1986; Menacherry et al., 1992; Smith et al., 1992).

On the other hand, the absolute recovery of an analyte is proportional to the perfusate flow rate, up to flow rates of 2 μl/min (Wages et al., 1986; Benveniste, 1989). Therefore, in the experiments where the total amount of analyte is measured (e.g., radioimmunoassay) it may be advantageous to use higher, rather than lower, flow rates (up to 2 μl/min), in order to achieve a sufficient quantity of sample for detection.

7.6.3. Microdialysis Probe Membrane Properties

Role of this factor in recovery of analyte has been already discussed in the previous chapter.

7.6.4. Analyte Properties

Kendrick (1989) was first to observe a strong negative linear relationship between molecular weight and the log percent of recovery, suggesting that one possible interpretation of the differences in relative recovery among analytes may be molecular weight. This is not surprising since the fact that when molecular weight increases, the diffusion coefficient decreases. He has also noticed that neuropeptides that had greater hydrophobic properties had lower flow rate and they responded slowly to changes in the external concentration of analyte, which means that substance structural characteristics may be a significant impediment to their collection by microdialysis.

7.6.6. Other factors that can influence recovery of analyte

Other factors, such as temperature or tissue factor, are significant for microdialysis experiments.

The relative recovery for different molecules depends on their diffusion coefficient, which is directly proportional to the temperature. On average, a 1% to 2% increase in the diffusion coefficient is observed for every degree (Celsius) of increase in temperature (Bard and Faulkner, 1980).

The extent to which relative recovery during in vivo microdialysis is affected by tissue factors depends on which medium (tissue, membrane, or perfusate) has the greatest resistance to diffusion, and that resistance depends on many factors as analyte characteristics, flow rate, etc.

7.6.7. Recovery after Surgery

After the probe has been positioned and surgery finished, there has to be a recovery period that is necessary to clean up the excess of neurotransmitter, the consequence of the leakage from nerve terminals damaged by the probe insertion. In the majority of studies in rats, the recovery period that lasts about 24 hours is used. When the microdialysis probe is implanted in mice, 48 hours recovery determines a more stable baseline of extracellular concentration of neurotransmitter.

It is possible to implant the same animal with two probes in two different regions of brain. 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.

7.6. Applications of Microdialysis Tecnique

Microdialysis has found its most important application in the field of neuroscience, and particularly in the field of neuropharmacology, although it can be used for measurement of endogenous molecules in almost every tissue. This technique makes possible to study not only the mechanism of drugs on the central level, but also the changes induced by natural stimuli. When it comes to brain research, this method is commonly used to measure neurotransmitters (e.g. dopamine, serotonin, norepinephrine, acetylcholine, glutamate, GABA) and their metabolites, as well as small neuromodulators (e.g. cAMP, cGMP, NO), amino acids (e.g. glycine, cysteine, tyrosine), and energy substrates (e.g. glucose, lactate, pyruvate).

Because of their role in the control of principal functions and behaviors, the biogenic amines dopamine and norepinephrine, together with their precursors and metabolites, have been involved in the pathophysiology of several neurological and psychiatric disorders, as biomarkers for disease development or progression, and targets for the development of novel therapeutic strategies. The possibility of direct detection of dopamine and norepinephrine in dialysate from freely moving animals has given an extraordinary advantage to researchers. Microdialysis is giving us unique possibility of continuously monitor of drug, neurotransmitter or their metabolite concentrations in the extracellular fluid of virtually any tissue over several hours, days, or even weeks. Moreover, use of Ringer solution allows sampling without fluid loss, thus microdialysis can be consequently used without disturbing the tissue conditions by local fluid loss, which can occur when using other techniques. Thanks to the semipermeable membrane, dialysate is free of cells, cellular debris and proteins, so further elaboration of dialysate is not needed.

Among applications of microdialysis, the most important are: (i) studies of functional neuroanatomy; (ii) studying the mechanism of action of known drugs, or development of new drugs and their pharmacological screening; (iii) pharmacokinetic studies and metabolism of drugs; (iv) behavioral studies (biochemical responses on 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 in clinical neurosciences.

But, there are some limitations of the use of microdialysis technique. Since this technique was developed, microdialysis probes have been developing to be smaller and more efficient. Despite these scientific advances, the invasive nature of this technique still presents some practical and ethical limitations. For example, it has been shown that implantation of a microdialysis probe can damage a tissue morphology resulting in acute implantation traumas that require sufficient recovery time. Additional factors, such as necrosis, inflammatory responses (Carson et al., 2015), or wound healing processes may influence experimental results, therefore they have to be taken into consideration for long-term sampling. For these reasons, it has been suggested to perform microdialysis experiments within an optimal time window, usually 24–48 hours after probe insertion (Di Chiara et al., 1996; Westernik et al., 1987).

Furthermore, use of the microdialysis technique is often limited by the determination of the probe's recovery, especially for in vivo experiments. The recovery is mostly dependent on the flow rate: the lower the flow rate, the higher the recovery. Nevertheless, in practice the flow rate cannot be decreased too much since the quantity of obtained dialysate will not be sufficient. It is therefore important to optimize the relationship between flow rate and the sensitivity of the analytical assay.

In this work microdialysis was used to assess the effects of drug of abuse and ketamine on noradrenergic neurotransmission. But our laboratory had collaborated with other lab studying Parkinson disease, and we contributed to this work by measuring the levels of L-DOPA in a blood of rats treated with this drug (Mulas et al., 2016).

THE AIM OF THE STUDY

Mental disorders are still the black hole of medicine with WHO (World Health Organization) assuming that every fourth person is affected by some mental disease at some point of their lives. By their data, around 450 million of people are currently affected and nearly two-thirds of them never seek for help due to stigma and discrimination (WHO). In many societies mental disease is a tabu and so many people will never seek for the help or even if they do, the existed therapies in many countries are not available to people without health insurance. But besides social and neglect by the side of governments, one other big problem is efficacy of existed treatment. Although there is available treatment, its efficacy and safety is the main concern. A numerous number of preclinical and clinical studies are being conducted in order to reveal the brain mechanisms of mental disorders. Despite substantial progress in identifying how certain behaviours are expressed, there is still long way to go ahead us.

The main focus of our laboratory was on the stress-related mental disorders as depression and drug abuse/addiction. Stress is a body's response to a stimulus that can be external or internal. From the anatomical and physiological point of view, the hypothalamus-pituitary-adrenal axis is the principal coordinator of the metabolic and behavioral body's response to stressful situations. A various stressors can cause hypothalamus release of the corticotropin-releasing factor, which stimulates the pituitary gland to produce and release adrenocorticotropic hormone; as a result, there is a production of different hormones involved in the series of physiological events induced by stress. And it has been shown that stress can influence facilitation and exacerbation of anxiety, substance use disorders and major depressive disorder (Hammen, 2005; Andersen and Teicher, 2009; Nugent et al., 2011), but the exact mechanism is not yet known. A region of interest with regard to stress and control of HPA axis is the Bed Nucleus of Strie Terminalis (BNST), a part of extended amygdala.

The BNST has both dopaminergic and norepinephrenic nerve stimulation. Noradrenaline is associated with stress response, while dopamine is associated with reward. The role of BNST's dopaminergic transmission and gratification circuits in a positive reinforcement and the motivation behind the addiction and abuse of substance is demonstrated by many studies (Park et al., 2013). These circuits are previously described in details, as well as the role of the BNST in the stress and reward systems.

On these premises, the aim of this study was to investigate the role of noradrenergic transmission in the BNST, in particular to evaluate the ability of drugs of abuse, such as nicotine, cocaine, amphetamine, morphine, ethanol and ketamine, to stimulate norepinephrine and dopamine in the BNST in freely moving rats through the technique of the microdialysis. These substances have different mechanism of action and all of them can increase dopamine output, although with different intensity, in areas of the extended amygdala, such as the ventral striatum and the BNST (Carboni et al., 2000). On the other hand, very little is known about the ability of these drugs to affect the norepinephrine transmission of the BNST, although norepinephrine antagonists have been involved in many aspects of addiction of many drugs of abuse (Mantsch et al., 2016, McElligott et al., 2013).

In this general picture, we found extremely interesting to investigate the effect of ketamine on the BNST transmission because ketamine, besides being a drug of abuse with peculiar features, has been claimed to possess antidepressant properties. In fact, being anxiety and depression often a part of the multifaceted domain of addiction, we wondered whether one drug of abuse, by interacting directly on the norepinephrine transmission in the BNST, could produce an alteration of a transmission that regulates a fundamental homeostatic equilibrium generated by the convergence of external and internal stimuli and whether this alteration is later translated into anxiety, depression and high sensibility to stress-induced reinstatement of drug seeking behaviour (McElligott et al., 2013).

MATERIALS AND METHODS

1. Animals

Male Sprague–Dawley (S.D.) rats weighing 230–250 g [Harlan, S. Pietro Natisone, Italy] were used in these experiments (Figure 13). The rats were housed in groups of 4 or 5 per cage, in standard temperature (22 ° C) and humidity (60%), with water and food at libitum and an artificial light and dark cycle of 12 hours (light, 8 AM to 8 PM).

Figure 13- SD rat with implanted fiber

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.

2. Microdialysis

In all our experiments we used the same procedure to assess the BNST. This technique was described in detail in the Introduction.

2.1. Microdialysis equipment

1. Microdialysis probe

2. A perfusion pump connected to a syringe of 2.5-5 mL through a polyethylene tube, which is capable of releasing the liquid with a flow of 0.1-10 μL / min

3. Perfusion ringer. The microdialysis fibers are perfused by an isotonic solution, whose composition should look as close as possible to the composition of the extracellular brain fluid.

4. A pin, which is located between the probe and the perfusion syringe and it allows rat to move freely, preventing the tubular twist.

2.1.1. Probes

Concentric dialysis probes were prepared with a 7mm piece of AN 69 (sodium methallyl sulfate copolymer) dialysism, 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.

2.2. Surgery

Rats were anesthetized with 400mg/Kg i.p. chloral hydrate and placed in stereotaxic apparatus (Figure 14).

Figure 14-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)] (Figure 15) 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.

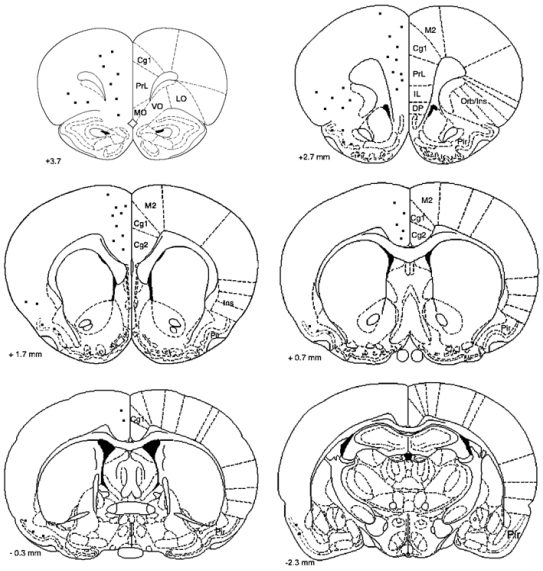

Figure 15- Atlas by Paxinos and Watson

2.4. Experiment

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 by HPLC in order to evaluate dopamine (for Ketamine) and norepinephrine sample content (for Ketamine and drug of abuse). The fiber outlet is connected to a short circular tube which collects the dialysate coming out of the fiber (Figure 16).

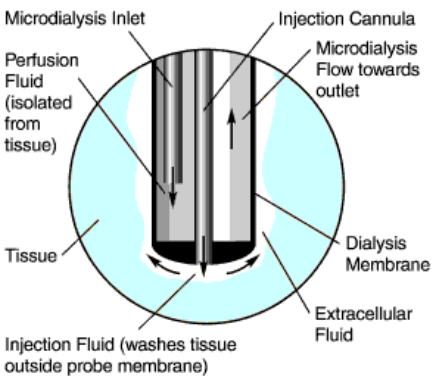

Figure 16- Microdialysis fiber

2.4.1. Analytical procedure of microdialysis

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 0.1M sodium acetate, 0.3 mM Na2EDTA, 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.

In these experiments we were interested in catecholaminergic neurotransmitters: norepinephrine (NA) and dopamine (DA). At this phase of the experiment we can distinguish 3 steps:

• Locating neurotransmitters in the chromatogram.

Preparation of different standard concentrations of Noradrenaline and Dopamine proceeds. Next step is injection of 10 μl of the solution containing the neurotransmitter (DA or NA or NA-DA) at a concentration of 100 μM (10-8 mM) and then location of the peak corresponding to that particular neurotransmitter in the chromatogram and measurement of the retention time.

• Detection of basal values.

In the HPLC, the samples recovered from untreated rats are identified in the chromatogram with the peaks corresponding to NA and DA. The area and height of the peak are measured. This step is repeated until obtaining height and constant area values that represent baseline values to which reference will be made when comparing the values obtained after a treatment. The neurotransmitter 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 were: 41.34 (±1.76; n =119) and 23.86 (±0.94; n =119) fmol/20 µl sample, respectively.

• Treatment with substances.

When the basal output of NA or DA reached stable values, rats were given (i.p., s.c.) a challenge dose of the drug tested or saline. Each implanted rat was challenged with a single dose of the test drug only once.

1. Drug of abuse.

Each drug was tested at doses of: morphine chlorhydrate (1.0-3.0 mg/kg s.c.), ethanol (0.5-1.0 g/kg i.p.), nicotine (0.2-0.4 mg/kg s.c.), amphetamine sulphate (0.5-0.5 mg/kg s.c.) and cocaine chlorhydrate (2.5-5.0 mg/kg i.p.), dissolved in the solution of saline and injected immediately. Four rats were administered with saline.

2. Ketamine

Each implanted rat received a single acute i.p. injection of ketamine, tested at doses of 10, 20, and 40 mg (4 times for each dose administered). Number of rats used for each testes dose is six. Five rats were administered with saline.

2.4.2. Origin of drugs

Nicotine tartrate, morphine HCl, amphetamine and cocaine HCl were obtained from Sigma (Milano, Italy), ethanol by Carlo Erba.

Ketamine hydrochloride (Ketalar) was purchased from by Farmaceutici Gellini, Milan, Italy, dissolved in saline and administered i.p. immediately.

3. Motility Experiment

In addition to microdialysis experiments of ketamine, the motility experiment was performed. Four rats, for each experimental group, were exposed individually, in a new environment for three 10 min periods in the time interval 15-45 min, after drug administration. Total and locomotion activity was evaluated through an “Open Field” meter (Columbus Instruments, Columbus OH, USA). Each 10 min period activity and global exposition period (30 min) activity were statistically analysed (ANOVA) for differences in total and locomotion activity between groups and within periods in each experimental group.

4. 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 sacrificed. 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) (Figure 15 and 17).

Figure 17-Oscillating microtome

The slices were then observed under the the microscope to determine the exact location of the fiber in the investigated area (BNST) (Figure 18. and 19.). Results from rats implanted outside the BNST were discarded.

Figure 18- Brain slices

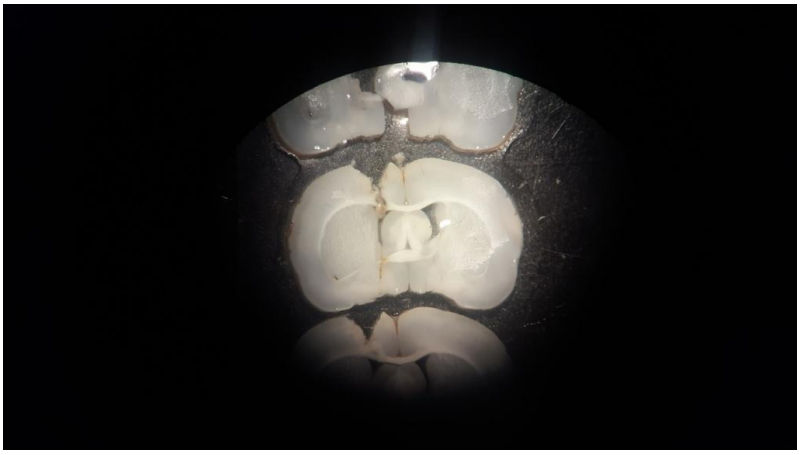

Figure 19- Brain slice under the microscope

5. 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

1. Effects of Nicotine on Norepinephrine and Dopamine Output in the BNST

1.1. Noradrenaline

Figure 20-A shows the effect of a treatment with increasing doses of nicotine (0.2 mg/kg and 0.4 mg/kg) or saline on the NE output in the BNST of SD rats.

Two-way ANOVA of the results obtained showed a significant dose-response effect ($F_{2,11}$ = 23,6 $p < 0,001$), a significant time effect ($F_{7,77}$ = 18,05 $p < 0,001$) and a significant overall interaction ($F_{14,77}$ = 10,4, $p < 0.001$).

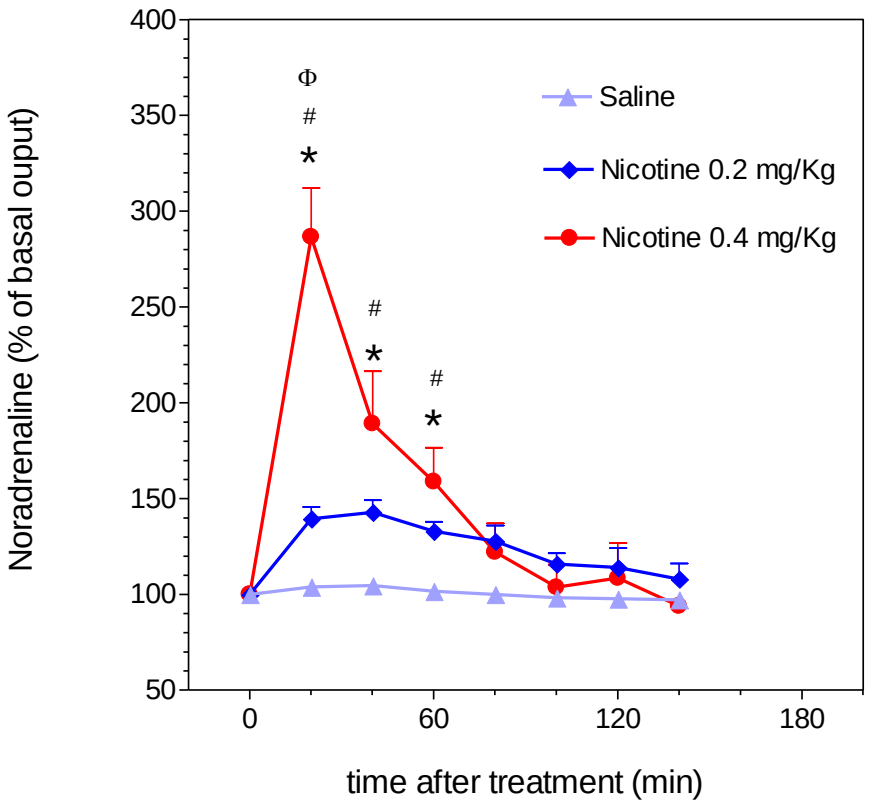

Figure 20- A Effect of nicotine on the BNST norepinephrine output

Effect of nicotine (0.2 and 0.4 mg/Kg s.c.) on the BNST NE output, expressed as a percentage of the basal output. Each point is the mean (±SE) of at least four determinatnts. *$p < 0.05$ from basal values; #$p < 0.05$ versus the corresponding time point after saline; ø$p < 0.05$ versus the corresponding time point of 0.2 mg/Kg.

POST-HOC (Turkey) analysis of extracellular levels of NA showed that the effect of 0.4 mg / kg of nicotine was significantly different from the effect of 0.2 mg / kg and the effect of saline. Furthermore, the output of NA after treatment with a dose of 0.4 mg / Kg in the range of 20-60 min is significantly higher than that measured before treatment (baseline) and that observed at the same time after treatment with saline. This analysis has also showed that NA output at the twentieth minute after treatment with 0.4 mg/Kg is significantly higher than one measured at the same time after treatment with a dose of 0.2 mg / Kg.

1.2. Dopamine

Figure 20-B shows the effect of treatment with increasing doses of nicotine (0.2 mg/kg and 0.4 mg/kg) or saline on the DA output in the BNST of SD rats.

Two-way ANOVA of the results obtained showed a significant dose-response effect ($F_{2,11}$ =34,2 $p < 0,001$), a significant time effect ($F_{7,77}$ = 10,3 $p < 0,001$) and a significant overall interaction ($F_{14,77}$ = 4,84 $p < 0,001$).

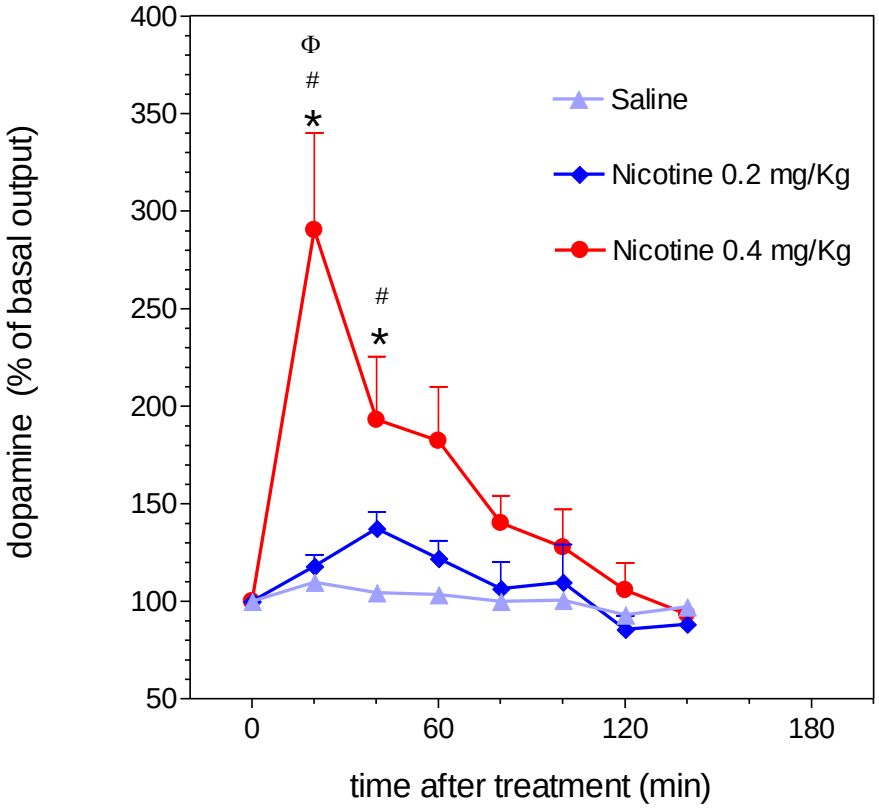

Figure 21- B Effect of nicotine on the BNST dopamine output

Effect of nicotine (0.2 and 0.4 mg/Kg s.c.) on the BNST dopamine output, expressed as a percentage of the basal output. Each point is the mean (±SE) of at least four determinatnts. *$p < 0.05$ from basal values; #$p < 0.05$ versus the corresponding time point after saline; ø $p < 0.05$ versus the corresponding time point of 0.2 mg/Kg.

POST-HOC (Turkey) analysis of extracellular levels of DA showed that the effect of 0.4 mg / kg dose was significantly different from the one of 0.2 mg / kg dose and the effect of the saline; the output of DA after the treatment with a dose of 0.4 mg / Kg in the range of 20-40 min is significantly higher than that measured before treatment (baseline) and that observed at the same time after treatment with saline. This analysis has also showed that DA output at the twentieth minute after treatment with 0.4 mg/Kg of nicotine is significantly higher than one measured at the same time after treatment with a dose of 0.2 mg / Kg.

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

2.1. Noradrenaline

Figure 21-A shows the effect of treatment with increasing doses of cocaine (2.5 mg/kg and 5 mg/kg) or saline on the NE output in the BNST of SD rats.

The statistical analysis of ANOVA results showed a significant dose-response effect ($F_{2,9}$ = 37.7, p <0.001), a significant time effect ($F_{7,63}$ = 25.9, p <0.001) and a significant overall interaction ($F_{14,63}$ = 9.47, p <0.001) for norepinephrine output.

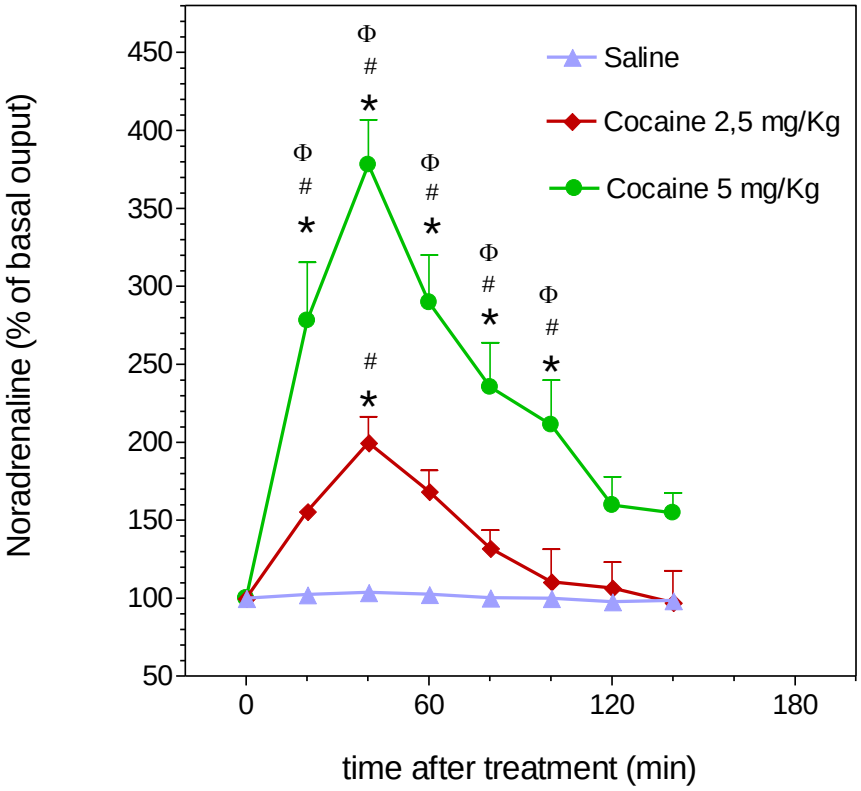

Figure 22 A- Effect of cocaine on the BNST norepinephrine output

Effect of cocaine (2.5 and 5 mg/Kg i.p.) on the BNST NE, expressed as a percentage of the 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 lower dose

The POST-HOC (Turkey) analysis of changes in extracellular levels of norepinephrine showed that the effect of 5 mg / kg dose of cocaine is significantly different from the effect of 2.5 mg / kg dose and the effect of saline. This analysis showed that the output of norepinephrine at fortieth minute after treatment with the 2.5 mg / kg dose is significantly higher than one measured prior to treatment (baseline) and at the same point of time after treatment with saline; the dose of 5 mg / kg has instead resulted in a significant increase of the output of NE in the range of 20-100 min, compared to the baseline and compared to the NE output in the same time range observed after treatment with saline. Finally, the analysis showed that the output of norepinephrine after treatment with 5 mg / kg in the range of 20-100 min is significantly greater than that measured at the same time after treatment with the dose of 2.5 mg / Kg.

2.2. Dopamine

Figure 21-B shows the effect of treatment with increasing doses of cocaine (2.5 mg/kg and 5 mg/kg) or saline on the DA output in the BNST of SD rats.

The statistical analysis of ANOVA results showed a significant dose-response effect ($F_{2,9}$ = 17, p <0.001), a significant time effect ($F_{7,63}$ = 18, p <0.001) and a significant overall interaction ($F_{14,63}$ = 6, p <0.001) for dopamine output.

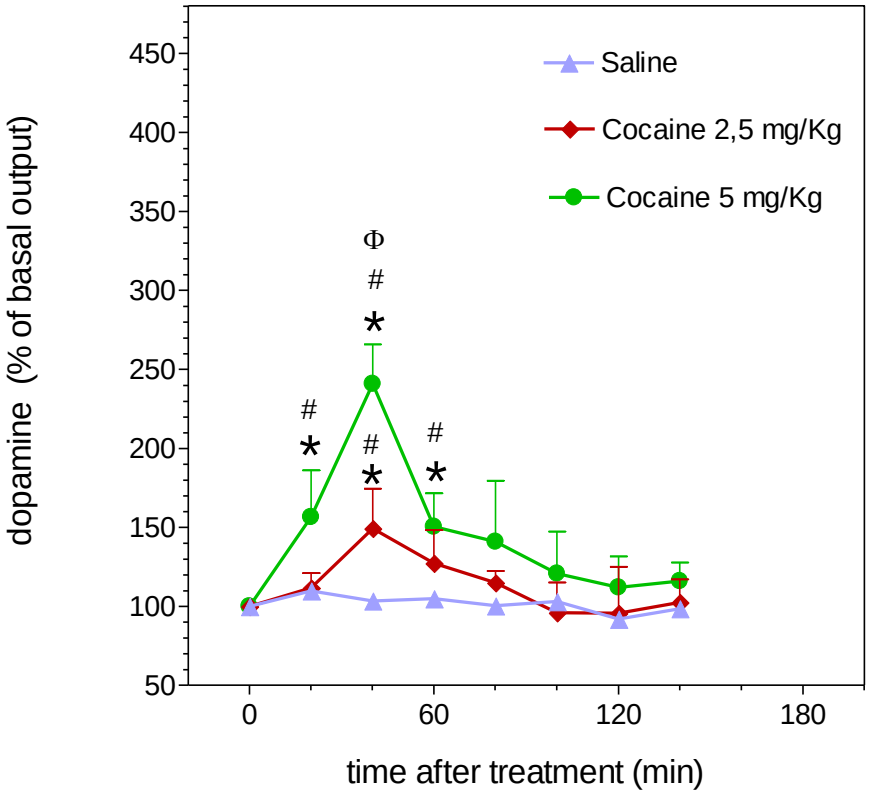

Figure 23 B- Effect of cocaine on the BNST dopamine output

Effect of cocaine (2.5 and 5 mg/Kg i.p.) on the BNST dopamine, expressed as a percentage of the 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 lower dose

The POST-HOC (Turkey) analysis of changes in extracellular levels of dopamine showed that the effect of 5 mg / kg dose of cocaine is significantly different from the effect of 2.5 mg / kg and the effect of saline. This analysis showed that the output of dopamine at the fortieth minute after the treatment with the 2.5 mg / kg dose is significantly higher than one measured prior to treatment (baseline) and at the same point of time after treatment with saline; the dose of 5 mg / kg has instead resulted in a significant increase of the output of DA in the range of 20-60 min, compared to baseline and compared to the DA output in the same time range observed after treatment with saline. Moreover, DA output at the fortieth minute after treatment with 5 mg/Kg of cocaine is significantly higher than one measured at the same time after treatment with a dose of 2 mg / Kg.

3. Effects of Morphine on Norepinephrine and Dopamine Output in the BNST

3.1.Noradrenaline

Figure 22-A shows the effect of treatment with increasing doses of morphine (1mg /kg and 3 mg/kg) or saline on the NE output in the BNST of SD rats.

The statistical analysis of ANOVA results showed a significant dose-response effect ($F_{2,13}$ = 15.58, p <0.001), a significant time effect ($F_{8,104}$ = 13.4, p <0.001) and a significant overall interaction ($F_{16,104}$ = 3.56, p <0.001) for norepinephrine output.

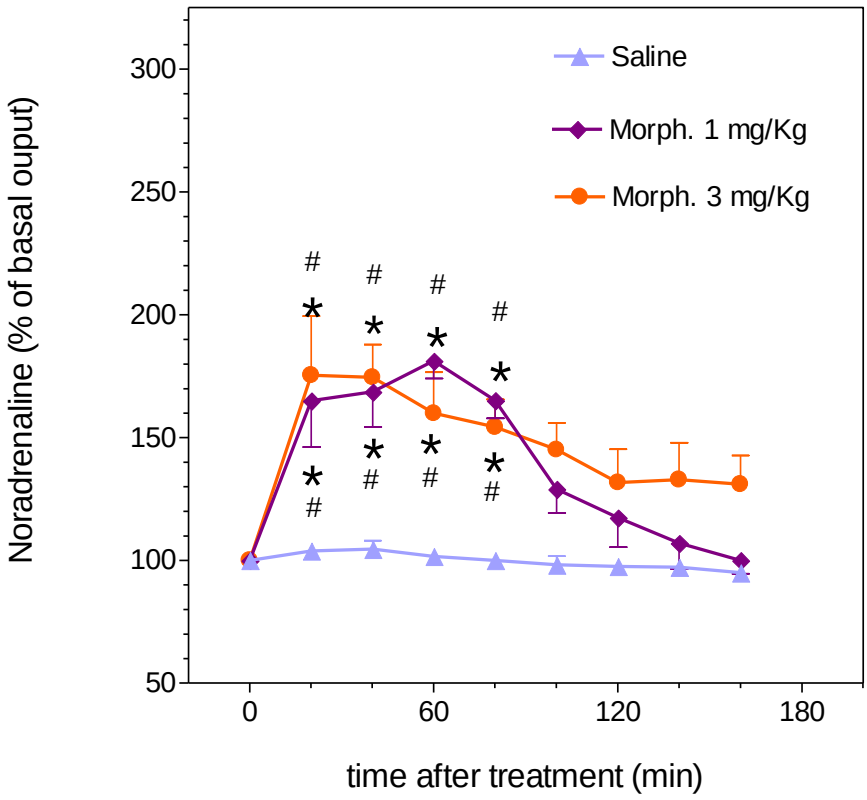

Figure 24 A- Effect of morphine on the BNST norepinephrine output

Effect of morphine (1 and 3 mg/Kg s.c.) on the BNST NE, expressed as a percentage of the 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 a lower dose.

The POST-HOC analysis (Tukey) of changes in extracellular levels of NA showed that the effects of 1 mg / kg and 3 mg / kg of morphine were significantly different from the effect of the saline. The above analysis showed that the output of NE after treatment with both doses over time of 20-60 minutes is significantly higher than that measured before treatment (basal) and compared to the NE output observed in the same time range after the saline treatment.

3.2.Dopamine

Figure 22-B shows the effect of treatment with increasing doses of morphine (1mg /kg and 3 mg/kg) or saline on the DA output in the BNST of SD rats.

The statistical analysis of ANOVA results showed a significant dose-response effect ($F_{2,12}$ = 7, p <0.001), a significant time effect ($F_{9,108}$ = 15.93, p <0.001) and a significant overall interaction ($F_{18,108}$ = 7.42, p <0.001) for dopamine output.

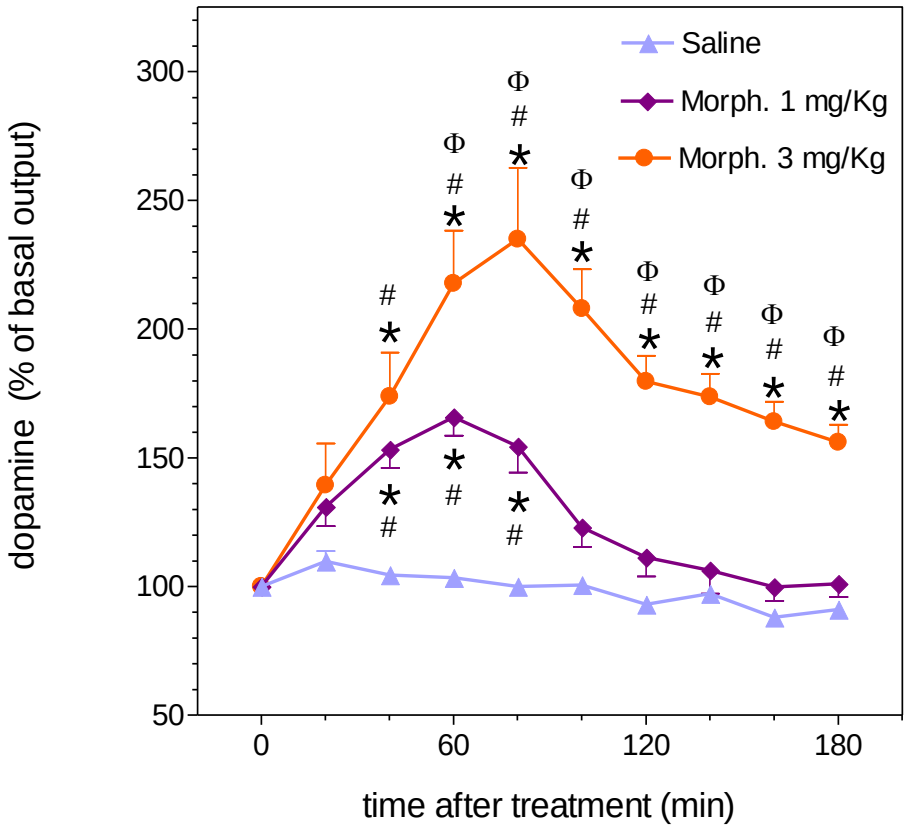

Figure 25 B- Effect of morphine on the BNST dopamine output

Effect of morphine (1 and 3 mg/Kg s.c.) on the BNST dopamine, expressed as a percentage of the 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 a lower dose.

The POST-HOC (Turkey) analysis of changes in extracellular levels of dopamine showed that the effect of 3 mg / kg dose of morphine is significantly different from the effect of 1 mg / kg and the effect of saline. This analysis showed that the output of dopamine from 40-60 minute after treatment with the dose of 1 mg / kg is significantly higher than one measured prior to treatment (baseline) and at the same point of time after treatment with saline; on the other hand, the dose of 3 mg / kg has resulted in a significant increase of the output of DA for much longer time, from 40-180 minute, compared to baseline and compared to the DA output in the same time range observed after treatment with saline. Moreover, this increase in DA output, from 60-180 minute, was statistically different from the increase cause by the dose of 1 mg/kg.

4. Effects of Amphetamine on Norepinephrine and Dopamine Output in the BNST

4.1.Noradrenaline

Figure 23-A shows the effect of treatment with increasing doses of amphetamine (0.25 mg/kg and 0.5 mg/kg) or saline on the NE output in the BNST of SD rats.

The statistical analysis of ANOVA results showed a significant dose-response effect ($F_{2,9}$= 50.69, p <0.001), a significant time effect ($F_{7,63}$ = 14.47, p <0.001) and a significant interaction ($F_{14,63}$ = 6.34, p <0.001) for NA output.

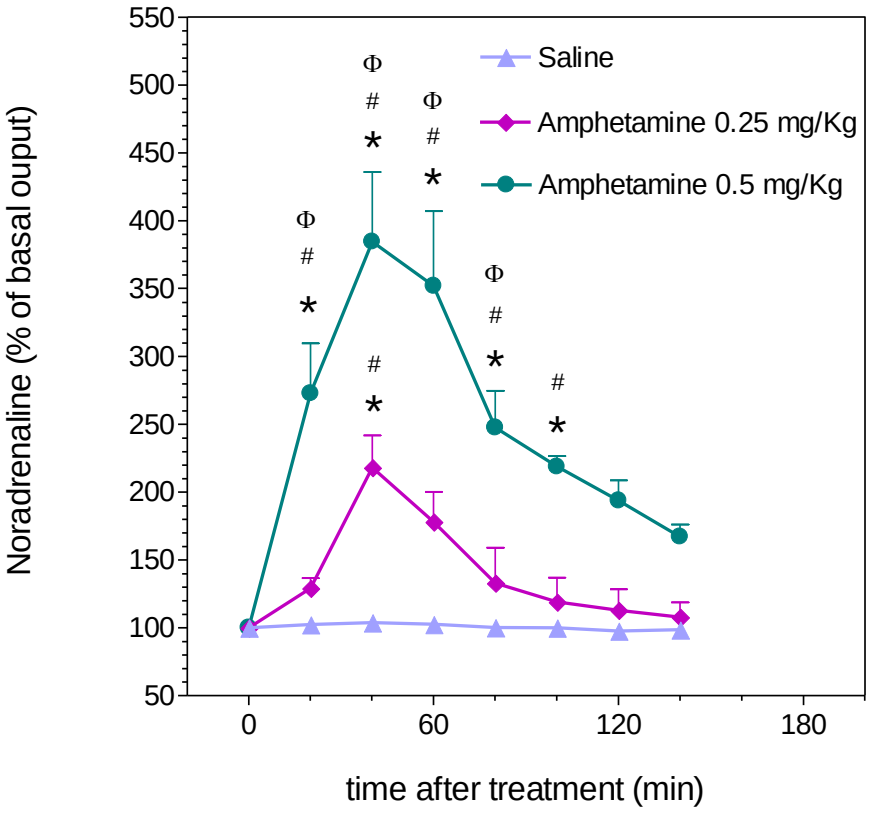

Figure 26 A- Effect of amphetamine on the BNST norepinephrine output

Effect of amphetamine (0.25 and 5 mg/Kg s.c.) on the BNST norepinephrine, expressed as a percentage of the 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 0.25 mg/Kg.

POST-HOC (Turkey) analysis of extracellular levels of noradrenaline showed that doses of 0.25 mg / Kg and 0.5 mg / kg were significantly different from each other and compared with the effect of saline. It showed that the output of noradrenaline after treatment with the dose of 0.25 mg / Kg at the fortieth minute is significantly higher than that measured prior to treatment (baseline) and that observed at the same time after treatment with saline; the 0.5 mg / kg dose, however, resulted in a significant increase in NE output in the range of 20-100 min compared to the baseline and compared to the NE output observed in the same interval after saline treatment. Finally, the analysis showed that the NE BNSToutput after treatment with 0.5 mg / kg in the 20-80 min interval is significantly higher than that measured at the same time after treatment with a dose of 0.25 mg / Kg.

4.2.Dopamine

Figure 23-B shows the effect of treatment with increasing doses of amphetamine (0.25 mg/kg and 0.5 mg/kg) or saline on the DA output in the BNST of SD rats.

The statistical analysis of ANOVA results showed a significant dose-response effect ($F_{2,9}$= 40.73, p <0.001), a significant time effect ($F_{7,63}$ = 22.49, p <0.001) and a significant interaction ($F_{14,63}$ = 13.46, p <0.001) for DA output.

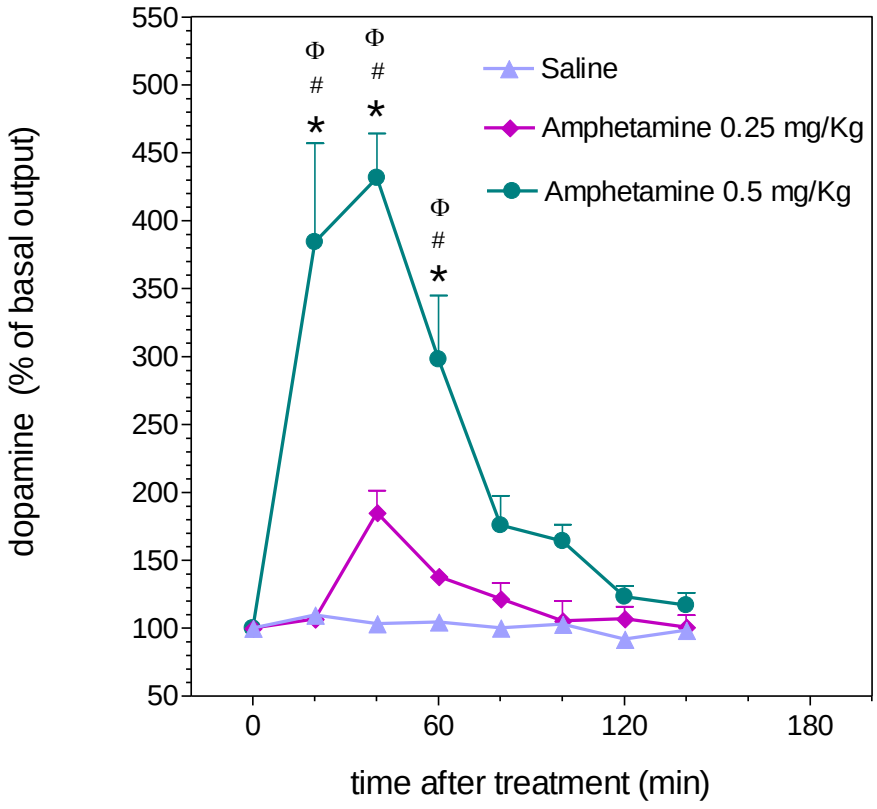

Figure 27 B- Effect of amphetamine on the BNST dopamine output

Effect of amphetamine (0.25 and 5 mg/Kg s.c.) on the BNST dopamine, expressed as a percentage of the 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 0.25 mg/Kg.

POST-HOC (Turkey) analysis of extracellular levels of dopamine showed that the output of dopamine after treatment with the dose of 0.25 mg / Kg wasn't significantly different from the output measured prior to treatment (baseline) and that observed after treatment with saline; on the other hand, the 0.5 mg / kg dose resulted in a significant increase in DA output in the range of 20-60 min compared to the baseline and compared to the DA output observed in the same interval after saline treatment. Finally, the analysis showed that the DA BNST output after treatment with 0.5 mg / kg in the 20-60 min interval is significantly higher than that measured at the same time after treatment with a dose of 0.25 mg / Kg.

5. Effects of Ethanol on Norepinephrine and Dopamine Output in the BNST

5.1.Noradrenaline

Figure 24- A shows the effect of treatment with increasing doses of ethanol (0.5 g/kg and 1 g/kg) or saline on the NE output in the BNST of SD rats.

Statistical analysis of ANOVA results showed a significant dose-response effect ($F_{2,12}$ = 5.49, p <0.05), a significant time effect ($F_{7,84}$ = 23.68, p <0.001) and a significant overall interaction ($F_{14,84}$ = 6.16 p <0.001) of ethanol on the NE output.

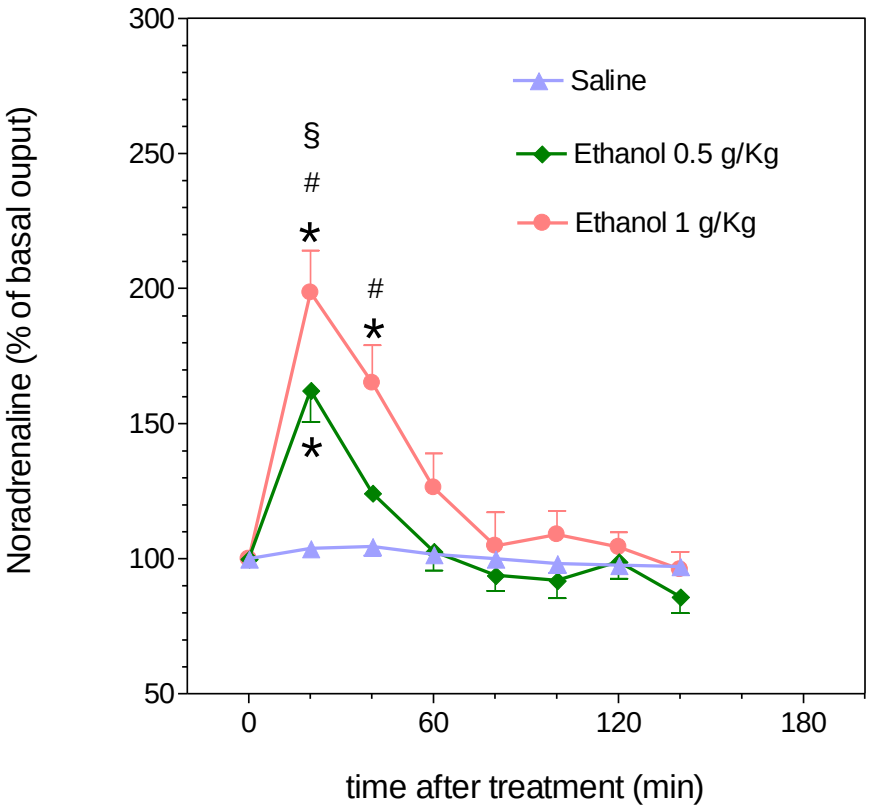

Figure 28 A- Effect of ethanol on the BNST norepinephrine output

Effect of ethanol (0.5 and 1 g/Kg i.p.) on the BNST norepinephrine, expressed as a percentage of the 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 0.5 mg/Kg

POST-HOC (Turkey) analysis of the extracellular levels of NE showed that the effect of the 1 g/Kg dose of ethanol is significant different from the effect of lower dose and the one of saline. These analyses showed that, 20 and 40 minutes after the administration of 1 g/Kg, NE output is significately higher than the one measured before the treatment, as well as the output observed after the treatment with saline. Furthermore, at the twentieth minute this effect was significally different from the effect of lower dose of ethanol on DA output. When it comes to the lower dose of 0.5 g/kg, analyses showed only one significant difference: at the twentheth minute after treatment the NE ouput was significately different from the baseline output.

5.2.Dopamine

Figure 24-B shows the effect of treatment with increasing doses of ethanol (0.5 g/kg and 1 g/kg) or saline on the DA output in the BNST of SD rats.

Statistical analysis of ANOVA results showed a significant dose-response effect ($F_{2,10}$ = 19.27, p <0.05), a significant time effect ($F_{7,70}$ = 23.3, p <0.001) and a significant overall interaction ($F_{14,70}$ = 6.5 p <0.001) of ethanol on the DA output.

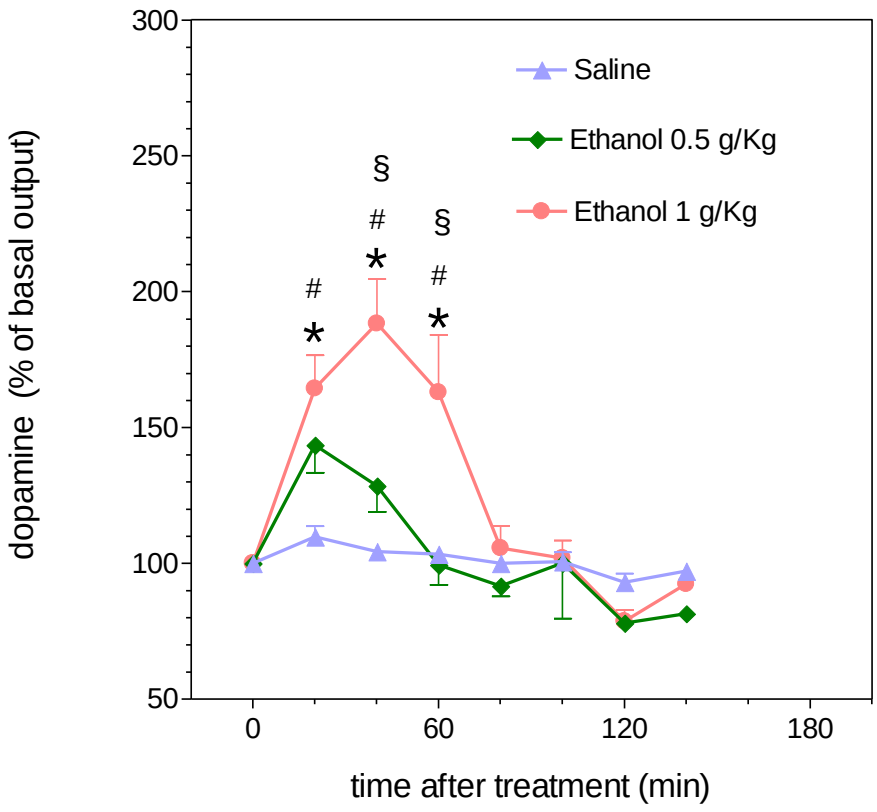

Figure 29 B- Effect of ethanol on the BNST dopamine output

Effect of ethanol (0.5 and 1 g/Kg i.p.) on the BNST dopamine, expressed as a percentage of the 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 0.5 g/Kg.

POST-HOC (Turkey) analysis of the extracellular levels of DA showed that the effect of the 1 g/Kg of ethanol is significantly different than the effect of a lower dose and the one of saline. It showed that, from 20-60 minutes after the administration of 1 g/Kg, DA output is significately higher than the one measured before the treatment, as well as the output observed after the treatment with saline. This increase was also significately different from the increase cause by lower dose from 20-60 minutes after the treatment. When it comes to the lower dose of 0.5 g/kg, analyses showed that there wasn´t significant difference between the DA output after treatment with ethanol and baseline and output after treatment with saline.

6. Effects of Ketamine on Norepinephrine and Dopamine Output in the BNST

Figures 25 and 26 show that ketamine increased norepinephrine (127, 155,186 %) and dopamine (114, 156, 176 %) output when administered at 10, 20, and 40 mg/Kg i.p., respectively. Two-way ANOVA of the results obtained showed a significant treatment effect ($F_{3,19}$ = 3.76, p < 0.02 and $F_{3,19}$ = 4.63, p< 0.01),), time effect ($F_{9,171}$ = 31.41, p< 0.001 and $F_{9,171}$ = 12,72, p < 0.001) and time-dose interaction ($F_{27,171}$ = 6,29, p< 0.001 and $F_{27,171}$ = 3,04, p< 0.001) for norepinephrine and dopamine, respectively.

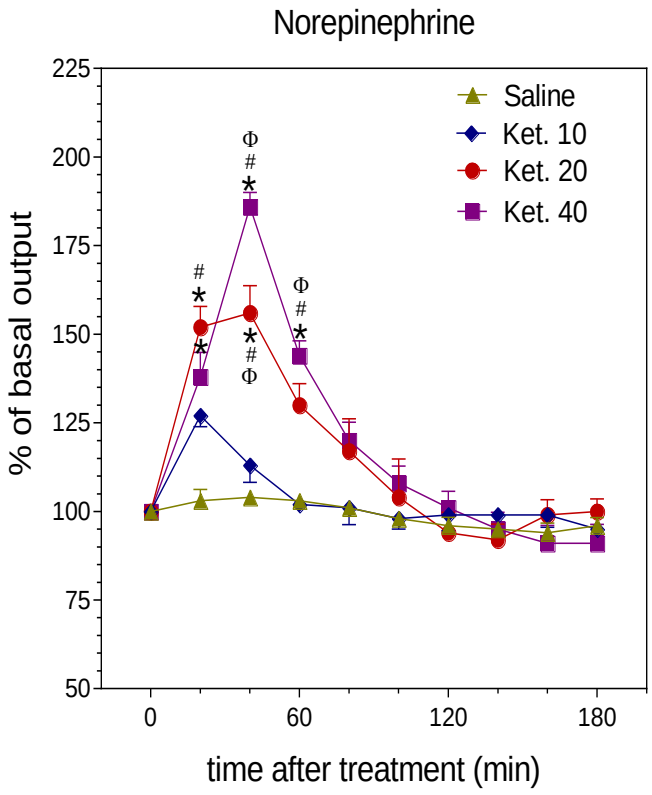

Figure 30 - Effect of Ketamine on the BNST NE output

Effect of Ketamine (10, 20 and 40 mg/Kg i.p.) on the BNST norepinephrine, expressed as a percentage of the 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 lower doses.

POST-HOC analysis showed that the increase of norepinephrine and dopamine output, obtained at doses of 20 and 40 mg/Kg were significantly higher than that produced by the dose of 10 mg/kg.

6.1. Norepinephrine

The above analysis showed that the output of NE, after the treatment with the dose of 20 mg / Kg in the time range from 20-40 min, is significantly higher than that measured prior to treatment (baseline) and that observed at the same time after treatment with saline; the dose of 40 mg / kg however resulted in a significant increase in NE output in the range of 20-60 min, compared to the baseline and compared to the output observed in the same interval after saline treatment. Finally, the analysis showed that the output of NE after treatment with 20 mg / kg at the fortieth minute is significantly higher than that measured at the same time after treatment with a dose of 10 mg / Kg; output of NA after the dose of 40 mg/Kg in the period from 40-60 minutes is significantly higher than that measured prior to treatment (baseline), and from the output measured at the same time after treatment with a dose of 10 and 20 mg / Kg and saline.

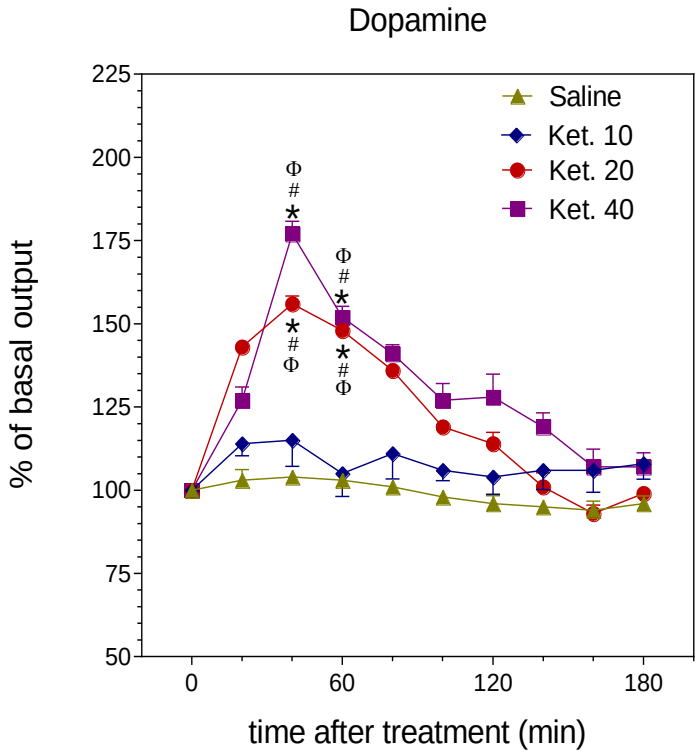

Figure 31 - Effect of Ketamine on the BNST DA output

Effect of Ketamine (10, 20 and 40 mg/Kg i.p.) on the BNST dopamine, expressed as a percentage of the 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 lower doses.

6.2. Dopamine

The POST-HOC analysis showed that the output of DA after treatment with the dose of 20 mg / Kg and 40 mg/Kg in the time range from 40-60 min is significantly higher than that measured prior to treatment (baseline) and that observed at the same time after treatment with saline. Moreover, the analysis showed that the output of DA after the treatment with 20 mg / kg is significantly higher in the period from 40-60 minutes than that measured at the same time after treatment with a dose of 10 mg / Kg; output of DA after the treatment with a dose of 40 mg/Kg in the period from 40-60 minutes is significantly higher than that measured at the same time range after treatment with a dose of 10 and 20 mg / Kg.

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DISCUSSION

1. Drugs of abuse and their effect on the BNST NA tansmission

This research demonstrates that the administration of each of the following substances of abuse (nicotine, cocaine, morphine, amphetamine and ethanol) resulted in a dose-dependent increase in the extracellular concentration (output) of noradrenaline in the BNST of awake, freely moving rats.

1.1. Nicotine

Nicotine addiction is associated with a very strong dependence; individuals who quit smoking have the highest occurrence of relapse and it is often triggered by a personal or environmental stressful situation. Considering the pivotal role of the BNST in modulating the HPA axis, we've wondered whether the effect of nicotine on NA transmission in the BNST could have a role in strengthening nicotine dependence and in resuming smoking after cessations, in particular after exposure to stress. Here we have observed that nicotine administration resulted in a significant increase in NA output; moreover, our lab previously showed that nicotine stimulated DA transmission in the BNST (Carboni et al., 2000), when we suggested that this effect was probably due to the stimulation of dopaminergic neurons of the VTA (Mereu et al., 1987; Pidoplichko et al., 1997), that project to the BNST and other various dopaminergic areas.

Nevertheless, nicotine modulates the release of multiple transmitters, including the one of NA. The first studies on nicotine and NA revealed that nicotine evokes NA release in hippocampus, both in vitro and in vivo (Brazell et al., 1991; Sacaan, Dunlop and Lloyd, 1995), as well as in the frontal cortex (Anderson et al., 2000; Summers and Giacobini, 1995). Furthermore, Singer and colleagues showed that acute nicotine increases NA release and turnover as indicated by the increase of metabolites in several brain areas (Singer et al., 2004). In addition, several microdialysis studies showed that acute nicotine increased NA

levels in mPFC and NAc shell and core (Liang et al., 2008) and that nicotinic agonists increased NA release in frontal cortex and hippocampus (Kennett et al., 2012). Another study found that exposure of the spinal cord to nicotine induced release of NA in spinal microdialysates (Li and Eisenach, 2002). Nicotine has been investigated as a possible treatment for ADHD and the withdrawal symptoms are experienced more intensely in ADHD patients who are attempting smoke cessation. This suggests that its effect on NE transmission, in areas such as the PFC and the hippocampus, may have an important role in cognition in healthy and unhealthy individuals (Potter and Newhouse., 2004; Pomerleau etal.,2003; Borodovitsyna et al., 2017).

Therefore, all studies listed above showed that nicotine has the ability to increase the NA output in a different brain areas and we saw the same effect of nicotine in the BNST; thus, we suggest that BNST might be the key element for the addictive nature of nicotine. The question is: which BNST neurocircuitries are involved in this effect of nicotine?

The BNST is the key element of withdrawal phase and stress-induced reinstatement of drug-seeking (Chapter No. 3: Abuse), and a recent review suggested that psychological stress facilitates the initiation of smoking, decreases motivation to quit and increases risk of relapse (Bruijnzeel, 2012). This might be due to changes in stress hormone signalling, such as NE, since nicotine withdrawal is characterized by depression-like symptomatology and a chronic administration of desipramine in rats rescued reward threshold elevations and decreased somatic signs of distress during nicotine withdrawal (Paterson et al., 2008). What has been tried in humans is the therapy with bupropion because it can help to maintain physiological measures of stress during nicotine withdrawal (Kotlyar et al., 2006). Interestingly, bubropion increases DA and NA output in the BNST in a dose dependent manner (Cadeddu et al., 2014). In adittion to this theory, one study showed that person who is strongly reactive to psychological stress is also strongly reactive to nicotine (Pomerleau et al., 1990). The

connection between anxiety disorders and nicotine was found and it seems that it is more likely for smoker to have an anxiety disorder than for non-smoker and that these smokers with an anxiety disorder are less motivated to quit (Johnson et al., 2000; Zvolensky et al., 2007). Therefore, we can hypothesize that nicotine affects anxiety-like behavior in a way that acute nicotine administration is decreasing and cessation of chronic nicotine administration is increasing anxiety-like behavior (George et al., 2007; Irvine et al., 2001).

One of possible explanation for these nicotine effects is the interaction between NE and CRF in the BNST. One of studies supporting this theory is the one of Zislis and colleagues (2007), where administration of the CRF (1/2) receptor antagonist D-Phe CRF (12-41) and clonidine, separately, decreased stress-induced reinstatement of nicotine-seeking behaviour. Furthermore, blockade of CRF1 receptors prevents dysphoria- and anxiety-like behavior associated with nicotine withdrawal (Bruijnzeel et al., 2009; Cohen et al., 2015; George et al., 2007), and both CRF1 and CRF2 receptors are present in the BNST.

Considering that the BNST belongs to the extended amygdala, we previously hypothesized that BNST DA transmission stimulation by nicotine contributes to the expression of the reinforcing properties of nicotine and could have a role in building (generating) nicotine dependence (Carboni et al., 2000). We suggest now that nicotine stimulation of NA transmission in the BNST is an essential stage of the process of maintaining nicotine dependence and in stress-induced HPA activation. In particular, repeated immobilization stress induces an enduring increase of CRH peptide expression in the CeA and the BNST (Santiba~nez et al., 2006) and a persistent increase in noradrenergic activity in the anterior aspects of the alBNST (Gonzales et al., 2017). Thus, smoking might repeatedly activate noradrenergic circuitry in the BNST leading to a coping response (receptor desensitization?) that after smoking cessation can be revaluated towards an opposite modification (sensitization); furthermore, this can expose the individual to stress-induced or cue-induced

relapse. Therefore, we here hypothesize that, after smoking cessation, even a mild increase of norepinephrine release in the BNST, independently of its origin, can activate a brain motivation circuitry that leads to relapse into smoking habit.

1.2. Cocaine

Despite the relevant investments in a research of cocaine use, dependence, abuse and addiction, cocaine is still a relevant health, social and economical problem. The property of blocking the dopamine transporter (DAT) has been considered the feature that supports the primary rewarding effect of cocaine (Heikkila et al., 1975; Ritz et al.,1987). Furthermore, an increase in dopamine transmission in specific brain areas such as the nucleus accumbens shell (Di Chiara et al. 2004) has been considered a key feature in cocaine addictive properties. Nevertheless, understanding the multifaceted world of cocaine use, abuse, dependence, withdrawal, detoxification and relapse requires an evaluation that goes beyond the role of DAT and NAcc shell. In particular, detoxification from cocaine addiction is a very difficult task and relapse is very common, even when it is sustained by a strong motivation. Detoxification is indeed accompanied by a very intense craving which can disappear but it can also resurface without warning. In addition, even after months of cocaine abstinence many former cocaine users cannot handle the stress and stress is often the main cause of relapse during this period. Unfortunately, there are no FDA approved drugs for cocaine addiction and although some experimental treatments are considered, there is a tremendous need of a better understanding of cocaine effects on brain neurocircuitry and transmitters involved.

Besides the ability to block DAT, cocaine blocks SERT and NET with comparable affinities (Ki values: 230–640 nmol/l, DAT; 140–740 nmol/l, SERT; 460–1600 nmol/l NET; Ritz et al. , 1990; Eshleman et al. , 1999; Rothman and Baumann, 2003; Han and Gu, 2006; Korpi et al., 2015). The role of these three transmitters in cocaine addiction has been investigated widely, but not evenly; in fact, in a past four decades the role of dopamine has received much more attention. These studies evidenced the pivotal role not only of the NAcc but even more

widely, of the extended amygdala. As it was explained in the Introduction, this area includes the shell of the NAcc, the BNST, the CeA, other amygdaloidal nuclei and the sublenticular subtantia innominate (Malenka et al., 2009). Our laboratory extended the investigation of cocaine effects on dopamine transmission to the extended amygdala and observed that cocaine increased, in dose-dependently manner, dopamine output in the BNST (Carboni et al., 2001). These results suggested that dopamine transmission in the BNST might have a role in the reinforcing properties of cocaine. With the aim of better understanding of the role that monoamine transporters play in the effect of cocaine, our laboratory also investigated the role of DAT and NET in other brain areas such as the NAcc shell, prefrontal cortex, caudate and BNST (Carboni et al., 2006). Our suggestion was that the blockade of DAT and NET can produce an additive effect on dopamine transmission and that this effect depends on the area investigated. Thus, by blocking both transporters, cocaine may produce peculiar effects that go beyond the single monoamine–transporter interaction. This feature can be particularly important in view of the use of specific receptor antagonists for managing cocaine addiction or preventing cocaine use relapse.

On this basis, we intended to go beyond DA transmission in NAcc shell, focusing on the pivotal role of stress and of other brain areas involved in cocaine detoxification. In particular, we thought that a further investigation of cocaine pharmacology and the involvement of specific, stress-coping brain areas could contribute to the knowledge of cocaine addiction mechanisms and subsequently to the definition of potential new pharmacological treatment.

A crucial role in the development and maintenance of anxiety and mood disorders has been given to extended amygdala, along with other distant brain areas. These two conditions are strictly linked with cocaine use and abuse (Beaulieu et al., 2014; Fox and Shackman, 2017). In particular, the effect of cocaine on this area can be considered crucial in the transition from drug-taking to addiction behaviour and in particular, the shift from positive drug

reinforcement to the negative reinforcement that involves dysphoria, anxiety and negative emotional states associated with cocaine abstinence/withdrawal (Alheid et al., 1998; Koob and Le Moal, 2001). Nevertheless, the remarkable complexity of monoamine brain innervation and the reciprocal influence of monoamine nuclei of origin in the brain (El Mansari et al., 2010) have probably hampered the identification of an efficacious pharmacological treatment for cocaine addiction.

In addition, it is quite interesting that the BNST is densely innervated by norepinephrine (Brownstein and Palkovitzs, 1984), serotonin (Phelix et al., 1992b) and dopamine (Phelix et al., 1994). All three monoamines make contacts with CRF neurons, supporting the role of the BNST in the adaptive response to stress (Morilak et al., 2005). Norepinephrine neurons make synaptic contacts with dendrites of CRF neurons in the ventrolateral BNST, dopamine neurons innervate soma and dendrites of CRF neurons in the dorsal part (Phelix et al., 1994), while serotonin neurons innervate CRF neurons in both areas (Phelix et al., 1992a). In turn, BNST CRF output reaches the hypothalamic ventromedial nucleus (Shin et al., 2008) and many other brain areas (Dabrowska et al., 2016). Consequently, cocaine, besides potentiating monoamine transmission in numerous brain areas such as NAcc shell and core, caudate and prefrontal cortex, can interact directly on DAT, NET and SERT in the BNST, producing a direct effect on the BNST CRF neurons and thus on the BNST output (Mc Farland et al., 2004; Ettemberg et al., 2015; Dabrowska et al. 2016). It has been shown that CRF transmission is involved in cocaine effects. In fact, cocaine can increase CRF concentration in blood (Rivier and Vale, 1987; Goeders, 1997; Sarnyai et al., 2001) and can change CRF peptide or mRNA levels within the extended amygdala (Maj et al., 2003; Richter et al., 1995; Zhou et al., 1996). These early observations were extended to one trait of cocaine addiction as Erb and Stewart (1999) reported that CRF antagonism completely blocked reinstatement of cocaine-seeking induced by presentation of foot-shock stress. On these premises and due to

knowledge on CRF-NE interaction in the BNST, it appeared interesting to focus on the effect of cocaine on norepinephrine transmission in the BNST: an area that, besides dopamine, is strongly involved in norepinephrine and CRF circuitry (Aston Jones et al. 1999; Delfs et al., 1998).

The Figure 21-A shows that cocaine, when administered in dose of 2.5 and 5 mg/Kg, increased the output of norepinephrine in the BNST in a time- and dose-dependent manner, producing a maximum increase of 400% of basal output at the 40th minute after administration of 5 mg/kg and of 200% for the dose of 2.5 mg/kg. The effect on BNST norepinephrine vanished within two hours even at the highest dose. Interestingly, the comparison of cocaine effects on norepinephrine with those of dopamine evidenced that cocaine produces a stronger effect on norepinephrine, since that on dopamine wasn't higher than 250% for the dose of 5 mg/kg and not significant for the dose of 2.5 mg/kg. The heterogeneity of the BNST, in terms of nuclei, transmitters and the size of the microdialysis fiber, hardly allows a specific measurement of transmitters in one specific nucleus. Thus, although we recovered the dialysate from both the ventral and dorsal BNST, we must refer the effects of cocaine on norepinephrine output to the ventral area, while dopamine is likely recovered mostly from the dorsal BNST. Although BNST norepinephrine originates almost exclusively from the A2 region of nucleus of tractus solitarious (NTS) (Aston Jones et al. 1999; Delfs et al., 1998), the interpretation of these results is somehow difficult because the BNST-CRF output involves different brain areas such as the BLA, the CeM (medial division of the central nucleus of the amygdala), the DRD (dorsal raphe nucleus – dorsal division), the LH (lateral hypothalamus), the NAcc shell, the PBP (parabrachial pigmented nucleus), the PVN, the PL, the PN (pontine nuclei), the RMC (red nucleus, magnocellular part), the SNC (substantia nigra pars compacta), the SNR (substantia nigra reticulate), the NTS and the VLPAG (ventrolateral periaqueductal grey) (Drabowska et al. 2016). Here, after taking into

account this complex network and considering the reciprocal interaction of monoamine transmission, we cannot rule out a role of norepinephrine in the reinforcing effects of cocaine.

The effect of cocaine on norepinephrine transmission and its involvement in generating the condition of an elevated sensibility to stress-induced relapse has received much attention (Erb and Stewart, 1999; Erb, 2010; Wise, 2012; Aguilar et al., 2009; Mantsch et al., 2016) because of its potential role in the development of a therapy for cocaine addiction. Accordingly, strict relationship between cocaine effects, stress, BNST-CRF circuitry activation and norepinephrine transmission might allow us to target any of the multiple steps involved in cocaine addiction, to investigate the potential substance for use in the treatment and in particular, to prevent stress-induced reinstatement of cocaine consumption.

Intriguingly, norepinephrine and CRF may be involved either in the reinforcing properties of drugs (Goeders et al., 2000; Piazza et al., 1996), or in the anxiogenic effects of drug withdrawal (Rodrigues et al., 1997) and reinstatement of drug seeking (Erb et al., 2000). In particular, intracerebroventricular injection of norepinephrine can induce drug seeking behaviour in cocaine abstinent rats previously trained to self-administer cocaine, but not in rats in which norepinephrine administration was preceded by the administration of the CRF receptor antagonist D-Phen CRF (12-41) (Brown et al., 2011). This observation requires a further deepening of norepinephrine-CRF role in cocaine addiction. In particular, we have to find out if this interaction is producing determinant effects at CRF output level or at CRF input level? Nobis and collaborators (2011), in a slice preparation, demonstrated that isoproterenol, through the adrenergic β_1 receptor activation within BNST, produces an enhancement excitatory synaptic transmission through an increase of glutamate presynaptic function in the dorsolateral BNST, an area innervated by dopamine, norepinephrine and additionaly by glutamate from the central nucleus of amygdala. The increase in sEPSP frequency, but not amplitude, was inhibited by the pre-application of the CRF1 antagonist

NB127914. These authors also showed that dopamine produced similar glutamate activation; furthermore, dopamine activation did not overlap with norepinephrine activation and dopamine could still produce a further enhancement of sEPSP under a period of maximal β_1 activation (Nobis et al., 2011). Moreover, they showed that either cocaine self- administration or cocaine chronic treatment disrupted the $CRFR_1$ modulation of β1 receptor activation, which means that the enhancement of glutamate transmission was disrupted; this effect returned after 10 days of cocaine withdrawal, although it can be reintroduced with a cocaine challenge (Nobis et al., 2011). In summary, these authors propose that dopamine and norepinephrine can modulate glutamate release in the dorso-lateral BNST through a CRF interneuron (most likely for dopamine) or external CRF innervation from central nucleus of amygdala (most likely for norepinephrine).

In addition to importance of CRF for the cocaine addiction there are studies showing that when self-administered, cocaine increases glucocorticoid secretion in rats (Galici et al., 2000), monkeys (Broadbear et al., 1999) and human cocaine addicts (Ward et al., 1999). When the glucocorticoid response to cocaine was eliminated through surgical adrenalectomy, but with the basal corticosterone replacement, and before rats were allowed to self-administrate cocaine for 14 days, this didn't have effect on cocaine intake; on the other hand, subsequent reinstatement in response to footshock or administration of CRF i.v. was completely abolished (Graf et al., 2011). By contrast, if adrenalectomy was done following the self-administration experiment, footshock- or CRF-induced cocaine seeking was unaffected. This suggests that there are neuroadaptations at the time of cocaine use as a result of elevated glucocorticoids that is responsible for vulnerability of addicts to stress-induced relapse. One of possible mechanism of glucocorticoid-induced neuroplasticity is its action on glucocorticoid receptors expressed in the brain area implicated in stress-induced cocaine seeking, as well as in CRF-expressing cells in the BNST (Cintra et al., 1987).

A dense set of BNST projections to the VTA (reviewed by Silberman and Winder, 2013) become activated during reinstatement of cocaine seeking (Mahler and Aston-Jones 2012). When disconnected, this pathway reduces cocaine preference and stress-induced cocaine seeking (Sartor and Aston-Jones, 2012; Vranjkovic et al., 2014). In a recent disconnection study by Sartor and Aston-Jones, a functional role of BNST-VTA pathway in cocaine seeking was proved by CPP test; after contralateral inhibition of the BNST of one hemisphere and the VTA of the other, they saw attenuated expression of CPP (Sartor and Aston-Jones, 2012). Vranjkovic and colleagues (2014) have proved that CRF is expressed in the vBNST neurons that innervate the VTA and that, after self-administration of cocaine in rats, the CRF antagonist antalarmin injection in this site prevented the reinstatement of cocaine seeking induced by a stressor, intermittent footshock. Moreover, they showed that β_2 adrenergic activation in the vBNST, that requires local CRF receptor activation, is both necessary for stress-induced reinstatement and sufficient to induce cocaine seeking. In addition, a disconnection approach showed that this CRF dependant β_2 receptors activation is regulating vBNST efferents that release CRF into the VTA, promoting in that way cocaine use.

As previously discussed, there are laboratory studies in human cocaine addicts (Jobes et al., 2011) and preclinical experiments in rodents (Erb et al., 2000; Mantsch et al., 2010) which are pointing out the role of NE signaling in the stress-induced relapse to cocaine use; giving the position and the features of the BNST, it is logical to assume that this very nucleus can be a location where increase of NE signalling regulates drug use.

Altogether, the neuronal peculiar feature of the BNST and its relationship with the reward and stress systems is giving to this nucleus a crucial role in all segments of cocaine transition: from use, over abuse and towards addiction. Thus, it can be hypothesized that the stressful experience of drug addiction can increase the intrinsic power of cocaine in altering the

delicate equilibrium of stress control in the BNST and that this part of the brain cannot be excluded as a potential target for cocaine therapy.

1.3. Morphine

The administration of morphine in a dose of 1 and 3 mg/Kg i.p. increased the output of norepinephrine in the BNST, the same effects that we saw when measuring dopamine. Quite surprising is that while the increase of dopamine was time- and dose-dependent, producing a maximal increase of 230% and 150% of basal values at the 80th min after administration for the dose of 3 and 1 mg/kg respectively, the increase of norepinephrine was higher for the dose of 1 mg/Kg (maximal increase: 180% at the 60th min after administration), as compared with the increase produced by the dose of 3 mg/Kg. The effect of morphine on the BNST norepinephrine vanished within two hours even at the highest dose, while the increase in dopamine output lasted longer.

The interpretation of these results requires an evaluation of how morphine can interact with opioid receptors localized in the norepinephrine circuitry that innervates the BNST, or how morphine interacts with other neuronal circuits that in turn may modulate norepinephrine innervation of the BNST. Unfortunately, very few studies have investigated opiate-norepinephrine interaction at the level of the BNST and they address the withdrawal aspect (Aston-Jones et al., 1999) and the stress-induced reinstatement of opiate abuse, rather than investigating the direct effect of morphine on NE and the potential role of NE in its reinforcing properties. Although the effect of morphine on norepinephrine output is somehow lower than that of cocaine, it still can be linked to several traits of opioid addiction. Objectively, the evidence that norepinephrine may mediate the reinforcing effects of opioids is poor, in particular when this theory is tested with self-administration experiments. Nevertheless, there are some evidences showing that norepinephrine receptor antagonists may mediate the effect of opiates on locomotion, sensitization and conditioned place-preference and that norepinephrine can modulate opiate induced dopamine release in crucial

brain areas (Weinshenker and Schroeder 2007). On the other hand, the involvement of norepinephrine in withdrawal related effects in opiate dependence and in stress-induced reinstatement of opiate abuse has been investigated (Wise and Koob, 2014; McElligott et al., 2013; Fox et al., 2017; Harris and Aston-Jones, 2007). In particular, the association of the CRF BNST transmission and opiate priming/stress-induced reinstatement of morphine CPP has been found (Wang et al., 2005), whereas Houshyar et al., (2003) found that intermittent morphine acts as a stressor on the mRNA BNST (Houshyar et al., 2003).

Nevertheless, what remains completely unexplored is the role of the repeated morphine stimulation of norepinephrine transmission in the BNST in terms of adaptation of BNST circuitry, as well as the effect of repeated norepinephrine stimulation of the same or parallel norepinephrine circuitry in the BNST, following the abstinence experience or following stress-induced craving for opiates. Fox et al. (2017) have investigated, through the fast-scan cyclic voltammetry in freely moving rats, the real time catecholamine overflow during acute morphine exposure and naloxone precipitated withdrawal in the NAcc and in the norepinephrine rich ventral BNST. These authors found an increase in dopamine output in the NAcc but not in norepinephrine output in the vBNST. Conversely, dopamine output decreased during withdrawal, while norepinephrine was released during specific withdrawal symptoms. Technical differences in the method used in this experiment do not allow us to discuss the absence of stimulation of norepinephrine output after acute morphine injection observed by Fox and coll., although the very high dose of morphine used (10 mg/Kg) and the apparent bell shaped curve we observed with the doses of 1 and 3 mg/kg suggest that the release of norepinephrine in the vBNST may have to be further investigated. Apparent contrasting results were also presented in the past. As far as a direct norepinephrine assessment in the BNST, it has been also found that naloxone induced opiate withdrawal in chronically morphine treated rats determined a significant decrease of norepinephrine content

in the BNST and in the amygdala (Van Bockstaele et al., 2008), while Fuentealba et al., (2000) observed that chronic morphine treatment (10 to 100 mg/Kg morphine twice a day for 6 days) increased norepinephrine levels in the vBNST and that naloxone precipitated further increases. In any case, understanding how morphine stimulates norepinephrine and dopamine output in the BNST and what is the consequence of the acute and chronic effects of morphine is not easy because of the widely diffuse presence of multiple opiate receptors in brain areas that are interconnected with the BNST (Poulin et al., 2009). BNST receives an innervation from the CeA neurons that release dinorphine on the BNST neurons that project to downstream targets such as PVN of hypothalamus, but also dorsal raphe and to VTA, that are involved in anxiety and reward related behaviours (Merchant et al., 2007). In particular, K opiate receptor can reduce GABAergic inhibitory synaptic transmission from CeA to the BNST (Li et al., 2012).

That opiate dependence increases vulnerability to stress is a well known fact (Kreek and Koob, 1998; Blatchford et al., 2005; Koob, 2008). This process is based on adaptations of stress-systems induced by opiates that may, in part, underlie this vulnerability (Buckingham and Cooper, 1984; el Daly, 1996; Kreek and Koob, 1998; Houshyar et al., 2004). One of these systems is the CRF system and opiate administration alters the stress-induced elevation of CRF mRNA levels in the BNST (Shalev et al., 2001). Specific effect of morpine within the BNST was seen after chronic morphine administration when it selectively strengthened a population of excitatory fibers entering the lateral division of the BNST and further projecting towards the VTA (Dumont et al., 2008). Moreover, study of Wang and colleagues (2006) showed how the CRF1 receptor antagonist CP-154,526 injection into the BNST attenuated footshock-induced reinstatement of morphine CPP, which demonstrates region-specific roles of brain CRF_1 receptors in reinstatement of morphine CPP. However, this shows that BNST is involved in a withdrawal and reinstatement of morphine abuse, but this

CRF changes may also be involved in a motivational process to continue drug use during dependence. Jaferi and colleagues (2009) were first to show, at the ultrastructural level, that after the chronic administration of morphine, the cytoplasmic and particularly mitochondria-associated CRF receptor density is increased in dendrites of the BNST in mice. Kash and colleagues (2008) hypothesied that dopamine could activate the CRF neurons in the dlBNST and therefore enhance excitatory neurotranmittion through CRF-GLU interaction since it is known that CRF is a modulator of GLU transmission (Ungless et al., 2003; Liu et al., 2004). We may hypothesize that NE can affect excitatory transmission of the BNST through the same mechanism; therefore, morphine may start one direct and rapid interaction between NE and CRF system in the BNST, regulating in this way the excitatory transmission and plasticity in the key brain region for reinforcement and reinstatement of drug-seeking behaviour.

1.4. Amphetamine

We showed here that the administration of amphetamine in a dose of 0.25 and 0.5 mg/Kg s.c. increased the output of norepinephrine (Figure 23-A). It was previously showed, and presented here in graphic 23-B, that amphetamine increased the dopamine output (Carboni et al., 2000) in the BNST. The increase of norepinephrine was time- and dose-dependent producing a maximal increase of about 400% and 220% at the 40th min after administration, for the dose of 0.5 and 0.25 mg/kg respectively. The output of norepinephrine, although similar to that of dopamine in terms of maximal increase (200% of basal), returned to basal levels later than that of dopamine (about 2 hours).

It is known that amphetamine depletes dopamine and norepinephrine vesicles, producing an excess of monoamine in the neuronal terminal which in turn, through the inversion of the transporter direction, increases the release of neurotransmitters in the synaptic space. Amphetamine differs from cocaine as it produces non exocytic stimulation-independent release of monoamine via reverse transport, independently of the depolarization of the terminal and calcium presence in the extracellular space (Carboni et al., 1989). Moreover, from a molecular point of view it is surprising that the action of amphetamine is very powerful, in terms of doses and increased output, as it can go up to 2000% of basal values when measured with the microdialysis technique (Fan and Hess, 2007). In this study we observed that a low dose of amphetamine produced a relevant increase in the output of norepinephrine in the BNST. We would like to evaluate the possible consequences of this increase, from the addiction standpoint, looking at a possible role of the BNST in the reinforcing effects of amphetamine and in the stress-induced relapse after abuse discontinuation, but evidence in literature are scarce.

Amphetamine produces several different behaviours, depending on dosage. In low doses, amphetamine produces hypermotility, whereas in higher doses it produces stereotyped behavior. Amphetamine is a dopamine, noradrenaline and serotonin indirect agonist that can interact with brain circuitry on multiple levels producing a wide range of effects. Although most of the evidences for the mechanism of action of amphetamine have been provided studying dopamine outflow, it is possible to extend our knowledge studying the VMAT2 vesicular transporter (Cliburn et al., 2017; Taylor et al., 2014). Essentially, amphetamine can increase dopamine output in different brain areas by preventing dopamine reuptake through DAT blockade (Ritz et al., 1987, Carboni et al., 1989, Kuczenski et al., 1991), or by entering the neuronal terminal either through DAT (Liang and Rutledge, 1982) or by membrane diffusion due to its lipophilic properties (Mack and Bonisch, 1979). In the neuron terminal amphetamine can bind to the vesicular monoamine transporter VMAT2 (Sulzer and Rayport, 1990) and dissipate the pH gradient that drives vesicular monoamine uptake (Sulzer et al., 1995), thereby generating dopamine efflux. In turn, dopamine can diffuse into the terminal cytoplasm and leave it through the inversion of DAT transport direction (Sulzer et al., 1995; Pifl et al., 1995; Sulzer et al., 2005). Thus, we can assume that the redistribution of norepinephrine and dopamine in the extravesicular compartment in the neuronal terminal by amphetamine and amphetamine analogues can be directly related to the increase of the output measured by microdialysis methods. On these bases, it is interesting to hypothesize that the strong increase in norepinephrine and dopamine output in the BNST may, in a long run, produce a dysregulation of the central noradrenergic system in the BNST; this effect might be due to the similar effect produced by chronic stress, the effect that has been implicated in the pathogenesis of depression and anxiety disorders (Goddard et al., 2010, Morilak and Frazer, 2004). In particular, repeated immobilization stress induces and endures the increase of CRH in the CeA and in the BNST (Santibanez et al., 2006), while it decreases the climbing

behaviour in the forced swim test, a noradrenergic mediated stress-coping behaviour, and an increased efficacy of behavioural response to desipramine (Hadweh et al., 2010). Our laboratory has recently investigated the effect of antidepressants on the norepinephrine and dopamine output in the BNST. It was shown that either the SSRI or the SNRI determined a dose-dependent increase of NE and DA, suggesting that the BNST may be strictly involved in the therapeutic effect of antidepressant, as well as in the aetiology of depression (Cadeddu et al., 2014). In this regard, the recent results of Gonzales et al., (2017) suggested that restrain stress induces and increases the norepinephrine release, synthesis and uptake in the anterior lateral BNST, leading to a decrease of norepinephrine synaptic availability and possibly to the mechanisms of adaptation of neurocircuitry, similar to those that characterize melancholic depression in humans: the persistent stress-system dysfunction (Ampuero et al. 2015, Wong et al., 2000). Considering that amphetamine produces an internalization of NET (Dipace et al., 2007), and in the long run a reduced availability of DAT and dopamine receptors in humans (Ashok et al., 2017), we might predict that this type of response can occur in the BNST and can have a role in the depression associated with psychostimulants in humans (Filip et al., 2013; Rusyniak, 2013, Homer et al., 2008).

1.5. Ethanol

The administration of 0.5 and 1 g/Kg i.p. of ethanol increased the output of norepinephrine in the BNST in a dose-dependent manner (Figure 24-A). The maximal increase of norepinephrine (190% of basal) was obtained 20 mins after the administration of 1 g/Kg; in our previous work, the maximal increase of dopamine (210% of basal; Figure 24-B) was obtained at the 40^{th} min after the administration of 1 g/Kg of ethanol. The increase in catecholamine output was short lasting as norepinephrine returned to the values not different from basal within 60 mins and dopamine within 80 mins. The fact that the low doses of ethanol can affect both transmitters suggests that at least part of the acute effects, as well as the effects of repeated doses of ethanol, are produced with the involvement of the BNST.

Ethanol has a complex mechanism of action since it is lacking in specific receptors, but its interaction with the GABAergic and glutamatergic system can affect most of the brain transmitters in many brain areas (Abrahao et al., 2017). At the same time, alcohol abuse is not causing dependence directly and animal studies and human observations have shown that alcohol sensitivity has a genetic component (Bell et al., 2001 and 2017). One more feature of ethanol pharmacology is linked to an initial stimulatory effect and a subsequent inhibitory effect, which might be crucial for its abusive potential (Cui and Koob, 2017).

The role of noradrenergic transmission in the effects of ethanol had been observed in the 60s and 70s, but later the role of dopamine took the lead in explaining the effects of ethanol, despite the fact that acute administration of ethanol is increasing the turnover of noradrenaline more than that of dopamine and greatly stimulates the activity of noradrenergic neurons (Aston-Jones et al., 1982). Additionally, DBH inhibitors suppress volunteer ethanol consumption while dopaminergic area lesions do not (Weinshenker and Schroeder 2007). It has been recently suggested that adolescent alcohol use may produce maladaptive decision-

making through a disruption in dopamine network dynamics via increased GABAergic transmission within the VTA (Schindler et al., 2016); moreover, chronic ethanol exposure might produce a profound and long-lasting changes in local inhibitory control of the CeA, resulting in an increase in the CRF1 neurons output of the CeA (Herman et al. 2016).

Taking this into consideration, it is not surprising that the extended amygdala (BNST), brain area rich in glutamate, GABA and catecholamine innervation is deeply involved in alcohol abuse and addiction.

The relationship between the effect of alcohol, norepinephrine transmission and the complex peptide-like systems such as NPY and CRF in the BNST has been reviewed by Kash et al., (2012). In particular, it has been reported that activation of α_1 adrenergic receptor leads to a depolarization of a subpopulation of BNSTal neurons and to an increase of GABA release (Dumont & Williams, 2004). Therefore, we can propose that the increase in norepinephrine BNST due to effect of acute ethanol can, in a long run, produce a crucial modification of the BNST neurocircuitry that can lead to alcohol dependence and addiction.

Furthermore, it has been proposed that alcohol can also produce its effect affecting directly memory processes, such as long term depression (LTD), through α_1 modulation of function in the BNST (McElligott et al., 2010). In this paper authors showed that four days of alcohol vapor exposure partially diminished the magnitude of NE mediated LTD. Moreover, they showed that activation of α_1 adrenergic receptor also leads to an increase in glutamatergic transmission, in a CRF-R_1 dependent manner (McElligott et al., 2010). Significance of α_1 receptors has been further confirmed in both preclinical models of alcohol dependence (Rasmussen et al., 2009; Walker and Koob, 2008; Walker et al., 2008), and clinical observations (Simpson et al., 2009). In addition, some studies have shown that α_2 adrenergic receptors are involved in alcohol dependence; in fact, it has been reported that clonidine and

guanfacine ameliorate withdrawal symptoms from alcohol, as well as from other drugs of abuse (Muzyk et al., 2010).

On the other hand, it seems that the CRF system is important for the transition from alcohol use and abuse to alcohol dependence (Heilig and Koob, 2007). It has been shown that repeated exposure to ethanol followed by withdrawal produces an upregulation of the CRF system in rats (Funk et al., 2007; Roberto et al., 2010), and mice (Eisenhardt et al., 2015). Furthermore, it was shown that CRF_1 antagonist can reverse increased ethanol intake by dependent rats and mice (Chu et al., 2007; Finn et al., 2007; Funk et al., 2007; Correia et al., 2015), suggesting a critical role of the CRF_1 system in the behavioral expression of alcohol dependence. Moreover, other studies suggest that CRF signaling is also recruited during binge-like ethanol drinking before dependence (Lowery-Gionta et al., 2010, 2012). Lowery-Gionta and colleagues (2012) showed that, upon binge-like ethanol consumption, CRF protein levels increased significantly in the CeA and VTA and that by blocking the CRF-R_1 in both the CeA and VTA, binge-like ethanol consumption was blunted. Additionally, selective inhibition of CRF neurons in the BNST which project to the VTA has the same effect (Pleil et al., 2015). Silberman et al. (2013) also suggested that the catecholaminergic neurotransmission in the BNST activates CRF BNST neurons, leading to an increase of glutamatergic neurotransmission and therefore excitation of the BNST afferents onto GABA BNST neurons. In turn, these neurons project onto VTA GABA neurons, providing disinhibition of VTA dopaminergic neurons (Figure 27.).

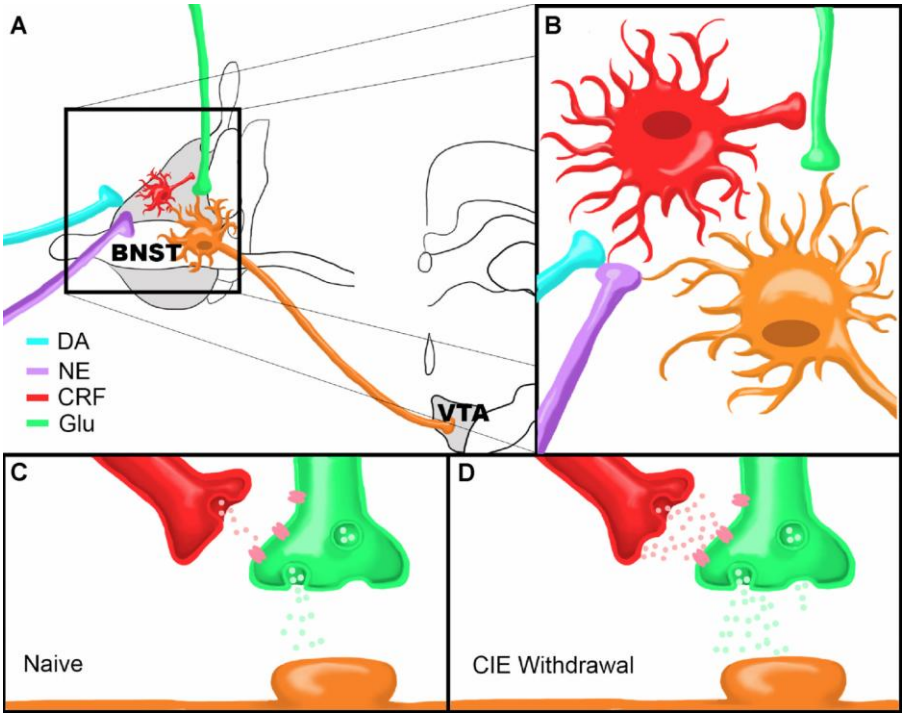

Figure 32- BNST, CRF, DA and NE

(Silberman et al., 2013)

"(A) Dopamine and norepinephrine afferents synapse onto CRF producing neurons in the BNST which in turn influence neurotransmitter release from glutamatergic afferents onto BNST neurons projecting to the VTA. (B) Close up view of proposed neurocircuitry described in A. (C–D) Model of CRF modulation of glutamatergic transmission onto a VTA-projecting BNST neuron in a drug-naïve state (C) or during acute ethanol withdrawal following CIE (D). Note that there are higher levels of CRF and glutamate release during withdrawal compared to the drug-naïve state".

In their new study Rinker and colleagues (2017) show that CRF BNST projections to the VTA, but not local VTA CRF neurons, are involved in modulating binge-like ethanol drinking. They showed that these BNST CRF projections are likely to make synapse with CRF/GABAergic VTA neurons, in both cases leading to excitation of DA VTA, and activation of these neurons promotes continued binge drinking (Rinker et al., 2016). Moreover, in the VTA, block of CRF- R_1 results in reduced ethanol intake, but only if there is uninterrupted CRF- R_2 signaling.

Overall, we can hypothesise that, by increasing the NE extracellular levels in the BNST, ethanol activates local CRF neurons projecting to the VTA, activating in this way the

local glutamatergic transmission and also CRF VTA projections, leading towards activation of the VTA neurons and mesolimbic DA reward pathway.

But BNST is not only involved in the activation of the mesolimbic DA pathway that results in a progression from moderate ethanol consumption to ethanol abuse and dependence. BNST also plays an important role in pre-existing anxiety disorders that contribute to the development of alcohol addiction. There are reports saying that 75% of individuals that abuse alcohol are currently or previously diagnosed with anxiety disorder (Kushner et al., 2000; Kushner et al., 2012; Swendsen et al., 2010), and Koob (2003) has suggested that alcohol abuse and therefore addiciton can be promoted by chronic anxiety symptoms.

2. Effects of Ketamine on the DA and NE Transmission in the BNST

The results of this experiment have been published in the journal of European Neuropsychopharmacology: Ketamine modulates catecholamine transmission in the bed nucleus of stria terminalis: The possible role of this region in the antidepressant effects of ketamine (2016, Appendix).

We showed that the acute systemic administration (i.p.) of ketamine increased NE and DA output in the BNST, in a time- and dose-dependent manner (Figure 26-A and B). The highest increase was observed 40 mins after treatment (85% and 75% above basal levels, for NE and DA respectively). Regarding the high sub anaesthetic dose of ketamine that was used in this experiment, we assessed the motor effects of all doses of ketamine (10, 20 and 40 mg/Kg) and there wasn't significant difference in either total or ambulatory activity between each dose of ketamine and saline.

We considered that the ketamine effect on dopamine and norepinephrine transmission might be due to direct effect on reuptake of catecholamine in the BNST, like it was previously considered by Tso et al. (2004), who used the fast cyclic voltammetry (FCV) technique. But, if we go beyond this hypothesis, we can also consider that ketamine produces its effect through its action on the GLU neurons of the BNST. The most striking observation about ketamine is that it can produce a rapid antidepressant effect through inhibition of glutamate NMDA receptor, as reviewed by Duman's group (Gerhard et al., 2016). They suggest that ketamine has stronger affinity for the NMDARs located on GABA neurons of the PFC, which in turn disinhibits pyramidal cells, producing a glutamate burst. This explanation could be updated taking into consideration the newest hypothesis proposed by Zanos and colleagues (2016), who suggest that ketamine is acting through its metabolite (2R-6R)-hydroxynorketamine, which would involve early and sustained activation of AMPARs, to

produce behavioural, electroencephalographic, electrophysiological and cellular antidepressant-related action in mice. Interestingly, when ketamine is self-administered by rats, it modulates αCaMKII expression and phosphorylation, a feature shared by other drugs of abuse with different mechanisms of action (Caffino et al., 2017), but not by antidepressants. It is thus puzzling to discover how this drug can play a role in both mood disorders and the reinforcing field. At this regard Caffino et al. (2016) proposed that a single ketamine infusion can increase mBDNF in the hippocampus and decrease it in the ventral striatum, while repeated infusion via self-administration decreased mBDNF in the hippocampus and in the ventral striatum. We can thus assume that ketamine, through the increase of norepinephrine and dopamine in the BNST can activate both the circuitry that produces the reinforcing and antidepressant effects by interacting directly with the glutamate innervation and the CRF input and output of the BNST (Bath et al., 2017).

But ketamine might also act through the BNST itself. In fact, glutamate projections from the ventral subiculum directed to the PVN relay in different BNST nuclei, where the excitatory signal from the hippocampus is transformed into inhibitory output to the PVN (Cullinan et al., 1993). In addition, dopamine and norepinephrine afferents to the BNST make synaptic contacts onto CRF neurons and as like it was explained before, these CRF neurons are influencing the release of glutamate onto GABA BNST neuron projecting to the VTA, activating DA VTA neurons. But one of the main goals of these experiments was to explain the fast antidepressant effect of ketamine comparing its effect and those of classical antidepressants onto catecholaminergic neurotransmission in the BNST. In the previous work of our laboratory it was observed that, among others, (i) desipramine, a norepinephrine transporter blocker (NET), (ii) citalopram, a selective serotonin transporter blocker (SERT), (iii) imipramine, a SERT and (through its metabolite desipramine) NET blocker and fourth, bupropion, a dopamine transporter (DAT) and NET blocker, independently of their

mechanism of action, strongly increased catecholamine output in the BNST (Cadeddu et al., 2014). Therefore, we observed that antidepressants and ketamine have a similar effect on norepinephrine and dopamine output in the BNST and although the mechanism of ketamine fast antidepressant effect still remains a puzzle, we might suggest that the catecholamine transmission in the BNST has a very complex role in regulating multiple function of this nucleus.

OVERALL CONCLUSIONS

The interesting finding of this work is that several drugs of abuse, although owning a distinct mechanism of action, stimulate norepinephrine output in the BNST. We evaluated the possibility that the increase in norepinephrine could have a role in the reinforcing effect of each substance tested, but the evidences to support this theory are not so strong. On the other hand, it is known that norepinephrine transmission has a role in the stress triggered drug reinstatement after detoxification from drug of abuse. Interestingly, the effect on norepinephrine output in the BNST of the different drugs of abuse tested is of a different entity and may be linked with the grade of susceptibility to stress-induced reinstatement. For instance, the comparison of cocaine effects on BNST output with those of morphine evidenced that cocaine produced a stronger effect on norepinephrine, while morphine produced the highest effect on dopamine. Trying to understand what the meaning of this difference could be, we examined the possibility that the increase of dopamine in the BNST could be mainly involved in the reinforcing properties of a drug of abuse, while the increase in norepinephrine could be rather linked to the stress-induced reinstatement of drug abuse. But, what is mostly unexplored is how morphine- or cocaine-induced norepinephrine increase modifies BNST neuronal circuitry in such a manner to expose it to hypersensitivity to stress, which strongly produces an uncontrollable need to administer a drug of abuse again, even if it was terminated after a process that inevitably has required an intense effort.

In addition, the fact that BNST sends a dense set of projections to the VTA (reviewed by Silberman and Winder, 2013), that become activated during reinstatement to cocaine seeking (Mahler and Aston-Jones 2012), suggests that the mechanisms of reinforcing (dopaminergic) and stress-induced reinstatement (noradrenergic) are intimately connected. Furthermore, BNST and its afferent and efferent projections which involve, among others, GABA, glutamate and CRF transmission, are likely to play a crucial role in all segments of drug of abuse transition, from use to abuse, addiction, detoxification and reinstatement. Thus, it can

be hypothesized that the stressful experience of drug addiction has the power of altering the delicate equilibrium of stress control in the BNST, involving also anxiety and depression circuits, as suggested by the results of ketamine experiment.

In conclusion it can be suggested that the afferent and efferent neuronal circuitry that involves the BNST could be a target for the search of therapeutic tools in drug abuse therapy (Koob and Mason, 2016), as well as in antidepressant therapy.

When it comes to ketamine, the increase in both DA and NE was lower but was not substantially different from that produced by classical antidepressants and we suggest that catecholamine increase in BNST is not likely to be related to a rapid ketamine antidepressant effect, though it might be related to its performance in predictive tests of antidepressant properties.

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APPENDIX

European Neuropsychopharmacology (▮▮▮▮) ▮, ▮▮▮–▮▮▮

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SHORT COMMUNICATION

Ketamine modulates catecholamine transmission in the bed nucleus of stria terminalis: The possible role of this region in the antidepressant effects of ketamine

Roberto Cadeddu[a], Dragana Jadzic[a], Ezio Carboni[a,b,*]

[a]*Department of Biomedical Sciences, University of Cagliari, Via Ospedale 72, 09126 Cagliari, Italy*
[b]*Neuroscience Institute, Section of Cagliari, National Research Council of Italy – CNR, Cagliari, Italy*

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Abstract
Since the therapeutic treatment of depression is far from being satisfactory, new therapeutic strategies ought to be pursued. In addition, further investigation on brain areas involved in the action mechanism of antidepressants can shed light on the aetiology of depression. We have previously reported that typical and atypical antidepressants strongly stimulate catecholamine transmission in the bed nucleus of stria terminalis (BNST). In this study, we have built on that work to examine the effect of ketamine, an unusual antidepressant that can produce a fast-acting and long-lasting antidepressant effect after administration of a single sub-anaesthetic dose. Ketamine is an antagonist of the ionotropic N-methyl-D-aspartate (NMDA) receptor but can also act through its metabolite (2R-6R)-hydroxynorketamine. Using the microdialysis technique in freely moving rats, we monitored the acute effect of ketamine on catecholamine release in the BNST to gain clues to its prompt antidepressant effect. Male Sprague-Dawley rats were implanted with a microdialysis probe in the BNST and 48 h later, were injected with ketamine (10, 20, and 40 mg/kg, i.p.). Ketamine increased norepinephrine (127%, 155%, 186%) and dopamine (114%, 156%, 176%) extracellular concentration above basal in a time and dose dependent manner, without significantly modifying motility. Since the effect of ketamine, although lower, was not substantially different from that produced by classical antidepressants, we suggest that catecholamine increase in BNST is not likely to be related to a rapid ketamine antidepressant effect, though it might be related to its performance in predictive tests of antidepressant properties.

*Correspondence to: Department of Biomedical Sciences, University of Cagliari, Via Ospedale 72, 09124 Cagliari, Italy. Tel.: +39 070 675 8672; fax: +39 070 675 8665; Mob.: +39 3804335413.

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Research paper

Neurosteroid dehydroepiandrosterone improves active avoidance retrieval and induces antidepressant-like behavior in rats

Janko Samardzic [a] ... Dubravka Svob Strac [d]

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Research Paper

Differential induction of dyskinesia and neuroinflammation by pulsatile versus continuous L-DOPA delivery in the 6-OHDA model of Parkinson's disease

Giovanna Mulas, PhD [a], Elena Espa, PhD [a], Sandro Fenu, PhD [a], Saturnino Spiga, PhD [b], Giovanni Cossu, MD [c], Elisabetta Pillai [a], Ezio Carboni, PhD [a], Gabriella Simbula, PhD [a], Dragana Jadžić [a], Fabrizio Angius, PhD [a], Stefano Spolitu, PhD [a], Barbara Batetta, PhD [a], Daniela Lecca, PhD [a], Andrea Giuffrida, PhD [d], Anna R. Carta, PhD [a,*]

[a] Department of Biomedical Sciences, University of Cagliari, Cittadella Universitaria di Monserrato, S.P. N. 8, 09042, Monserrato, Cagliari, Italy
[b] Department of Life and Environmental Sciences, University of Cagliari, via Fiorelli 1, Cagliari, Italy
[c] Department of Neurology, AOB "G. Brotzu" General Hospital, via Peretti 2, Cagliari, Italy
[d] Department of Pharmacology, UT Health Science Center San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, United States

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ABSTRACT

Neuroinflammation is associated with L-DOPA treatment in Parkinson's disease (PD), suggesting a role in L-DOPA-induced dyskinesia (LID), however it is unclear whether increased inflammation is specifically related to the dyskinetic outcome of L-DOPA treatment. Diversely from oral L-DOPA, continuous intrajejunal L-DOPA infusion is associated with very low dyskinetic outcome in PD patients. We reproduced these regimens of administration in 6-OHDA-lesioned hemiparkinsonian rats, where dyskinetic responses and striatal neuroinflammation induced by chronic pulsatile (DOPAp) or continuous (DOPAc) L-DOPA were compared. Moreover, we investigated the contribution of a peripheral inflammatory challenge with lipopolysaccharide (LPS), to DOPAp-induced dyskinetic and neuroinflammatory responses. Rats 6-OHDA-infused in the medial forebrain bundle received two weeks treatment with DOPAp, DOPAc via subcutaneous osmotic minipumps, or DOPAp followed by DOPAc. L-DOPA plasma levels were measured in all experimental groups. An independent group of rats received one peripheral dose of LPS 24 h before DOPAp treatment. Abnormal involuntary movements (AIMs) were evaluated as a rat model of LID. Immunoreactivity (IR) for OX-42, microglial and neuronal TNF-α, iNOS and GFAP was quantified in denervated and contralateral striatum. In addition, serum TNF-α was measured. The 6-OHDA denervation induced a mild microgliosis in the striatum two weeks after neurotoxin infusion, and increased TNF-α IR in microglia. Rats receiving the DOPAp treatment developed AIMs and displayed increased striatal OX-42, microglial TNF-α, iNOS and GFAP. Moreover, TNF-α IR was also increased in a subpopulation of striatal neurons. Conversely, DOPAc did not induce AIMs or inflammatory responses in either drug-naïve animals or rats that were previously dyskinetic when exposed to DOPAp. Serum TNF-α was not altered by any L-DOPA treatment. LPS pre-treatment increased the degree of DOPAp-induced AIMs and striatal IR for OX-42, TNF-α, iNOS and GFAP. Altogether the present findings indicate that in the 6-OHDA model, chronic L-DOPA induces striatal inflammatory responses, which however depend upon the administration regimen and the dyskinetic outcome of drug treatment. The potentiation of dyskinetic responses by LPS suggests a reciprocal causal link between neuroinflammation and LID.

1. Introduction

L-DOPA therapy represents the gold standard for patients with Parkinson's disease (PD), however long-term administration results in treatment-related motor complications, including L-DOPA-induced dyskinesia (LID). L-DOPA dose, administration route and regimen are considered the key factors affecting LID onset (Rascol et al., 2015). Unlike pulsatile L-DOPA, continuous L-DOPA-carbidopa intrajejunal infusion (LCIG) has shown good efficacy in treating PD symptoms and reducing the intensity of motor fluctuations and LID (Antonini et al., 2016; Olanow et al., 2014). Yet, the underlying reasons for these divergent effects are still largely unclear. Over the last decade, concerns have been raised regarding the possible toxic effect of L-DOPA, and the

* Corresponding author.
E-mail address: acarta@unica.it (A.R. Carta).

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