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TITLE

"Effect of medial prefrontal cortex 6-OHDA lesions on behavioral and neurochemical responses to gustatory stimuli"

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To my parents, Stergios & Anna Bimpisidis,

to whom I owe everything

Contents

Abbreviations

5-HT	**5-H**ydroxy**t**ryptamine (serotonin)
6-OHDA	**6-h**ydr**o**xy**d**op**a**mine
ABC	**A**vidin **B**iotin **C**omplex
AC	**A**nterion **C**ingulate
Ach	**Ac**etyl**h**oline
ACSF	**A**rtificial **C**erebro**s**pinal **F**luid
AdC	**Ad**enylyl **C**yclase
BBB	**B**lood **B**rain **B**arrier
CCK	**Ch**ole**C**ysto**K**inin
Cg	**C**ingulate **G**yrus
CPP	**C**onditioned **P**lace **P**reference
CPU	**C**audate **Pu**tamen
CSF	**C**erebro**s**pinal **F**luid
DA	**D**opamine
DAB	3,3'-**di**amino**b**enzidine
DAT	**D**op**a**mine **T**ransporter
DMI	**D**es**m**ethyl**i**mipramine - Desipramine
FC	**F**rontal **C**ortex
GABA	**g**amma-**A**mino**b**utyric **A**cid
Glu	**Glu**tamate

GPCR	**G - P**rotein **C**oupled **R**eceptor
HPLC	**H**igh **P**erformance **L**iquid **C**hromatography
HRP	**H**orse**r**adish **P**eroxidase
i.o.	**i**ntra**o**ral
i.v.	**i**ntra**v**enous
ICSS	**I**ntra**c**ranial **S**elf **S**timulation
ID	**I**nside **D**iameter
IL	**I**nfra**l**imbic cortex
LC	**L**ocus **C**oeruleus
MAO	**M**ono**a**min**o**xidase
MD	**M**edio**d**orsal thalamic nucleus
mPFC	**m**edial **P**re**F**rontal **C**ortex
NA	**N**or**a**drenaline
NAT	**N**or**a**drenaline **T**ransporter
NAc	**N**ucleus **Ac**cumbens
NMDA	***N*-M**ethyl-**D-A**spartate
OD	**O**utside **D**iameter
PBS	**P**hosphate **B**uffer **S**olution
PET	**P**ositron **E**mission **T**omography
PFC	**P**re**F**rontal **C**ortex
PFA	**P**ara**f**orm**a**ldehyde
PL	**P**re**l**imbic cortex

PPTg	**P**edunculo**p**ontine **T**e**g**mental nucleus
SA	**S**elf **A**dministration
SNc	**S**ubstantia **N**igra pars **C**ompacta
SOM	**Som**atostatin
SP	**S**ubstance **P**
TBS	**T**ris-**B**uffered **S**aline
TH	**T**yrosine **H**ydroxylase
VTA	**V**entral **T**egmental **A**rea

A. Introduction

The experiments that constitute the results of the present thesis were held out at the Department of Toxicology (Faculty of Pharmacy, University of Cagliari, Italy) under the supervision of Professor Gaetano Di Chiara and with the scientific collaboration of Dr. Maria Antonietta De Luca.

As indicated in the title, the thesis discusses the role of the medial prefrontal cortex (mPFC) dopamine (DA) in the nucleus accumbens (NAc) neurochemical and behavioral responses to taste stimuli. Nucleus Accumbens DA has been implicated in reward and motivational processes while disturbances in DAergic transmission in the mPFC have been involved in several disorders such as schizophrenia and depression. In particular, an hypodopaminergic state in the mPFC has been proposed to mediate the negative symptoms of schizophrenia. Furthermore, disturbances in cognitive functions related to mPFC DA neurotransmission have been reported to occur in substance abuse and in compulsive disorders in general.

Studying the responsiveness of mesolimbic DA after lesions of mPFC DAergic input can provide important information on the nature of the interaction between these DAergic systems and help understanding the functional organization in the circuits studied. Furthermore, it might shed light on aspects of behavior that are often neglected, such as disturbances in motivational learning. These studies can in turn lead to a better understanding of the mechanism of action of current medications of schizophrenia and depression and to develop new therapeutic strategies for these conditions.

A.1 Dopamine and Dopaminergic Systems

Dopamine is a biogenic amine that derives from the aminoacid tyrosine (see figure A.1) and a neurotransmitter that acts through G-protein-coupled receptors (GPCRs) named D_1 to D_5 according to their coupling with specific G proteins and their ability to stimulate or inhibit adenylyl cyclase (AdC): D_1-like family of receptors

(which includes D_1 & D_5 receptors) that stimulate the synthesis of AdC and D_2-like family (D_2, D_3 & D_4 receptors) that inhibit it (**Webster, 2001**).

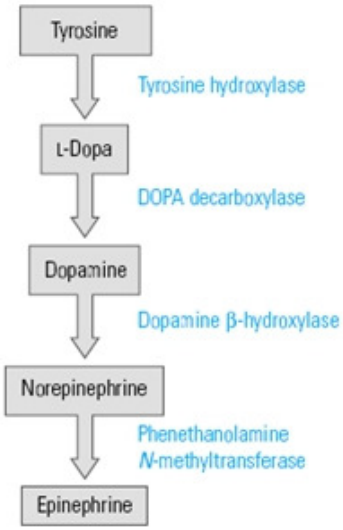


Figure A.1 The biosynthetic pathway of DA (source: Kolb & Whishaw, 2003)

There have been identified three groups of anatomically and functionally different subgroups of DA midbrain neurons that give rise to the three out of four major DAergic systems of the mammalian brain (**Carlsson et al., 1962; Dahlström & Fuxe, 1964**). The *nigrostriatal* or *mesostriatal* pathway rises from the cell bodies of the neurons of the substantia nigra pars compacta (SNc, lateral A9 group) which project primary to the dorsal striatum. This pathway has prominent role in the control of voluntary movement and is also related to the initiation and execution of habitual behavior, while its selective degeneration is responsible for the motor symptoms observed in patients with Parkinson's disease. The cell bodies of the *mesolimbic* and the *mesocortical* systems are located in the ventral tegmental area (VTA – medial A10 group & retrorubal field – A8 group, respectively) and project to the limbic system (NAc, central nucleus of amygdala and septum, entorhinal cortex, olfactory tubercules) and the medial prefrontal cortex (mPFC) respectively. These pathways are involved in regulation of cognition, emotions and reward (see below) and have been implicated in pathological conditions as drug addiction, depression and schizophrenia. The *tuberoinfundibular* system consists of DAergic neurons located in

the wall of the third ventricle (A12 & A14 cell groups), within the arcuate nucleus of the hypothalamus, that project to the median eminence and are involved in endocrine control. There, DA acts as a prolactin release inhibiting factor in the hypophysial portal circulation. Dopaminergic projections are also sent from the dorsal hypothalamus (A11 & A13 groups) to the spinal cord and neurons containing DA can also be found in the olfactory system (A15 cells in the olfactory tubercule and A16 in the olfactory bulb) and in the retina (A17 group of cells) (**DeLong, 2000; Kandel, 2000; Saper, 2000; Schwartz, 2000; Dianne et al., 2008**) (see figure A.2).

Dopamine transmission takes place in two independent but interactive releasing modes with distinct temporal properties: a fast, transient, "phasic" release dependent on action potentials and a constant, "tonic", action potential-independent release. Tonic DA is released slowly with a prolonged onset and the whole process takes long periods of time - from minutes to hours. This kind of release is regulated by presynaptic glutamatergic afferents that synapse with the DAergic terminals and underlies the basal levels of DA in subcortical areas. On the other hand, phasic DA release is triggered by behaviorally relevant stimuli (**Grace, 1991, 2000; Gonon, 1997; Dreher & Burnod, 2002**).

The aim of the studies presented in the current thesis was to investigate the role of the distinct DAergic systems in reward processes and the interaction between these systems. Despite the fact that the nigrostriatal pathway, apart from its role on the coordination of movement, has also been demonstrated to participate in some forms or aspects of reward functions (**Young et al., 1992; Ito et al., 2002**), the studies of the present thesis focused on more traditionally "reward circuits" i.e. the mesolimbic and mesocortical DAergic systems and in particular the projections from VTA to the mPFC and to the NAc. Below, there will be presented briefly some anatomical information and theories regarding the role of DA in these areas and after these information there will be discussed findings from the literature suggesting interaction between cortical and subcortical DA. At the end of the chapter will be presented the aim of the study.

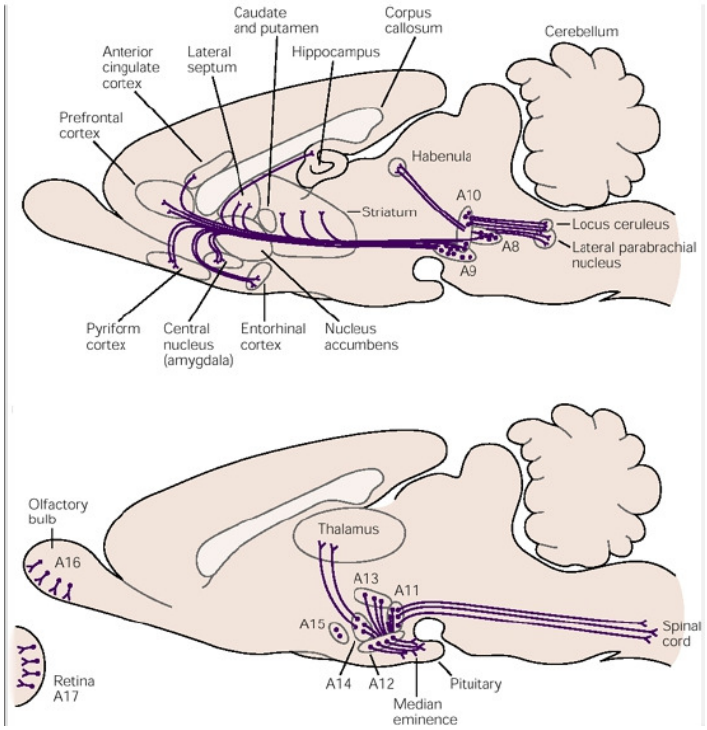


Figure A.2 Dopaminergic pathways in the rat brain (**from Schwartz, 2000**)

A.2 Nucleus Accumbens

A.2.1 Introduction

Nucleus Accumbens is a small nucleus ventral to the head of the caudate nucleus (see figures A.2, A.3, B.6) and together with the ventromedial part of the caudate-putamen and the olfactory tubercules comprise the anatomical complex often referred as "*ventral striatum"* (**Nakano, 2000**). Nucleus Accumbens is described as an "integral, but specialized part of the striatal complex" (**Heimer e al., 1991**) and consists mainly (90-95%) of medium spiny neurons; on the spines and the bodies of them synapse afferent fibers from interneurons, the cortex and other areas (see below) (**Somogyi et al., 1979; Freund et al., 1984; Smith & Bolam, 1990**).

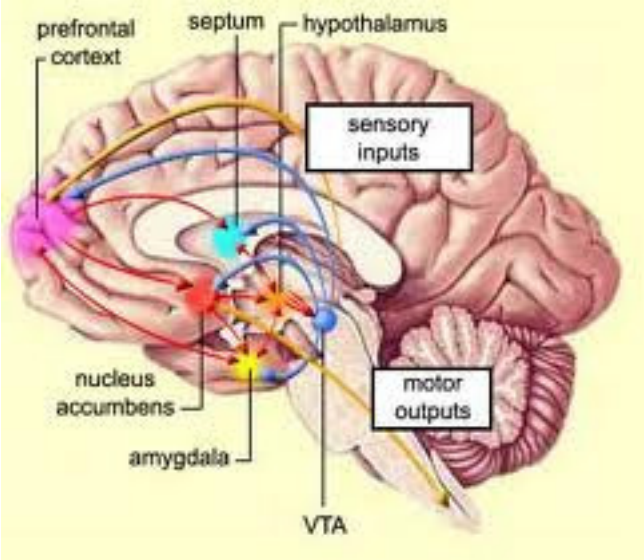


Figure A.3 Position of the Nucleus Accumbens (NAc) in the human brain (**from library.thinkquest.org**)

For long time since its first description, NAc was considered as a modified appendage of the striatum, rather than a discrete nuclear entity (**Zahm & Brog, 1992**). It was in the middle '80s when the studies of Groenewegen & Russchen (**1984**) and Zaborszky et al. (**1985**) demonstrated significant differences between the projections of the lateral and the medial NAc and introduced the division of the NAc into two histochemically distinct compartments: the "core" and the "shell".

Further anatomical (**Heimer et al., 1991; Zahm & Brog, 1992; Zahm & Heimer, 1993**), histochemical (**Jongen-Relo et al., 1993; Jongen-Relo et al., 1994**), behavioral (**Deutch & Cameron, 1992; Prinssen et al., 1994, Pierce & Kalivas, 1995; Corbit & Balleine, 2011**) and neurochemical (**Pontieri et al., 1995, 1996; Tanda et al., 1997; Bassareo et al., 2002**) studies established scientifically the division of NAc in these two compartments, the ventro-medial "shell" and dorsolateral "core" which have distinct structure, properties and physiological roles. Core is considered as part of the nigrostriatal DAergic pathway and is involved mostly in motor behavior while the shell comprises part of the mesolimbic dopaminergic pathway and mesolimbic system and is involved in reward processes, emotions and cognitive functions (**Alexander & Crutcher, 1990; Alheid et al., 1990, Smith & Bolam, 1990; Deutch & Cameron, 1992**). Histological, biochemical and connectional methods have shown that the NAc shell is characterized by typical striatal features but also by

characteristics of those of the extended amygdala, suggesting that the NAc shell comprises a transitional or integrative zone between the two areas (**Heimer et al., 1991**).

As mentioned above (see section A.1), NAc is the main target of the mesolimbic dopaminergic system - it receives dense DAergic innervation from the VTA. It also receives inputs from a lot of cortical and non cortical areas, including the hippocampal formation, the mPFC, the insular cortex, the amygdala and the thalamus and is characterized as a "portal" of prefrontal inputs to the striatum (**Nakano, 2000**). The most prominent projections of the NAc reach the pallidum, the substantia nigra and the VTA (**Swanson & Cowan, 1975; Williams et al., 1977; Nauta et al., 1978**).

The two distinct compartments of the nucleus receive afferents from and project to different areas of the brain. Tract-tracing methods have shown that both shell and core areas project to pallidal, hypothalamic and mesencephalic areas but core projections arrive more prominently to the substantia nigra while those of the shell to the VTA. In addition, shell projects also to the extended amygdala unlike core (**Heimer et al., 1991**).

Several neuroactive/neuromodulatory substances have been detected in the NAc as acetylcholine (Ach), DA, serotonin (5-HT), noradrenaline (NA), somatostatin (SOM), gamma-aminobutyric acid (GABA), substance P (SP), glutamate (Glu), neurotensin, Leu-enkephalin, Met-enkephalin, cholecystokinin (CCK), melatonin, histamine, galanine and neuropeptide Y (**Voorn et al., 1989; Alheid et al., 1990; Graybiel, 1990; Selden et al., 1994, Parent et al., 1995**).

Nucleus Accumbens is suggested to be an interface between limbic and motor functions (**Mogenson et al., 1980; Mogenson & Yang, 1991**) and to be involved in several aspects of behavior as motivation, reward, water and food intake, sexual behavior, emotions and cognitive functions and to play a major role on drug dependence (**Wise, 1987; Alexander & Crutcher, 1990; Alheid et al., 1990, Smith & Bolam, 1990, Di Chiara, 2002; Di Chiara et al., 2004; Di Chiara & Bassareo, 2007; Anselme, 2009**). It has been suggested that the shell of the NAc has more limbic functions (more specific role in emotion and motivation) while the core

compartment is involved mainly in the motor expression of motivated behaviors (**Zahm & Brog, 1992; Bassareo & Di Chiara, 1999; Brauer et al., 2000**).

A.2.2 The Role of Dopamine in the Nucleus Accumbens

The role of DA in general and in the mesolimbic system in particular has been a subject of debate for almost 30 years. Nowadays it is generally accepted that the mesolimbic DAergic system plays a critical role in conventional and drug reward, motivation and reinforcement, but the exact nature of this role is still unclear.

One of the first studies to link DA to motivation by conventional rewards was made by Ungerstedt (**1971**) who showed that lesion of the nigrostriatal DA system by 6-hydroxydopamine (6-OHDA) induced severe aphagia and adipsia in rats. Further experiments with DA receptor antagonists led to the "*anhedonia hypothesis*" that viewed DA as the substrate of the hedonic/pleasant effects of all rewards - from drugs of abuse to natural stimuli as sex and food (**Yokel & Wise, 1975; Wise, 1980, 1982**). This theory remained influential for a long time among scientists and among the media and public opinion as well. Koob and Le Moal (**1997, 2001**), influenced by Wise's and others contemporary theories of DA role in reward, speculated that drug exposure changes the "*reward set point*" of the organism so that withdrawal in depended individuals results in reduced DAergic transmission and in a state of anhedonia.

However, the "anhedonia hypothesis" was thought to be unable to explain the observation that pharmacological blockade of DA receptors does not suppress hedonic responses estimated by the taste reactivity paradigm (see next chapter, section B.6.1) (**Peciña et al. 1997; Berridge & Robinson, 1998; Di Chiara et al., 2004**). Thus, food reward has been found to be independent of DA transmission (**Salamone et al., 1997; Berridge & Robinson, 1998**) and DA is also released by aversive events and stress (**McCullough & Salamone, 1992; Salamone, 1994; Kalivas & Duffy, 1995**). Microdialysis and *in vivo* voltammetry studies have shown that DA transmission can be activated in anticipation of the consumption of drug or food rewards or by

presentation of reward-conditioned stimuli (**Blackburn et al., 1989; Phillips et al., 1993; Bassareo et al., 2007**). In line with these studies are the observations of Schultz and his colleagues (see below) that DA neurons fire in response to the presentation of stimuli conditioned to rewards.

Further studies on the properties of DAergic transmission showed that the role of DA in reward is not simply that of mediating pleasure elicited by a rewarding stimulus. Due to the spatial and temporal characteristics of its release and diffusion in the extracellular space, localization and transduction mechanisms of its receptors (extrasynaptic release and transduction by GPCR), DA exerts a delayed, tonic influence, acting "in parallel" with stimuli that follow its release, "*modulating their ability to elicit a response*", rather than "i*n series*" with them (**Di Chiara & Bassareo, 2007**).

Berridge and his colleagues proposed a theory, in which DA has a role different from that of mediating the hedonic properties of rewards. This role is that of attributing incentive salience to conditioned stimuli. They distinguish two components of rewarding stimuli: "*liking*", the unconscious hedonic evaluation of a stimulus, and "*wanting*", "*the underlying core process that instigates goal-directed behavior, attraction to the stimulus, and consumption of the goal object*" (**Berridge & Robinson, 1998 pp 313**). According to this theory, DA participates in "wanting" and not in ''liking'' (**Berridge et al., 1989; Robinson & Berridge, 1993; Berridge, 1996; Berridge & Robinson, 1998**).

However, it has been argued (**Di Chiara, 2002**) that, although DA is not the substrate of the hedonia associated to food consumption, it is nonetheless the substrate of the appetitive properties of food-conditioned incentive stimuli acting during the preparatory phase of feeding behavior. This kind of hedonia, characterized by euphoria and behavioral arousal ("*incentive arousal*", **Di Chiara, 2002**) is mimicked by cocaine and amphetamine psychostimulants and is thought to be related to stimulation of DA transmission in the NAc shell (**Di Chiara, 2002**). On this basis, two kinds of hedonia have been distinguished, a DA-independent consummatory or stimulus-bound hedonia, such as that associated to food taste, and a DA-dependent preparatory or state-hedonia, associated to the incentive

arousal state of food search and approach. These two kinds of hedonia correspond to distinct phases of motivated behavior, appetitive/preparatory and, respectively, consummatory, elicited by distinct stimuli, distal (visual, acoustic, olfactory) and, respectively, proximal (tactile, proprioceptive, taste) and associated to distinct behavioral response patterns of search, exploration and approach independent from the specific reward (appetitive behavior) and, respectively, reward specific motor responses (consummatory responses).

Furthurmore, according to Salamone and colleagues "wanting" can be distinguished into two separate components, "*directional*" and "*activational*", where directional aspects can be appetitive in nature and activational aspects include "*the initiation and sustaining of instrumental actions or the tendency to work for food*"; "*the tendency to work for motivational stimulus, and overcome response constraints*" (**Salamone & Correa, 2002 pp 1 & 15**). According to these authors, DA in the NAc is involved in instrumental action in tasks with a high degree of "*work-related response costs*" (**Salamone et al., 2001; Salamone & Correa, 2002**).

A popular theory of the role of DA is the "*prediction error hypothesis"* of Schultz and colleagues. This theory is based on correlative evidence arising from extracellular recording of putative DA neurons in the monkey VTA. These studies showed that food and liquid rewards elicit phasic firing in most midbrain DA neurons. These neurons are activated by the unpredicted occurrence of reward or of stimuli conditioned to it. Presentation of stimuli conditioned to food reward prevents the activation of DA units by food itself. Thus, a fully predicted reward doesn't elicit a response. On the other hand, omission of a predicted reward induces depression of DA neurons. Therefore, according to the "reward-prediction error" hypothesis, DA codes for the occurrence of an unpredicted reward and for the omission of a predicted one (**Schultz et al., 1993; Schultz et al., 1997; Schultz, 2007**). Although this hypothesis can account for the phasic activity of DA neurons in response to rewards and their conditioned stimuli, it does not necessarily applies to the action of DA on its receptive neurons. In fact, the temporal and spatial properties of DA mediated signals are quite different from those of a fast transmitter acting via ionotropic receptors (**Di Chiara & Bassareo, 2007**). Thus, by the time DA is released,

diffuses in the extracellular space, reaches its receptors and activates them, the phasic response of DA neurons is transformed into a tonic influence on a widely distributed range of DA sensitive neural elements. Thus, the computational properties that are attributed to DA by Schultz theory do not fit with those of a neuromodulator like DA but rather with those of a fast, ionotropic excitatory transmitter acting like glutamate. As a matter of fact, evidence is now being provided that glutamate is a co-transmitter of DA in mesolimbic neurons (**Lavin et al., 2005**). According to Schultz and colleagues, DA, released as a result of an error in the prediction of reward, acts as a teaching signal in associative and hebbian learning functions. However, the evidence provided in support of this theory is only circumstantial.

Unfamiliar palatable food stimulates DA release in the shell and core and in the mPFC but only in the NAc shell this effect undergoes habituation following repeated exposure. This property has been related to a role of DA in associative learning mediated by food reward. Accordingly, DA release in the NAc shell by food taste would serve to consolidate the memory trace of taste stimulus, thus allowing association with its post-ingestive consequences, as in a taste aversion paradigm (**Fenu et al., 2001; Di Chiara & Bassareo, 2007**). These and other observations are the basis of the view that DA is involved in the acquisition of incentive-motivational properties by stimuli conditioned to rewards (incentive learning). The DA subsystem specifically involved in this process is NAc shell DA (**Di Chiara et al., 2004; Di Chiara & Bassareo, 2007**). The influence of NAc shell DA on incentive learning is retrograde, affecting the association of stimuli that precede or are coincident with reward. Drugs of abuse increase DA preferentially in the NAc shell and this effect is thought to facilitate the association between drug reward and stimuli that predict their occurrence. Since DA is preferentially released in the NAc shell in response to drug-reward, drug-conditioned stimuli should also release DA in the same area. This is indeed what has been found with brain microdialysis after stimuli conditioned to drugs of abuse (**Bassareo et al., 2007**). Food-conditioned stimuli elicit a different pattern of DA activation, since they stimulate NAc core but not shell DA (**Bassareo et al., 2011**). Both food- and drug-conditioned stimuli, however, stimulate DA release in

the mPFC. Therefore, food-conditioned and drug-conditioned stimuli differentially stimulate DA transmission in the NAc shell and core. These differential properties of drug- versus food-conditioned stimuli have been assigned an important role in the ability of drugs to elicit addiction (**Bassareo et al., 2007, 2011**).

Finally, an *"anticipatory dynamics model"* (and its "*uncertainty processing theory of motivation"* revised form) for the role of DA in motivation and reward has been recently proposed. According to this theory, phasic DA release is induced by events that comprise "*uncertainty with respect to the stimulus arrival*". Dopamine in limbic and cortical areas facilitates anticipation which is necessary for the organism to deal with uncertainty. Nucleus Accumbens (and the core compartment in particular) is the region where signals from other corticolimbic areas regarding anticipation converge. Due to its connections with motor areas can fast reduce uncertainty by adopting appropriate motor responses to environmental challenges. The theory also includes another important role for DA: the facilitation of task related attention which serves for motivational specificity (towards a particular reward) to take place. This "*anticipatory attention*" occurs through neuronal pathways co-utilizing DA & Ach (**Anselme, 2009, 2010**).

A.3 Prefrontal and Medial Prefrontal Cortex

A.3.1 Introduction

The prefrontal cortex (PFC) consists part of the frontal cortex (FC - along with motor and premotor cortices) and is located in the anterior part of it. Originally, Jersey Rose and Clinton Woolsey in the late '50s, nominated as "prefrontal cortex" the area of the frontal lobes of mammals of all species that has strong reciprocal projections with the mediodorsal thalamic nucleus (MD) (**Groenewegen et al., 1990; Kolb & Whishaw, 2003; Steketee, 2003, 2005**). Prefrontal cortex is divided in medial and lateral parts and more specifically into three main regions: the *dorsolateral* PFC (Broadmann areas 9 & 46), the inferior ventral PFC (areas 11, 12, 13 & 14 – also

known as *orbitofrontal* cortex) and the *medial prefrontal* (mPFC) cortex which is considered part of the anterior cingulate (AC) area (**Kolb & Whishaw, 2003**) (see figure A.4).

Medial prefrontal cortex is located along the medial wall of the brain hemispheres anterior and dorsal to the genu of the corpus callosum (**Heidbreder & Groenewegen, 2003**) and is an area well known to interact through excitatory aminoacid (EAA) efferents with subcortical areas (**Thierry et al., 1983; Christie et al., 1985; Sesack et al., 1989; Sesack & Pickel, 1992**). It consists of the infralimbic (IL), prelimbic (PL - Cingulate gyrus 3 - Cg3 of the anterior cingulate), dorsal and ventral anterior cingulate (Cg1 & Cg2) and medial precentral (frontal area 1) cortices from ventral to dorsal (**van Eden & Uylings, 1985; Groenewegen et al., 1990; van Eden et al., 1992; Steketee, 2003, 2005**) (see figure A.5).

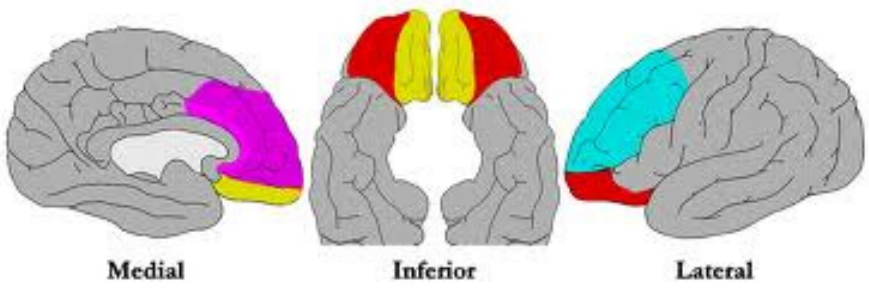


Figure A.4 Schematic representation of the prefrontal cortex (PFC) and its subdivisions in the human brain (**from focus.psychiatryonline.org**)

Medial prefrontal cortex receives the most prominent cortical afferents from the contralateral mPFC and the most prominent subcortical afferents from the MD (as mentioned above). Both of these afferent connections are glutamatergic in nature. Additional glutamatergic input arrives to mPFC from the hippocampus & the amygdala (**Condé et al., 1990, 1995; Groenewegen et al., 1990; Ray & Price, 1992; Kuroda et al., 1993**). Furthermore, a source of NA in the mPFC originates from the locus coeruleus (LC) and, as already mentioned, of DA from the VTA (**Thierry et al., 1973; Björklund et al., 1978**; **Foote et al., 1983**). Other neuroactive substances as GABA (mostly from interneurons), endogenous opioids (there exist mostly δ-receptors and lower levels of μ- & κ) and cannabinoids (CB1 receptors), 5-HT and

neurotensin are also present in the mPFC (**Mansour et al., 1994, Moldrich & Wenger, 2000; Tzschentke, 2001**). As already mentioned above, one of the most prominent connections of mPFC is with the MD and a direct feedback loop between the two brain regions has been described (**Kuroda et al., 1993**). Furthermore, it provides heavy innervation to subcortical areas as the NAc and the VTA (**Thierry et al., 1983; Christie et al., 1985; Sesack et al., 1989; Sesack & Pickel, 1992**).

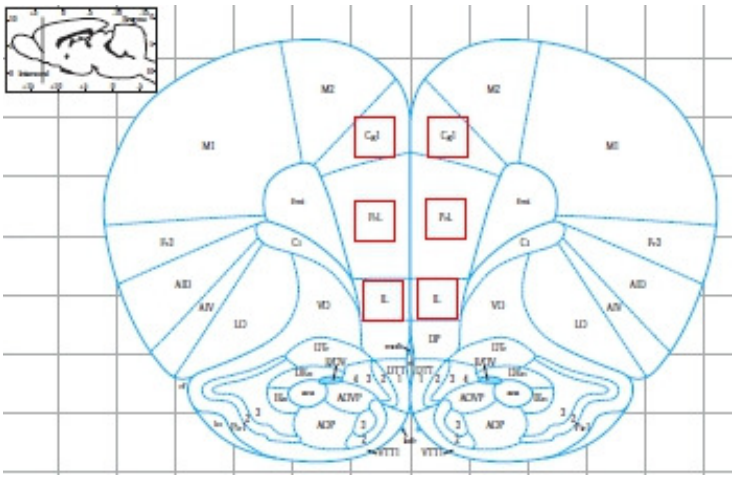


Figure A.5 The medial prefrontal cortex in the rat brain. Red squares indicate infralimbic (IL), prelimbic (PL) and anterior cingulate (Cg1) cortices from ventral to dorsal (**From Paxinos & Watson, 2007**)

Each subarea of the mPFC appears to have distinct afferent and efferent connections which might also suggest different functional roles. For instance, the subareas of the mPFC show topographical organization regarding their efferents where more dorsal parts (anterior cingulate and dorsal prelimbic areas) project to the striatum and dorsal parts of the NAc, while the more ventral subareas (ventral prelimbic and infralimbic cortices) send fibers to ventral aspects of the NAc, (i.e. shell) (**Sesack et al., 1989; Berendse et al., 1992**). As for the afferents, IL cortex receives inputs from the hippocampus and the brain stem (VTA) but not from the MD, which makes it, according to some authors, part other than the traditional mPFC. As already mentioned, its efferents target principally NAc shell but also the rostral MD, the hypothalamus, the amygdala & the brain stem nuclei. Prelimbic cortex receives inputs from MD, hippocampus and the VTA. It receives the densest

DAergic input among the other mPFC subareas and it also has the strongest projection to the VTA. It also projects to the NAc core (as mentioned above), rostral MD and basolateral amygdala among other forebrain structures. The anterior cingulate subarea receives inputs from the caudal MD thalamus, from other neocortical areas and from the VTA. It projects mainly to the dorsal striatum, the caudal MD and to other neocortical and brainstem areas (**Thierry et al., 1973; Lindvall et al., 1978; Beckstead, 1979; Sesack et al., 1989; Groenewegen et al., 1990, 1999; Hurley et al., 1991; Berendse et al., 1992; Tzschentke, 2001**).

Being a heterogeneous area, PFC has been reported to be involved in a great variety of functions-behaviors, as planning of voluntary movement, adjustment of behavior in relation to space, arousal and attention, temporal sequencing of actions, planning of forthcoming behavior based on previously acquired information, response selection and response inhibition, stress responses, mood, spatial and associative learning, memory retrieval and working memory (with a special role of DA in the mPFC – see below) (**Pinel, 2000; Tzschentke, 2001; Curtis & D'Esposito, 2003; Kolb & Whishaw, 2003; Ramnani & Owen, 2004; Surmeier, 2007**).

Disturbances in the structure and function of PFC have been reported to exist in schizophrenic patients, in individuals with unipolar depression and bipolar disorder. It has been suggested that decreased DAergic activity in the mPFC is responsible for the negative symptoms that occur in schizophrenia while in depression the reduced activity of the area is correlated with the disturbances in memory and attention observed in the disorder (**Blumberg et al., 1999; Webster, 2001**). Regarding substance abuse disorders it has been suggested that long term abuse of substances might induce changes in important cognitive/executive functions as decision making and correct judgment (**Powell, 2004**). It has also been proposed that dysfunction of the PFC (more specifically in the orbitofrontal cortex) due to substance abuse might induce inability to achieve control over the use of the substance, incapability to cease its intake despite the harmful consequences for the individual (**Porrino & Lyons, 2000**), disturbed ability in comparing the future beneficial effects from the cessation and the harmful effects of continuation of the abuse of substance as well

as disturbed impulse control and relapse (**London et al., 2000; Kalivas & Volkow, 2005; Garavan & Hester, 2007**).

The mPFC has been implicated in many of the functions mentioned above and is generally accepted that participates in "higher order cognitive functions" as attentional processes, working memory and behavioral flexibility (**Heidbreder & Groenewegen, 2003**). Some researchers propose a clear distinction between ventral and dorsal subdivisions/subareas of the mPFC because of common phylogenetic origin but also due to anatomical, neurochemical and functional differences observed in the areas. Moreover, lesion, transient or permanent inactivation and antagonist-using studies suggest a different role for each subarea of the mPFC. Thus, lesions and pharmacological manipulations of the dorsal part of the mPFC (AC and dorsal PL cortices) can alter the performance in several tasks. Among others they have been shown to increase conditioned fear responses, to impair the acquisition of avoidance tasks, to block the expression of cocaine sensitization and to produce memory impairments for egocentric responses. On the other hand, lesions of the ventral part of the mPFC (ventral PL and IL cortices) have been shown to impair the extinction of fear responses; to produce impairments in passive avoidance, working memory, shifting to different cue modalities for reinforcement and reversal learning; to block conditioned place preference (CPP) to cocaine; and to attenuate the development of sensitization to cocaine but not amphetamine. This region seems to play a role in the reinstatement of cocaine self administration as its inactivation blocks the drug seeking behavior. Furthermore, these lesions impair performance in elevated plus maze, taste aversion and behavioral flexibility tests. In conclusion it is stated that the dorsal part of the mPFC is mainly involved in temporal sequencing regarding behavioral responses and the ventral part in set shifting/behavioral flexibility, in guidance of behavior in threatening or aversive conditions and in preparatory processes of reacting time performance. For more information regarding the functional and anatomical dissociation between the dorsal and ventral parts of the mPFC the author suggests the review of Heidbreder & Groenewegen (**2003**). This thesis aims to point out the role of DA in mPFC and not of the whole

region (or its subdivisions) per se. Limited information exists for the role of DA in a subregion-specific manner and this information will be discussed on the next section.

A.3.2 The Role of Dopamine in the Medial Prefrontal Cortex

The DAegic innervation of the mPFC is less dense than this of the NAc or the striatum; the basal extracellular and tissue levels of DA are much lower in the mPFC (**Garris et al., 1993; Ihalainen, 1999; Tzschentke, 2001**). The distribution of DA per se and of the other elements of the DAergic system as the dopamine transporter (DAT) also differs along the ventrodorsal axis of the mPFC. The DAT is more densely distributed in the AC than in the PL cortex (**Sesack et al., 1998**) and DA terminals are denser in the PL than the other subregions of the mPFC (**Thierry et al., 1973; van Eden et al., 1987**).

Dopaminergic terminals from VTA innervate the deep layers of mPFC pyramidal cells (**Berger et al., 1991; Dreher & Burnod, 2002**) and in turn these pyramidal neurons send glutamatergic efferents to the VTA (**Taber et al., 1995**). In addition, DA terminals synapse with GABAergic interneurons in the mPFC, modulating their function (**Penit-Soria et al., 1987; Pirot et al., 1992**). Dopamine in the mPFC serves as an inhibitory neuromodulator and through the synaptic distribution of the DAergic fibers this action can be direct on the pyramidal neurons and/or indirect by modulating the GABAergic interneurons' activity which synapse with the pyramidal neurons (**Ferron et al., 1984; Godbout et al., 1991; Pirot, 1992; Sesack et al., 1995**). Thus, it has been shown that, DA or DA agonists administered locally and chemical or electrical stimulation of the VTA and the subsequent release of DA in the mPFC result in inhibition of the mPFC pyramidal cells (**Bernardi et al., 1982; Ferron et al., 1984; Thierry et al., 1986; Peterson et al., 1987; Mantz et al., 1988; Yang & Mogenson, 1990; Karreman & Moghaddam, 1996**). Despite this clear evidence of an inhibitory role of DA in the mPFC, the data in the literature suggest a more complex role of DA in the area. Thus, several findings suggest that the effect of DA on

prefrontal cells depends on the cell type and its state, i.e. the level of activity due to other inputs (for further information, see **Tzschentke, 2001**).

Dopamine in general seems to have a non specific function and because of that, DA in the mPFC might play the same role in all the processes mentioned already for the NAc (**Anselme, 2009**). Today it is widely accepted that DA in the mPFC has a modulatory role and changes in the DAergic transmission in the area do not produce a clear behavioral output.

Despite the fact that mesocortical DAergic system is the one with the higher responsiveness to stress, both acute and chronic, with respect to the other DAergic systems (**Thierry et al., 1976; Blanc et al., 1980; Deutch et al., 1985; Jedema & Moghaddam, 1994; Cuadra et al., 1999**), DA in the mPFC is also increased by other salient stimuli, such as novelty, food and conditioned to food stimuli (**Feenstra & Botterblom, 1996; Bassareo & Di Chiara, 1997; Bassareo et al., 2002; 2007**).

As in the case of DA in the NAc, DA in the mPFC seems to play a role in reward processes as well, despite the fact that this role is not clear yet. Nevertheless, the increase of DA in the mPFC doesn't seem to be induced by the consumption of a reward per se, but seems to be associated with "novelty, excitement, expectancy and operant and classical conditioned responses" and to code for generic motivational value (**Bassareo & Di Chiara, 1997; Tzschentke, 2001; Bassareo et al., 2002**). Di Chiara and collegues suggest that unlike DA in the NAc, and the shell compartment in particular, DA in the mPFC is not related to the ability of some drugs to induce abusive behaviors. In fact, drugs that increase DA (or NA) preferentially in the mPFC are not abused by humans or they have low abuse potential (**Tanda et al., 1994, 1996; Bassareo et al., 1996**).

A great amount of work has been held out to unravel the role of DA in the mPFC in working memory, i.e. the ability to retain and manipulate information temporarily. Thus, the projection from VTA to mPFC is said to play a pivotal role in working memory function, as it has been demonstrated in studies with monkeys and rats performing the "delayed alternation task", a test that measures the intact function of this higher order cognitive function. The ability of both D_1 agonists and antagonists to impair performance suggests that the influence of D_1 receptor

occupation on working memory follows an inverted U-shaped curve (**Arnsten, 1997; Dreher & Burnod, 2002**). The role of DA on working memory has been also repeatedly demonstrated in humans (**Luciana et al., 1992**) and predominantly in the PFC in non-human primates (**Sawaguchi & Goldman-Rakic, 1994**). As in the case of NAc DA regarding reward, several theories tried to explain the exact role and function of mesocortical DAergic projections in working memory.

Briefly, Cohen and collegues (**Servan-Schreiber et al., 1990; Cohen & Servan-Schreiber, 1992; Braver et al., 1999**) have proposed a model of DA function in the mPFC according to which DA serves to increase signal-to-noise ratio and that way to facilitate both excitatory and inhibitory inputs to the cells of this region. Dopamine's role is to gate access of information to active memory, i.e. to provide updating of useful, task-relevant information but also to protect from irrelevant information interference. These effects occur in the synapse by "*transient potentiation of both excitatory afferent and local inhibitory input*". According to this theory, cognitive deficits in disorders that are characterized from a cortical hypodopaminergic state as in schizophrenia result from a failure in "gating" incoming information, from an increase in "noise". The "noise" results in disturbances in updating and maintaining important and context relevant information in the active/working memory – cognitive characteristics of vital importance for cognitive control (**Braver et al., 1999**).

In means of conjoining reward processes and higher order cognitive functions as working memory and decision making, Durstewitz and collegues proposed a different model (**Durstewitz et al., 1999; Durstewitz & Seamans, 2002**). According to these authors, DA in the mPFC serves to increase "*the robustness of representations encoding goal-related information*" by reducing the impact of intervening stimuli.

As mentioned above in the section for DA system characteristics, DA in the mPFC is also released in phasic ("extrinsic modulation", in terms of neural networking) and tonic ("intrinsic modulation") fashions. The source of phasic release is the firing of DAergic neurons located in the VTA; tonic release is induced by glutamatergic synapses (by local glutamatergic networks in the mPFC or by subcortical efferents) on the DAergic terminals in the mPFC independent of VTA neurons' firing (**Takahata**

& Moghaddam, 1998; Dreher & Burnod, 2002). Attempting to fit these release properties of DA in a model, Dreher & Burnod (**2002**), suggested that DA in the mPFC serves to induce a threshold that restricts excitatory inputs to pyramidal neurons. The phasic and tonic modes of release induce this restriction in different time scales - seconds and minutes/hours respectively. Thus, phasic DA is modeled as a temporary restricting threshold of the external inputs arriving on apical dendrites of mPFC that follows their presentation. On the other hand, the control of mPFC on the VTA neurons firing, serves for phasic DA release to adapt to the "temporal structure" and the requirements of the task being performed (**Dreher & Purnod, 2002**).

Limited data exist regarding the role of DA in each subarea of the mPFC in behavior. Hitchcott and collegues (**2007**) have shown that DA in the ventral parts of the mPFC (i.e. the IL cortex) is necessary for adjustment of behavior in relation to outcome expectancy. Thus, when DA was injected in the IL cortex, instrumental behavior was sensitive to outcome devaluation; DA in this area promoted shifting of behavior from stimulus-response conditions to action-outcome associations. The authors discussed the possibility that DA in the IL part of the mPFC plays an important role in attentional processes and this in turn had major impact in the behavioral manifestations observed during their experiments (**Hitchcott et al., 2007**). In another recent study, Naneix et al. (**2009**) made specific DAergic lesions in infralimbic and prelimbic cortices of adult rats. Using behavioral paradigms they showed that DAergic dennervation within each area is not necessary for the acquisition of an instrumental response to receive a reward. However, animals with DAergic lesions in the PL showed deficits to adapt their instrumental behavior to contingency changes between behavior expression and reward delivery. This might also be related to the role of DA in behavioral flexibility associated with DA in the mPFC (**Naneix et al., 2009**). More studies are necessary to clarify the role of DA in each subregion of the mPFC on several aspects of behavior.

Concluding the data regarding the role of DA in the mPFC, it worth mentioning the fact that there are researchers that support that there is non-specific role of DA on behavior (see **Anselme, 2009**), which partially is true as can also be seen from the data mentioned above. Thus, it is suggested that DA can serve in all three major

functions where is supposed to be involved – motor, reward, cognitive – in a unitary mode in which the DA system serves to help the organism to "*learn about, predict and respond appropriately to events that lead to reward*" (**Braver et al., 1999**).

A.4 Evidence of Interaction Between Cortical and Subcortical Dopamine

Dopaminergic terminals arising from the VTA innervate the deep layers of mPFC pyramidal cells (**Berger et al., 1991; Dreher & Burnod, 2002**) which project through glutamatergic efferents to subcortical areas as the VTA and the NAc (**Taber et al., 1995; Carr et al., 1999**). In addition, as already mentioned DA terminals from VTA synapse with GABAergic interneurons in the mPFC modulating their function (**Penit-Soria et al., 1987; Pirot et al., 1992**). These structural characteristics serve to provide direct and indirect inhibitory control of DA from the VTA on the activity of pyramidal neurons of the mPFC which in turn modulates the activity of subcortical areas including NAc and VTA. Several studies have shown that mPFC and NAc strongly interact regarding DA transmission and subsequently in several aspects of behavior.

Evidence for general cortical – subcortical interaction was provided by early studies which showed that damage in the rat cortex can enhance the locomotor activity induced by amphetamine (**Adler, 1961; Iversen, 1971**) and the stereotypies induced by apomorphine (**Scatton et al., 1982**).

Further experiments pointed out a specific role of the prefrontal cortex in controlling subcortical DA. Thus, chemical or electrical stimulation of the mPFC was shown to increase the firing rate and to change the firing pattern of DA neurons in the VTA (**Gariano & Groves, 1988; Chergui et al., 1993; Murase et al., 1993**) and to increase DA levels in the NAc via *N*-methyl-D-aspartate (NMDA) receptor activation in the VTA (**Taber et al., 1995; Taber & Fibiger, 1995; Karreman & Moghaddam, 1996; You et al., 1998**). Oppositely, temporary inactivation of the mPFC was shown to reduce firing rate and burst firing in DA neurons located in the VTA (**Svensson & Tung, 1989; Murase et al., 1993**).

Through these studies it was made clear that interaction between mPFC and subcortical areas as the NAc and the VTA exists. Furthermore, a great amount of evidence suggests that mPFC and NAc show opposite characteristics regarding DAergic transmission and the way DA is released in each area during several aspects of behavior. Regarding behavior, augmentation of DAergic transmission in the mPFC follows reduction in spontaneous or drug-induced locomotor behavior; when administered locally in the mPFC, apomorphine and amphetamine decrease spontaneous and amphetamine induced activity (**Vezina et al., 1991; Broersen et al., 1999; Lacroix et al., 2000**). On the other hand, D_1 antagonists injected in the mPFC increase the locomotion induced by intra-NAc amphetamine injections (**Vezina et al., 1991**). While in these studies there was no effect on locomotion of the microinjections of the substances in the mPFC alone, in some other studies a mild increase in locomotion was reported after microinjections of amphetamine (**Carr & White, 1986**). Increasing the DA levels by intra-mPFC injections of DA reuptake inhibitors was also found to decrease novelty-induced locomotion, an effect mediated by both types of DA receptors (**Radcliffe & Erwin, 1996**) while in other studies is reported that only D_1 receptors are involved in these phenomena (**Lacroix et al., 2000**). Finally, with respect to locomotion, it was found that systemic injection of cocaine induces positive correlation between locomotion and DA levels in NAc and negative correlation between locomotion and DA levels in the mPFC (**Hedou et al., 1999**).

With respect to neurochemistry the pattern discussed above (increases of DA in the mPFC result in decreases in induced locomotion and vice versa) is observed also in DA concentrations: increasing DAergic transmission in the mPFC leads to decreased DAergic activity in the NAc and decreased DAergic transmission in the mPFC produces increased levels of DAergic activity in the NAc. Thus, it has been shown that locally administered amphetamine, cocaine or apomorphine in the mPFC decrease the DA levels and DA metabolites in the NAc while DA antagonists have the opposite effect (**Louilot et al., 1989; Jaskiw et al., 1991; Kolachana et al., 1995**).

Additional evidence was provided by combined behavioral and neurochemical data which revealed that acquisition of intravenous (i.v.) amphetamine self

administration (SA) increased DAergic activity in the NAc combined and decreased the extracellular DA in the mPFC (**Piazza et al., 1991**). Similarly, the inverse relationship between prefrontal and accumbal DA was observed by Vanderschuren and collegues (**1999**) who reported that sensitization occurs after a single injection of amphetamine and this effect induces an increase in locomotion and DA release in the NAc in response to a challenge injection. Sensitization was found to be depended on withdrawal duration – the bigger the withdrawal, the bigger the increase of DA and the locomotion induced by the challenge injection. Opposite effects were observed regarding mPFC DA (**Vanderschuren et al., 1999**). In the intracranial self stimulation (ICSS) paradigm it has been shown that miroinjections of a D_1 or D_2 antagonists in the NAc, increase ICSS reward thresholds of VTA stimulation (i.e. make the stimulation less regarding) while injections in the mPFC decrease the threshold (**Duvauchelle et al., 1992, 1998**).

Studies utilizing 6-OHDA lesions of the mPFC are in line with these data and the animals that have been lesioned demonstrate higher behavioral and neurochemical (in terms of increases of DA in the NAc) responses to pharmacological and natural stimulation. Thus, lesioned animals demonstrate higher haloperidol induced tyrosine hydroxylase (TH) activity (**Rosin et al., 1992**) and enhanced amphetamine-induced (**Banks & Gratton, 1995**) and K^+ stimulated (**Thompson & Moss, 1995**) DA release in the NAc. These lesions can induce enhanced motor stimulant and stereotyping responses after repeated injections of amphetamine (**Banks & Gratton, 1995; Espejo & Miñano, 2001**) and after acute injections of cocaine (**Beyer & Steketee, 1999**) or methylphenidate (**Wanchoo et al., 2010**). The effects of lesions seem to be specific for the DAergic responses of NAc since it has been reported that they do not affect the increase of DA in the striatum after amphetamine injections (**King & Finlay, 1995)**. The lesion-specific effect in NAc DAergic responsiveness could account for the results of the groups of Schenk (**1991**) and McGregor (**1996**) who showed that lesioned animals are more sensitive to the reinforcing effects of lower doses of cocaine (**Schenk et al., 1991; McGregor et al., 1996**). Nevertheless, Martin-Iverson and collegues (**1986**) reported that the lesions do not influence i.v. SA of cocaine. Similar results were obtained by Lecesse & Lyness (**1987**) regarding amphetamine

SA. Similarly contradictory are the results of SA studies where the D1 antagonist SCH23390 was injected in the mPFC. Thus, there was shown to have no effect on intracranial SA of cocaine when co-administered with cocaine (**Goeders et al., 1986**) but it was shown to reduce the rewarding effects of cocaine when it is self administered i.v. (**McGregor & Roberts, 1995**)**.** These differences might be explained in terms of doses used, schedules tested and the diffusion of the substane.

Ablation of DA in the mPFC appears to have profound effects on responses of animals to stressors i.e. they seem to increase the sensitivity of mesoaccumbal DAergic terminals to mild stressors. Thus, lesions induce increase of DA in the NAc which normally is not observed in response to a mild stressor (**Deutch et al., 1990**) or higher increases than sham operated animals (**King et al., 1997**). As in the case of drugs, the DAergic stimulation from stress in the lesioned animals seems to be selective for the NAc as no increases in DA were observed in the dorsal striatum after the stressor (**Deutch et al., 1990; King & Finlay, 1995**). A behavioral correlate for these findings might be the observation of Espejo (**1997**) that 6-OHDA lesions of the mPFC induce anxiogenic behavioral responses in rats under specific testing procedures. As in the case of stress, the lesions have been shown to enhance the mesolimbic DA release after repeated exposure to gustatory stimuli (**Mitchell & Gratton, 1992**).

King et al. (**1997**) have demonstrated that the lesions have differential effect on NAc core & shell DA transmission. Thus, they showed that basal levels and stress induced increases of DA were not observed in the NAc core. On the other hand the lesions increased basal levels and augmented the stress-induced DA levels in the NAc shell. When administered systemically, amphetamine increased DA levels in the NAc shell of lesioned animals as in the case of control rats but, surprisingly, the lesions attenuated the DA increase in the NAc core.

Several studies have shown that 6-OHDA lesions of the mPFC can increase the basal levels of DA and DA metabolites in subcortical areas as NAc and striatum (**Carter & Pycock, 1978; 1980; Pycock et al., 1980; Martin-Iverson et al., 1986; Leccese & Lyness, 1987; Louilot et al., 1989; Kurachi et al., 1995**). Some studies have even shown that there is increased local blood flow in the NAc and in the striatum

after the lesions, an observation which indicates increased metabolic activity in these areas (**Suzuki et al., 1995**). On the other hand there is also a considerable amount of publications where no differences in basal levels of DA and its metabolites in subcortical areas (mainly NAc and striatum) where observed between lesioned and sham operated animals (**Joyce et al., 1983; Oades et al., 1986; Schenk et al., 1991; Jones & Robbins, 1992; Rosin et al., 1992; Bubser, 1994; King & Finlay, 1995; McGregor et al., 1996**). These contradictory findings could be explained in terms of methodological differences, for instance different doses of 6-OHDA and the coordinates used between the different laboratories which might result in different magnitude and extension of the lesion.

In conclusion, all these data, apart from some discrepancies, suggest a strong interaction between cortical and subcortical DA, and especially between mPFC and NAc. Thus, it is observed that increased DAergic flow in one area results in decreased DAergic transmission to the other and vice versa, i.e. the decrease of DA in one area results in increases of DA in the other. These observations led to the suggestion that the mesocortical DAergic system exerts an inhibitory control on the mesolimbic DAergic transmission - DA in the mPFC acts as negative feedback in controlling DA in the NAc (**Tzschentke, 2001**). The mechanism for this relation is not totally clear yet but it is almost sure that involves the glutamatergic projections from the mPFC to the VTA. For instance, in the case of lesion studies, the increases in basal and induced DAergic transmission in the NAc might result due to the disinhibition of the glutamatergic pyramidal projecting neurons of the mPFC after the DA depletion. The increase of glutamate in subcortical areas would result to increase of phasic DA release on subcortical sites through stimulation of DAergic cell bodies (**Grace**, **1991**). This influence of mPFC on VTA DA release could be mediated by direct glutamatergic synapses on DAergic VTA cells (**McCormick et al., 1985; Overton & Clark, 1997**) but also indirectly by prefrontal glutamatergic synapses on non DAergic cells in the VTA and with other regions as the pedunculopontine tegmental nucleus (PPTg) which has direct excitatory synapses with the VTA (**Tzschentke, 2001**). It is also possible that other neurotransmitter systems are involved.

The effect of the disturbances of DAergic transmission (and its subsequent inhibitory influence on subcortical DA) on behavior has been already discussed by others. Thus, it has been speculated that disruption of the DAergic inhibitory control of the mPFC could lead to stress-induced psychotic symptoms in schizophrenic patients (**Weinberger, 1987**), to increased vulnerability to the addictive effects of drugs (**Deminière et al., 1989; Piazza et al., 1991**) or to general alternations of the DAergic responses to "pharmacological or environmental challenges of the system" that lead to potentiation of reinforcer efficacy (**McGregor et al., 1996**).

A.5 Aim of the studies

Previous studies from our laboratory have demonstrated that first exposure to palatable food increases DAergic transmission in NAc shell and core and in the mPFC; second exposure to the same taste stimulus fails to increase DA in the NAc shell – a phenomenon termed one-trial habituation – while it still increases DA in the NAc core and in the mPFC (**Bassareo & Di Chiara, 1997; Bassareo & Di Chiara, 1999; Bassareo et al., 2002**). Thus, it has been proposed that DA in the NAc shell codes for novelty and salience of a stimulus while DA in the NAc core and in the mPFC codes for generic motivational value (**Bassareo et al., 2002**).

While extracellular DA, as measured by positron emission tomography (PET) scan and microdialysis studies has been shown to be increased by drugs of abuse in rats, non human primates and humans, there exists a different pattern of influencing the DAergic release in the terminal regions of mesocortical and mesolimbic systems (**Di Chiara, 2002**). Thus, drugs of abuse, regardless of the contingency to the response to take the drug, preferentially increase DA in the NAc shell as compared to the core, but no one-trial habituation is observed to this increase (**Pontieri et al., 1995, 1996; Pontieri et al., 1996; Cadoni et al., 2005; Lecca et al., 2006a,b, 2007**). It has been suggested that these phenomena reflect abnormal Pavlovian learning regarding the conditioned stimuli paired with the drug intake (**Di Chiara et al., 2004; Di Chiara, 2005; Di Chiara & Bassareo, 2007**).

In a recent study, we (**De Luca et al., 2011a**) observed that sensitization to morphine can alter the DAergic responses to gustatory stimuli (both appetitive and aversive). Thus, increased and delayed DAergic flow in the NAc core of the sensitized animals was elicited after the first exposure to the stimulus while after the second exposure to the same stimulus abolishment of the habituation phenomenon was observed in the NAc shell. The second exposure to the same stimulus also produced a higher increase of DA in NAc core and induced habituation in DA increase in the mPFC of sensitized to morphine animals. These long term changes in DAergic response of different terminal areas were proposed to have implications in motivational processes and probably to result in increased incentive arousal and learning (**De Luca et al., 2011a**). The results of these studies are schematically represented on the table A.1.

As mentioned in the previous sections, DA in cortical and subcortical areas strongly interacts; DAergic transmission in the mPFC exerts an inhibitory tone in the NAc DA release. Studies utilizing 6-OHDA lesions of the mPFC have shown that after the lesions, the animals show sensitized like behaviors in response to chemical and natural stimuli. Since in the literature exists only one study on the DAergic responses of lesioned animals to gustatory stimuli (**Mitchell & Gratton, 1992**) and without the distinction of DA responses in NAc shell and core, we wanted to further investigate these phenomena. More specifically, we wanted to investigate the role of 6-OHDA lesions in the mPFC on the DAergic responses of NAc shell and core after the 1st and 2nd exposure to gustatory stimuli. Furthermore, we utilized behavioral experimental procedures to clarify the effects of the lesions on related aspects of behavior as locomotor activity and hedonic reactions.

Table A.1 Schematic representation of the results of previous studies from the laboratory (**Bassareo et al., 2002; De Luca et al., 2011a**)

Stimulus	NAc shell DA	NAc core DA	mPFC DA
Taste stimulus 1st exposure	↑	↑	↑
Taste stimulus 2nd exposure	habituation	↑	↑
Taste stimulus 1st exposure after morphine sensitization	↑	↑ (higher and delayed)	↑
Taste stimulus 2nd exposure after morphine sensitization	↑	↑ (higher)	habituation

B. Materials & Methods

B.1 Subjects

In all the experiments the subjects were male Sprague-Dawley rats weighing 250-275 g (Charles River, Calco, Italy). They were housed in group of six per cage with standard food (MIL topi e ratti, GLP diets, Stefano Morini, S. Polo D'Enza, RE, Italy) and water ad libitum, for at least one week in the central animal room of the Toxicology Department of the University of Cagliari, under constant temperature (23 C°), humidity (60%) and a 12 h light/dark cycle (light from 8 a.m. to 8 p.m.). All animal experimentation has been conducted in accordance with the statement revised and approved by the Society for Neuroscience in January 1995 and with the guidelines for care and use of experimental animals of the European Economic Commission (EEC Council 86/609; DL: 27.01.1992, N° 116).

B.2 Substances

Six-hydroxydopamine hydrochloride (6-OHDA, Sigma-Aldrich, Milan, Italy) was used to lesion the DAergic terminals in the mPFC. It was dissolved in saline containing 0.2 % ascorbic acid at a concentration of 4mg/ml. A total volume of 1 µl was microinjected in the mPFC for each one of the 4 microinjections at a constant rate of 0.2 µl per minute.

Desipramine hydrochloride (DMI - desmethylimipramine, 25 mg/kg, i.p.; Sigma Chemical Co., St Louis, MO, USA) was administered 30 minutes prior anaesthesia to protect the NAergic terminals from the 6-OHDA toxicity (see below). It was dissolved in saline and injected in a volume of 3ml/kg body weight.

The appetitive gustatory stimulus used in the study was a solution of chocolate syrup (Nesquik Squeeze©, Nestle, S.A., Vevey, Switzerland) and tap water (1:1). The chocolate syrup contained sucrose, water, cacao, corn syrup, citric acid, salt, potassium sorbate and artificial flavor and it was administered intraorally through a

cannula (see below).

B.3 Microdialysis probe and oral catheter construction

Due to the temporal distance between the 6-OHDA lesions and the microdialysis experiments, it was not possible to use probes of acute type because of gliosis formation around the probe (see below, section B.5.1) and the subsequent problems with DA recovery. Thus, after the 6-OHDA lesions, in the same operating session, the animals were also implanted with a guide cannula for microdialysis probes.

The probes utilized were of vertical-concentric type and were constructed as previously described from Lecca et al. (**2006**). The whole construction was based on a Push–Pull connector (Plastics One) where two silica capillary [140 µm outside diameter (OD), 50 µm inside diameter (ID), Polymicrotechnologies Inc., Arizona, USA] were inserted and stabilized into the inlet and the outlet needles. At the tip of the probe the capillaries were surrounded by AN69 membrane (Hospal, Dasco, Italy) stabilized by epoxy glue, with an active dialyzing portion of 1.5 mm (see figure B.1). The membrane was made from polyacrilonitril and met the criterions for use in microdialysis experiments: it was inactive, biocompatible and permitted the recovery of DA and other substances of small molecular weight (**Horn & Engelmann, 2001**). The probes protruded by 4 mm from the guide cannulae.

Figure B.1 Chronic microdialysis probe

The oral catheters were constructed from a 22-gauge stainless steel needle and polyethylene (PE) tubing (Polyethylene tubing, Portex limited, Hythe, Kent, England - ID 0.58 mm and OD 0.96 mm). The 22-gauge stainless steel needle was cut on one side (length 2 cm), was blunted and inserted in the PE tubing which was ending with a perforated circular disk with a hole to permit the flow of the gustatory solution.

B.4 Surgery

Six-hydroxydopamine is a hydroxylated derivative of DA (see figure B.2) and the most commonly used neurotoxin for producing lesions of DAergic neurons. It was isolated for the first time by Senoh & Witkop on 1959 (**1959a,b**) and the first studies about its biological actions were carried out from the group of Porter and Stone (**Porter et al., 1963; Stone et al., 1963**) who showed that 6-OHDA decreases the amount of noradrenaline in the autonomous nerve system in the heart. There is evidence that 6-OHDA exists as an endogenous toxin in the rodent and human brain (**Senoh & Witkop, 1959b; Andrew et al., 1993**).

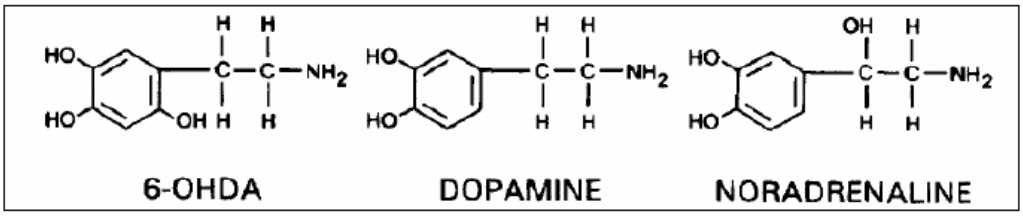


Figure B.2 Chemical structure of six-hydroxydopamine (6-OHDA), dopamine (DA) & noradrenaline (NA)

As can be seen in Figure B.2, the chemical structure of 6-OHDA is very similar to both DA and NA. Because of that, 6-OHDA is transferred by the DA and NA membrane reuptake transporters in the intracellular space of the catecholaminergic

neurons where it accumulates and produces its toxic effects (**Schwarting & Huston, 1996a**).

As it can be understood, a selective blocker for DA or NA transporter (DAT & NAT respectively), such as DMI and benztropine respectively, can be used to target DAergic or NAergic terminals more selectively. When greater toxicity is needed, it can be achieved by the use of a monoaminoxidase (MAO) inhibitor (e.g. pargyline) that prevents the degradation of the toxic metabolites of 6-OHDA by this enzyme.

It has been shown, both in vivo and in vitro, that as soon as 6-OHDA enters the cell, due to its great instability, it gets quickly oxidized to produce a series of substances as free radicals and H_2O_2. These products are very toxic for the cell and can alter its vital functions affecting proteins, membrane lipids and nucleic acids. Subsequently the oxidative stress leads to cell death (**Kumar et al., 1995; Schwarting & Huston, 1996b**). Furthermore, 6-OHDA inhibits the mitochondrial respiratory chain complexes I & IV (**Glinka et al., 1997**).

As in the case of DA, 6-OHDA cannot cross the blood brain barrier (BBB) and the toxin has to be delivered intracranially to act on the brain. The purpose of the present study was to specifically lesion the DAergic terminals in the mPFC region. As mentioned above, in order to spare noradrenergic terminals, the rats were treated with DMI (25 mg/kg, i.p.) 30 minutes prior to anaesthetic injection (chloral hydrate, 250 mg/kg, 3ml/kg in distilled water, i.p., Carlo Erba, Italy).

After anaesthetized, the animals were placed in a stereotaxic apparatus (David Kopf Instruments) and were subjected to two bilateral 6-OHDA (4μg/μl, 6-OHDA hydrochloride, Sigma, in 0.2 % ascorbic acid saline) or vehicle microinjections (0.2 μl/min, 1 μl for each injection) in the mPFC [3.2/2.7 mm anterior from bregma, ±0.6/±0.7 mm from midline and -5.0/-2.5 mm from dura with a flat scull (**Paxinos & Watson, 2007** see figure B.3**)**]. The injector was left in place for additional 5 min to permit the diffusion of the toxin/vehicle.

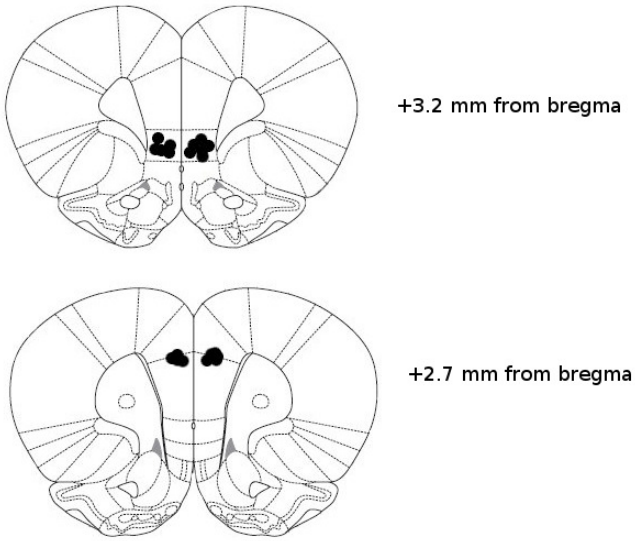


Figure B.3 6-OHDA/vehicle infusion sites (adopted from **Paxinos & Watson, 2007)**

After the toxin/vehicle injections, microdialysis probe guide cannulae (see figure B.4) were implanted above NAc shell (AP: 1.9, ML: 0.9 from bregma and V: -3.8 mm from dura) or core (AP: 1.2, ML: 1.7 from bregma and V: -3.6 from dura) according to the atlas of Paxinos and Watson (**2007**) and secured with glasionomeric cement (CX-Plus, Shofu Inc., Tokyo, Japan). At the end of the surgery session the animals were housed in two and were brought back in the main colony room.

Figure B.4 Guide cannula for microdialysis probes used in the studies

After one week of post-operative recovery, the animals were anesthetized (chloral hydrate, 240 mg/kg, 12ml/kg, i.p., Carlo Erba, Italy) and were implanted with intraoral catheters. The oral catheter was inserted at the level of the first molar and the PE tubing was passed along the skull (see figure B.5) where it was fixed with glasionomeric cement (CX-Plus, Shofu Inc., Tokyo, Japan) according to the method of De Luca et al. (**2011a**). After the operation the animals were put in hemispheric bowls.

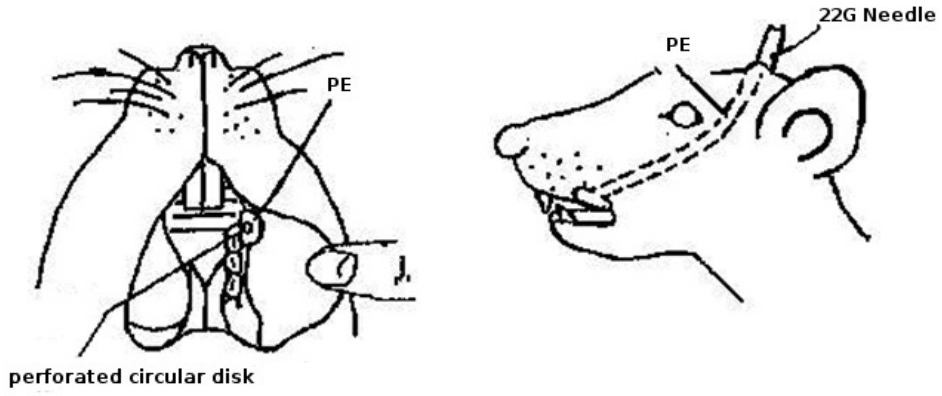


Figure B.5 Schematic representation of the position of implantation of the intraoral catheter in the rat

After recovery from the anesthetic, animals belonging to the pre-exposed group were treated with the appetitive sweet solution (Nesquik Squeeze©-water 1:1, 1ml, 0.2ml/min) and the rats belonging to the naïve group were treated intraorally with tap water.

B.5 Microdialysis Studies

B.5.A Introduction

Microdialysis is used to measure concentration of compounds in the extracellular fluid of living tissue by means of an implanted microdialysis probe. In vivo brain

microdialysis is a method that permits the collections of samples from the extracellular space of any desired brain region while the animal is freely moving. Several experimental treatments (drug administration, environmental and behavioral manipulations) can be utilized to observe variations from the basal levels regarding several neurotransmitters – neuromodulators (**Horn & Engelmann, 2001**).

The method of *in vivo* brain microdialysis is based on the diffusion of substances between the extracellular space in the brain and an artificial cerebrospinal fluid (ACSF – Ringer's solution) through a microdialysis probe equipped with a semipermeable membrane (**Ungersted, 1984**; **Hernandez et al., 1986; Di Chiara, 1990**). The diffusion takes place because of a concentration gradient between the cerebrospinal fluid (CSF) of the brain and an artificial one pumped at very low flow rate through the probe. The probe is implanted in the brain stereotaxically or is inserted through a guide cannula. Conversely, every substance that can pass through the semipermeable membrane can be delivered to the area where the probe is implanted, through the microdialysis probe. This method is called "retrodialysis" (**Horn & Engelmann, 2001**).

The advantages of *in vivo* trancerebral microdialysis include the minimization of tissue damage and the avoidance of mechanical pressure of the brain region under investigation (the major disadvantage while using the older "push pull" method); the collection of relatively pure samples for analysis; the avoidance of disregulation of the electrolytic balance of the brain. In addition, in vivo microdialysis can be used in freely moving animals and so can help to associate alternations in behavior with changes in brain neurochemistry. Finally, the capability to administrate substance locally through the probe (see above – "retrodialysis") can be a very useful tool for studying the effect of several substances on the region surrounding the probe. Retrodialysis avoids the high blood concentrations of the substance and its effects in non desired brain areas (**Di Chiara, 1990; Westerink, 1995; Horn & Engelmann, 2001**).

However, *in vivo* microdialysis is an invasive method in which "the external diameter of the probes is around one thousand times bigger than the synapses where a neurotransmitter is studied", which means that the amount of substance in

a sample does not represent the released substance from the actual locus of release (**Di Chiara, 1990**). Another disadvantage of the method is that it cannot be used consecutively for more than 3-4 days due to glia formation and the subsequent blocking of the pores of the probe's membrane. This limitation can be surpassed by using guide cannulae as in the present study. Finally, another limitation of the method is the poor time resolution, limitation that depends on the analysis equipment available in each laboratory (**Westernick, 1995**).

Despite the fact that new methods for monitoring extracellular DA have been developed (e.g. *in vivo* voltammetry) microdialysis is still a useful tool for monitoring changes in DA levels in a time frame of minutes. So the method monitors the DA levels that correspond to the tonic modality of DA release (see first part, section A.1) where methods as *in vivo* voltammetry monitor the phasic release of DA (**Di Chiara & Bassareo, 2007**).

During microdialysis experiments the samples can be analyzed using a variety of methods. In the present study I used the method proposed by Imperato & Di Chiara (**1984**), i.e. the direct injection of samples in a High Performance Liquid Chromatography (HPLC) apparatus equipped with a reverse phase column and a coulometric detector (ESA, Coulochem II, Bedford, MA) for quantification of neurotransmitters. It has been shown to be a reliable and suitable method for the *in vivo* study of neurotransmitter release and their metabolites (**Imperato & Di Chiara, 1984**).

The HPLC apparatus consists of a mobile (buffer solution) and a solid phase (column). The mobile phase "pushes" the sample to pass through a chromatography column containing particles that interact differently with each substance and this way affecting their retention time, i.e time needed to move out from the column. After retention the substance is quantified. The lab where the experiments took place was equipped with an HPLC adjusted to an electrochemical detector for the quantification of DA. Thus, after separated from other substances, DA was oxidized and then reduced for the production of a chromatographic peak. The unknown peak was compared to a peak of a known size, so the DA in the sample could be quantified.

In particular, during the experiments of the present study, the levels of DA in the NAc shell and core of 6-OHDA and sham operated animals were monitored during basal conditions and after intraoral (i.o.) chocolate infusion. Thus, on the day following of the intraoral catheter implantation, concentric microdialysis probes (**Lecca et al., 2006b** see also above section B.3) were inserted by hand through the guide cannulae, connected to an infusion pump and perfused with Ringer's solution (147 mM NaCl, 4 mM KCl, 2.2 mM $CaCl_2$) at a constant rate of 1 µl/min. Samples were starting being collected approximately 30 min after the connection of the probes to the pumps for the counterbalancing of the flow through the probe. Thereafter, dialysate samples of 10 µl were taken every 10 min and immediately injected into a high-performance liquid chromatography equipped with a reverse phase column (LC-18 DB, 15 cm, 5 µm particle size, Supelco) and a coulometric detector (ESA, Coulochem II, Bedford, MA) to quantify DA. The first electrode of the detector was set at + 125 mV (oxidation) and the second at - 175 mV (reduction). The composition of the mobile phase was: 50 mM NaH_2PO_4, 0.1 mM Na_2-EDTA, 0.5 mM n-octyl sodium sulfate, 15% (v/v) methanol, pH 5.5 and was pumped by a Jasco pump (Cremella, LC, Italy). The sensitivity of the assay for DA was 5 fmol/sample.

When DA in three consecutive samples didn't differ more than 10 %, the values were considered as the basal levels of DA and the animal was treated with a sweet solution (Nesquik Squeeze©-water 1:1, 1ml, 0.2ml/min). Dopamine levels were being monitored for two more hours after the treatment.

B.5.B Histology

At the end of the microdialysis experiment, a group of rats was sacrificed by a lethal dose of saturated chloral hydrate solution (3ml/kg, i.p.). The guide cannulae were removed and the brains were extracted and placed on a frozen saline surface. The hemisphere in which the guide cannula was implanted was dissected and was later cut by a vibratome (Vibratome, Campden Instruments, 2Biological Instruments, Besozzo, Italy) in serial coronal slices (100 µm thick) at the level of NAc, oriented

according to the atlas of Paxinos & Watson (**2007**). The sections were put in well plates, examined under a microscope and the location of the probes was reconstructed and referred to the atlas of Paxinos & Watson (**2007**) (see figure B.6). The brain hemisphere without the probe and the mPFC specifically, was processed for the quantification of neurotransmitters in the tissue in order to evaluate the magnitude of 6-OHDA lesion (see below).

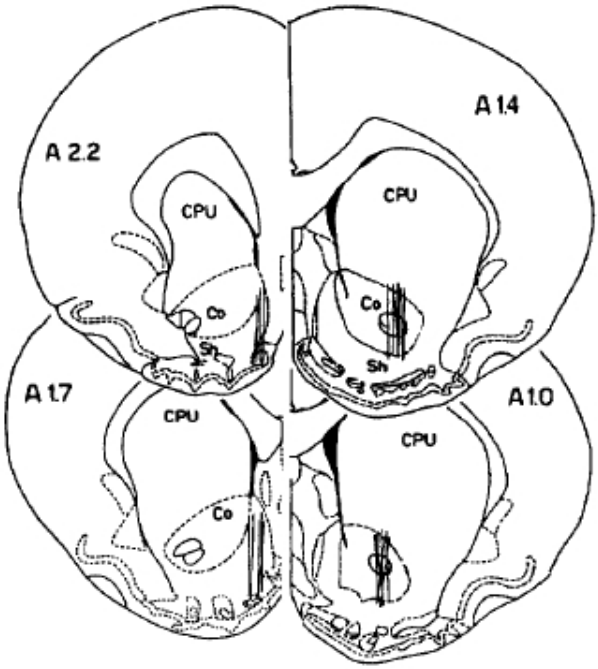


Figure B.6 Localization of dialysis probes (dialysis portion) within the NAc with shell and core compartments (A - anterior coordinate; CPU - caudate putamen; Co - NAc core; Sh - NAc shell Reconstructed from Paxinos & Watson, 2007)

Another group of the subjects was deeply anaesthetized with chloral hydrate (saturated solution) and transcardially perfused with 50ml of saline, followed by 100ml of paraformaldehyde (PFA) solution (4 % in Phospate Buffer Solution - PBS).

The aim of the perfusion procedure is to remove the blood from the brain and to fix the tissue. Briefly, after the animal is anaesthetized, the thorax is withdrawn and the stern is cut in a manner that the beating heart of the animal is exposed. A small dissection is made in the right atrium of the heart and from the left ventricle is administered firstly the saline and then the PFA solution. The heart works as a

"pump" and provides to the systemic circulation the saline and the PFA solution to remove all the blood from the body. After the procedure, the brains of the subjects were removed and the hemisphere implanted with guide cannula was stored and processed for histological examination as described above. The rest of the brain was postfixed (in 4 % PFA in PBS) for the immunohistochemical studies (see below).

Only animals with correct implantation of the probe were included in the statistical analysis.

B.6 Behavioral Studies

B.6.1 Taste Reactivity Test

Taste reactivity test has been utilized as an operational estimate of hedonic valence (positive or negative) and hedonic impact of tastes (**Grill & Norgren, 1978; Berridge, 2000**). This behavioral test was introduced by Grill and Norgren on 1978. They showed that passive deliverance of solutions in the mouth of rats produces particular behavioral reactions to each one solution, reproducible between subjects. Thus, palatable solutions (e.g. sucrose solutions) elicit a series of stereotype hedonic reactions to rats: lateral and rhythmic tongue protrusions, paw licks, face washing and increases in locomotion (see figure B.7). Bitter tastes, as quinine solution, elicit typical aversive reactions: gapes, chin rubs, face washing, flailing of the forelimbs and paw tread (see figure B.8). After neutral solutions, neutral reactions were observed: rhythmic mouth movements and passive drip of the solution (**Grill & Norgren, 1978; Berridge, 2000; Bassareo et al., 2002; De Luca et al., 2011a,b**).

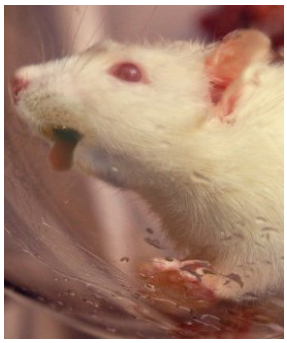
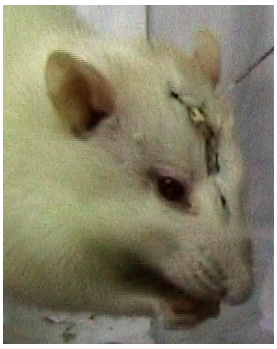
A)  B) 

Figure B.7 Hedonic Reactions to appetitive stimuli: A) tongue protrusion, B) paw licks (unpublished photographs from the archives of the laboratory of the Department of Toxicology of the University of Cagliari)

A) B)

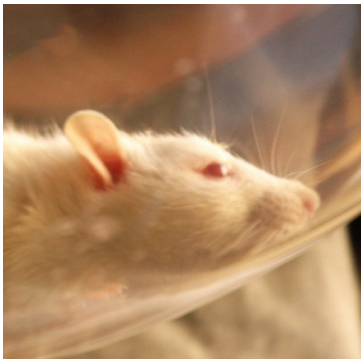
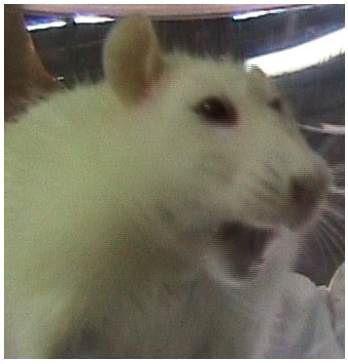
 

Figure B.8 Typical aversive reactions: A) chin rub, B) gape (unpublished photographs from the archives of the laboratory of the Department of Toxicology of the University of Cagliari)

Interestingly, it has been shown that these typical taste reactions to palatable and aversive stimuli can be observed in other species as well; they have been shown to be homologous between rats, human and other primate infants (**Steiner et al., 2001**). Thus, it is suggested that the taste reactivity test can be utilized for the direct assessment of the hedonic value of gustatory stimuli and to dissociate “liking” and “wanting” for a gustatory stimulus which are distinct behavioral characteristics that

their nature generated excessive debate in the past, regarding their recording and measurement (**Berridge, 1996; Berridge, 2000**).

In the present study, behavior was recorded during the infusion of solutions through the oral catheter at the same session when the microdialysis study was taking place. The oral catheter was connected to an infusion pump and a solution (chocolate, quinine or tap water) was pumped at a constant rate of 0.2ml/min, for a total amount of 1ml. During the taste reactivity test the animals were videotaped and the positive hedonic (ingestive) taste reactivity patterns were scored. Positive hedonic reactions consisted of lateral and rhythmic tongue protrusions, paw licks, face washing and locomotion (**Grill & Norgren, 1978; Berridge & Robinson, 1998**). Each lateral and rhythmic tongue protrusion was counted as individual event and each event was scored with one point. The rest of the events were scored with one point if the duration of the event was between 1 and 5 seconds and with two points if the duration of the event was more than 5 seconds.

B.6.2 Estimation of Motor Activity

Locomotor activity is associated with a great number of other types of behavior and because of that it is of high importance for the organism. Studying locomotor activity in intact subjects can provide substantial information about the neurobiological substrate of this behavior, as long as for the reasons of causing motor deficits. In rodents, measures of locomotor activity can provide important information on potential mechanisms of drug action. The functional outcome of this behavior can be influenced by a variety of experimental manipulations with models of CNS injury or disease included. The study of locomotor activity can also provide information about learning and memory processes but also about anxiety (**Decker, 2001**).

In the present study, the subjects that participated in the studies of motor activity were subjected to 6-OHDA lesions or sham operations as described above (see section B.4). As in the case of microdialysis studies, one week after the lesions the

animals were anesthetized again, were implanted with intraoral catheters and, after recovery from the anesthetic, the rats that belonged to the pre-exposed group were treated with the appetitive sweet solution (Nesquik Squeeze©-water 1:1, 1ml, 0.2ml/min) while the ones that belonged to the naive group were treated intraorally with tap water.

The estimation of motor activity was taking place the next day from the intraoral cannula implantation in a quiet, isolated room. The rats were place in the room 30 minutes before the experiment in order to get familiar with the novel environment. After this period they were put individually in the motility chambers.

The measurement of the motor activity was recorded automatically in cages equipped with two pairs of infrared photocell emitters and detectors situated along the long axis of each cage (Opto-Varimex; Columbus Instruments, Columbus, Ohio, USA). Interruption of a photocell beam was detected by a counter that recorded the total number of photocell beam interruptions. The counter recorded two types of motor activity: locomotor (ambulatory) activity, due to the locomotion of the rat along the long axis of the cage, and total motor activity, which represented locomotor activity plus non-finalized movements. The counter recognized the stereotyped movements because of the continuous interruption of the same photocell beam, whereas locomotor activity along the cage produced interruption of different photocell beams.

For half an hour (habituation period) the motor activity was recorder and these values were considered to represent the basalmotor activity. After that period of time the rats were treated with the chocolate solution or tap water (1ml/5min, i.o.) and their locomotor and total motor behavior was recorded for 30 more minutes.

B.7 Lesion Evaluation

B.7.1 Concentration of neurotransmitters in the tissue – Introduction

Regional tissue levels of neurotransmitters were measured in order to evaluate the magnitude of lesion and the impact of 6-OHDA injections on other neurotransmitter systems. The method described below is used to quantify neurotransmitters located in the intracellular space.

After the end of the experiments, a group of rats was sacrificed by a lethal dose of saturated chloral hydrate solution (3ml/kg, i.p.) as described above. From the rats participated in the microdialysis studies the guide cannulae were removed and the brains were extracted and placed on a frozen saline surface. The hemisphere in which the guide cannula was implanted was dissected and subjected to histological examination as described in the section B.5.B.

From the hemisphere without probe implanted and from the brains of a group of rats participated in the motor activity studies, the olfactory tubercules were removed and a coronal cut was made at 4.20 mm AP from bregma, using the "*Rat Brain Atlas*" as a reference (**Paxinos & Watson, 2007**). An additional coronal cut was made at 3.00 mm AP from bregma. From the latter remaining slice the mPFC was isolated, i.e. the IL, PL and Cg1 cortices (see part A, section A.3.1) and stored in 1ml vials.

The samples were sonicated in 250 μl of 0.2 M perchloric acid for 15 seconds in order to be homogenized. Then, they were centrifugated at 11000 spins per min for 15 min at 4° C and the supernatant was filtered (0.6 μm). After filtration, 20 μl samples were injected into an HPLC apparatus equipped with a reversed-phase column (C8, 3.5 μm particle sizes, Waters, Milford, MA, USA) and a coulomentric detector (Coulochem II, ESA Inc., Bedford, MA, USA) to quantify simultaneously NA, DA and 5-HT. The first electrode was set at -90 mV and the second electrode at +280 mV. The composition of the mobile phase was 75 mM NaH_2PO_4, 20 mM EDTA, 1 mM sodiumdecanesulphonate, 0.01% triethylamine and 15% methanol (pH 5.70) and was pumped through the column with a Jasco pump (Cremella, LC, Italy).

B.7.2 Immunohistochemistry Studies – Introduction

As described above, a group of the rats participated in the microdialysis experiments and another from those participated in the motor activity studies was deeply anesthetized with chloral hydrate (240 mg/kg, i.p.) and transcardially perfused with 50ml of saline, followed by 100ml of 4% paraformaldehyde solution. The brains were then removed and the hemisphere with the guide cannula from the rats participated in the microdialysis experiments was dissected and stored for histological examination (see above, section B.5.B). The other hemisphere, or the entire brain in the case of the rats participated in the motor activity studies, was postfixed for 2 days in PFA solution (4 % in PBS).

After 2 days, the brains were cut in 40-μm-thick serial coronal slices on a vibratome (Technical Products International, St Louis, MO, USA) which then were processed for tyrosine hydroxylase (TH) immunohistohemistry.

Immunohistochemistry is a technique that combines methods from immunology, histology and biochemistry and is used for the detection and study of distribution of several proteins in cells or tissues. It is based on the principal of the specific binding between antibody and antigen. Experiments using immunohistochemistry were described for the first time in 1940's but from that time the method has been modified to optimize efficiency regarding the protein binding and tissue fixation and the methods for tracing and counting the signal. Today, the most common practice is to use antibodies that are conjugated to an enzyme or to a fluorophore. In the first case, which was also used in the present experiments, after the antigen-antibody binding, the enzyme reacts with a specific substrate to produce a color that can be observed under an ordinary light microscope. In our case that enzyme was horseradish peroxidase (HRP) (**Bolak & Van Noorden, 2003**). The chromogenic substrate for HRP was 3,3'-diaminobenzidine (DAB) which produced a light brown coloration of the TH-positive fibers.

Signal amplification is of crucial importance for the analysis of the images. In the present study, indirect immunohistochemistry method was used, which means that a specific antibody binds to the target protein, which in turn binds to several

secondary antibodies conjugated to HRP to produce coloration. For further signal amplification can be used several other methods as the so called biotin-avidin complex (ABC), which was also used in the present study. According to this method, the secondary antibody is biotinylated and incubated to labeled (with the enzyme – in our case HRP) avidin-biotin reagent, in order to form large complexes with great numbers of the enzyme. As a result, the enzyme in presence of DAB produce higher amount of coloration visible to be analyzed (see figure B.9) (**Ramos-Vara, 2005**).

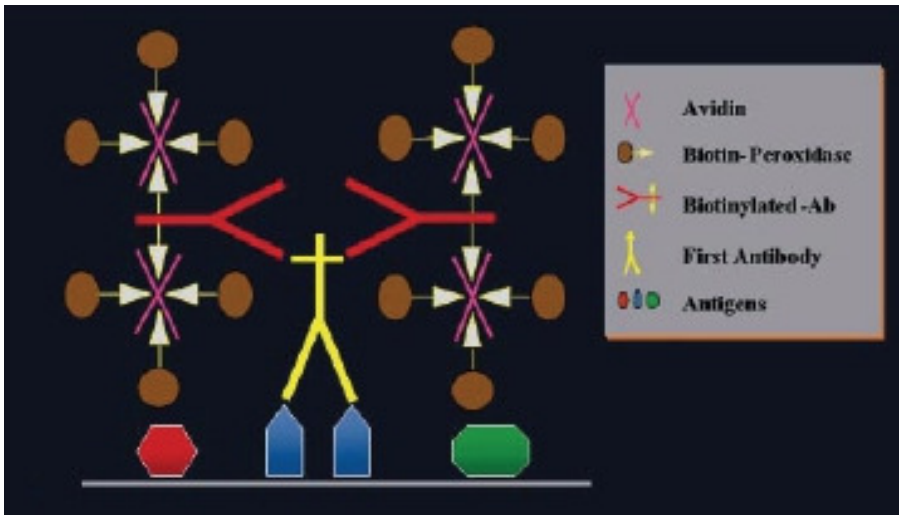


Figure B.9 Schematical representation of the immunohistochemical reaction (from Ramos-Vara, 2005)

In the present study, free floating immunohistochemistry was used with a monoclonal antibody specific for tyrosin hydroxylase (TH) for visualizing the DAergic terminals/fibers in the mPFC of lesioned and sham operated animals.

The 40-µm-thick serial sections of the mPFC (see above) were put in well plates and then rinsed for 10 min in

1. tris-buffered saline 50 mM (TBS) and Triton X-100 0.1%

2. TBS and Triton X-100 0.1%, containing H2O2 (0.85% p/v)

3. TBS and Triton X-100 0.1% and

4. 5% normal donkey serum (NDS) (Jackson Immunoresearch, EU, UK) in TBS and Triton X-100 0.1%.

Triton X-100 is a deterget which is used to reduce surface tension and subsequently to allow the use of less reagent to achieve better coverage of the sample. The samples were incubated with normal donkey serum in order to block the reactive sites to which the primary or secondary antibodies may otherwise bind to and so to reduce background staining.

After the procedure described above, the sections were incubated for 40 h with a mouse monoclonal primary antibody against TH (T1299, Sigma) at a dilution of 1:1000 in TBS. The reaction was amplified using biotinylated secondary antisera (Jackson Immunoresearch, EU, UK) and visualized by standard avidin–biotin–HRP (Vector Laboratories). Staining was obtained by the oxidation of peroxidases induced by H2O2 in the presence of DAB (Sigma). After 3 rinses of 10 min each in TBS, the sections were mounted on super frost plus glass slides (Thermo Scientific).

After drying and dehydrating with ascending series of alcohols, sections were mounted with Eukitt (Fluka) and examined through a Zeiss AxioScope.A1.

C. Results

C.1 Microdialysis Studies

Basal values

Basal values of DA, expressed as fmoles/10-µl sample (mean±SEM), were: NAc shell of sham 38±3 (n=14), NAc shell of lesioned 43±5 (n=14), NAc core of sham 48±6 (n=11), NAc core of lesioned animals 47±5 (n=14).

Effect of mPFC 6-OHDA lesion on the NAc shell DA responsiveness to chocolate in naive or pre-exposed rats

Panel A of figure C.1 shows that chocolate (1ml/5min, i.o.) increased NAc shell extracellular DA both in sham and in 6-OHDA lesioned naive animals. Two-way ANOVA revealed a significant effect of time [$F_{(12,180)}$=6.33; p<0.001]. Tukey's post hoc test revealed a statistically significant increase in DA with respect to basal values 10 minutes after chocolate in the lesioned group; no statistical significant differences between the two groups were observed.

Panel B of figure C.1 shows the effect of i.o. infusion of chocolate on NAc shell DA of sham and 6-OHDA lesioned in the mPFC animals, pre-exposed to chocolate. Two-way ANOVA showed a significant effect of group [$F_{(1,13)}$=6.42; p<0.03] and time [$F_{(12,156)}$=2.05; p<0.03]. Tukey's post hoc test revealed a significant increase of DA with respect to basal values 10 minutes after chocolate in the lesioned group and a statistically significant difference between the lesioned and sham groups in the same period of time.

Two-way ANOVA between sham naive and sham pre-exposed groups showed a significant effect of group [$F_{(1,13)}$=9.74; p<0.01], time [$F_{(12,156)}$=4.22; p<0.001] and group x time interaction [$F_{(12,156)}$=2.65; p<0.01]. Tukey's post hoc test revealed significant differences in DA levels between the groups at 10 and 20 min after chocolate treatment. In the lesioned animals, two-way ANOVA between naive and

pre-exposed animals revealed a significant effect of time [$F_{(12,180)}$=4.07; p<0.001] but no differences between the groups.

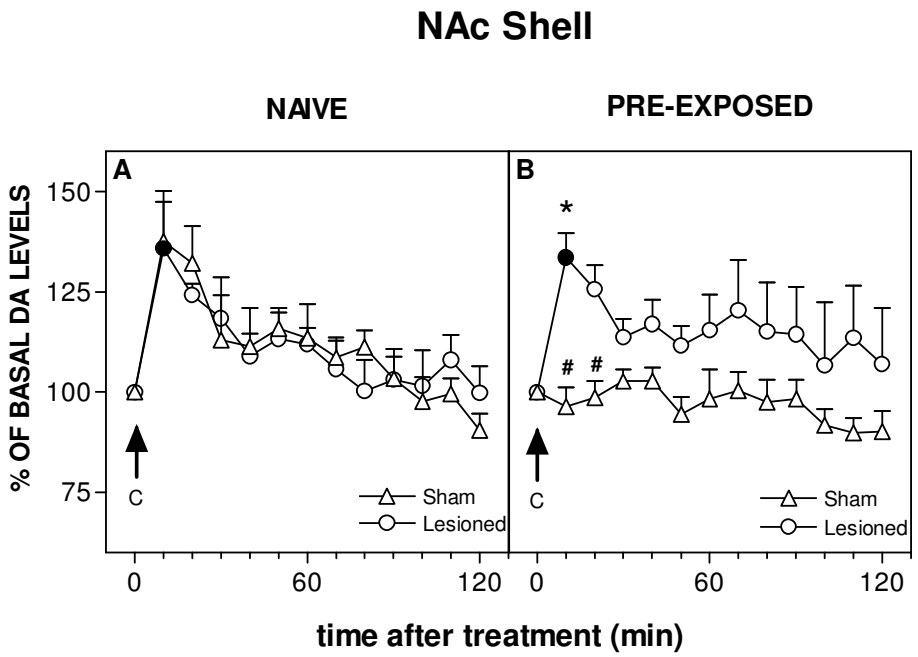


Figure C.1 Effect of i.o. chocolate on DA transmission in NAc shell in sham (*circles*) and 6-OHDA lesioned (*triangles*) in naive (*panel A*) and 24-h taste pre-exposed animals (*panel B*). Results are expressed as mean±SEM of change in DA extracellular levels expressed as percentage of basal values. The arrow indicates the start of chocolate infusion (*C*, 1ml/5 min). Solid symbols: p<0.05 vs. the respective basal values (two-way ANOVA); *p<0.05 vs. sham pre-exposed group; #p<0.05 vs. sham naive group (two-way ANOVA) (sham naive n=6, lesioned naive n=11, sham pre-exposed n=9, lesioned pre-exposed n=6)

Effect of mPFC 6-OHDA lesions on the NAc core DA responsiveness to chocolate in naive or pre-exposed rats

Panel A of figure C.2 shows that chocolate (1ml/5min, i.o.) increased NAc core extracellular DA both in sham and in 6-OHDA lesioned naive animals. Two-way ANOVA revealed a significant effect of group [$F_{(1,12)}$=8.66; p<0.02], time

[$F_{(12,144)}$=4.33; p<0.001] and group x time interaction [$F_{(12,144)}$=3.58; p<0.001]. Tukey's post hoc test revealed a statistically significant increase in DA with respect to basal values after chocolate in the lesioned group and statistically significant differences in the DA levels between the groups.

Panel B of figure C.2 shows the effect of i.o. infusion of chocolate on NAc core DA of sham and 6-OHDA lesioned in the mPFC animals pre-exposed to chocolate. Two-way ANOVA showed a significant effect of time [$F_{(12,192)}$=5.75; p<0.001]. Tukey's post hoc test didn't reveal any statistically significant differences between the groups.

Two-way ANOVA between sham naive and sham pre-exposed groups showed a significant effect of time [$F_{(12,156)}$=3.32; p<0.001] but no statistically significant differences between the groups. In the group of lesioned animals, two-way ANOVA between naive and pre-exposed rats revealed a significant effect of group [$F_{(1,15)}$=11,41; p<0.005], time [$F_{(12,180)}$=5.71; p<0.001] and group x time interaction [$F_{(12,180)}$=4.96; p<0.001]. Tukey's post hoc test revealed statistically significant differences between the groups.

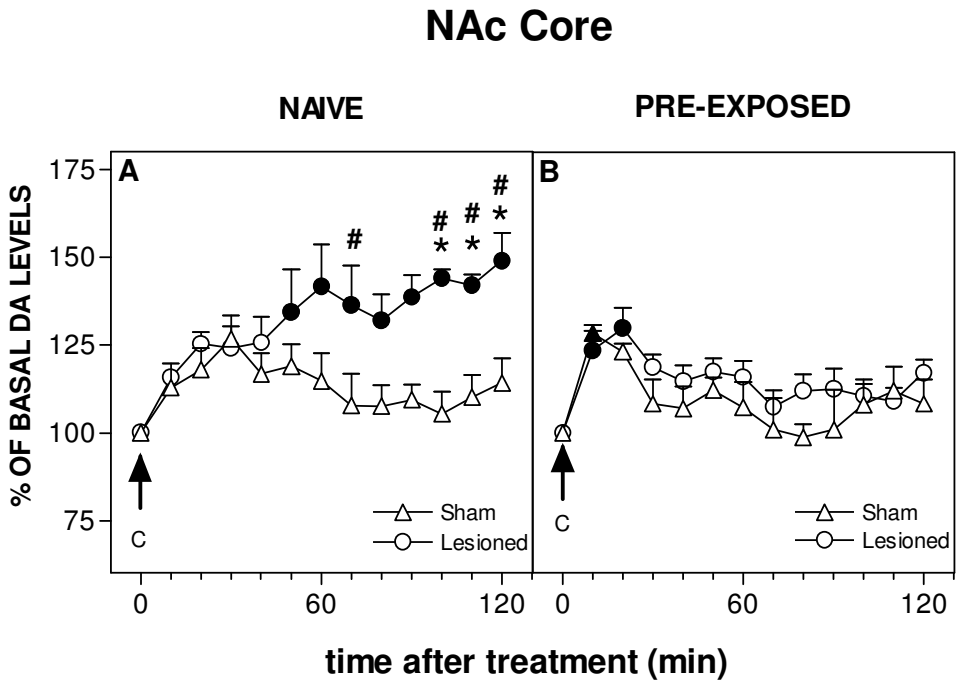


Figure C.2 Effect of i.o. chocolate on DA transmission in NAc core in sham (*circles*) and 6-OHDA lesioned (*triangles*) in naive (*panel A*) and 24-h taste pre-exposed animals (*panel B*). Results are expressed as mean±SEM of change in DA extracellular levels expressed as the percentage of basal values. The arrow indicates the start of chocolate infusion (*C*, 1ml/5 min). Solid symbols: $p<0.05$ vs. the respective basal values (two-way ANOVA); *$p<0.05$ vs. sham naive group; #$p<0.05$ vs. sham naive group (two way ANOVA); (sham naive n=8, lesioned naive n=6, sham pre-exposed n=7, lesioned pre-exposed n=11)

C.2 Behavioral Studies

C.2.1 Taste Reactivity Test

Effect of 6-OHDA lesions in mPFC on taste reactivity to chocolate in naive and taste pre-exposed rats

Behavioral taste reactions to chocolate in naive sham, naive lesioned, pre-exposed sham and pre-exposed lesioned rats during the microdialysis experiment

are shown in figure C.3. Unpaired *t*-test did not show any statistical significant differences between sham naive and sham pre-exposed, lesioned naive and lesioned pre-exposed, sham naive and lesioned naive and between sham pre-exposed and lesioned pre-exposed animals.

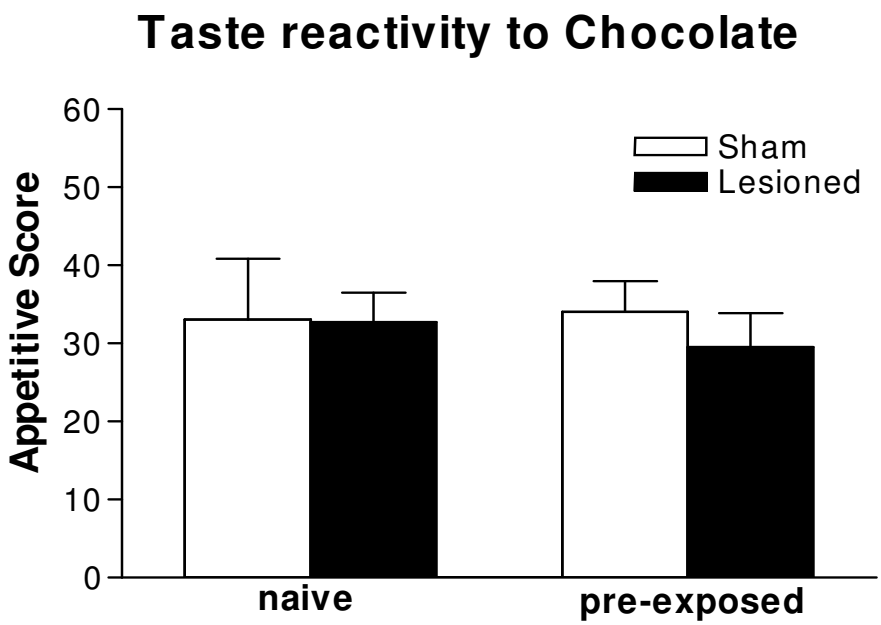


Figure C.3 Behavioral appetitive score to chocolate in sham (clear bars) or 6-OHDA lesioned in the mPFC rats (black bars), naive or 24-h pre-exposed to taste. Results are expressed as mean±SEM of behavioral score; (sham naive n=22, sham pre-exposed n=18, lesioned naive n=27, lesioned pre-exposed n=16)

C.2.2 Estimation of Motor activity

Effect of 6-OHDA lesions in the mPFC on basal and chocolate-induced locomotor and total motor activity

Cortical DA depletion did not produce differences during the habituation period in total motor (panel A) and locomotor (panel B) activity (unpaired t-test) as shown in figure C.4.

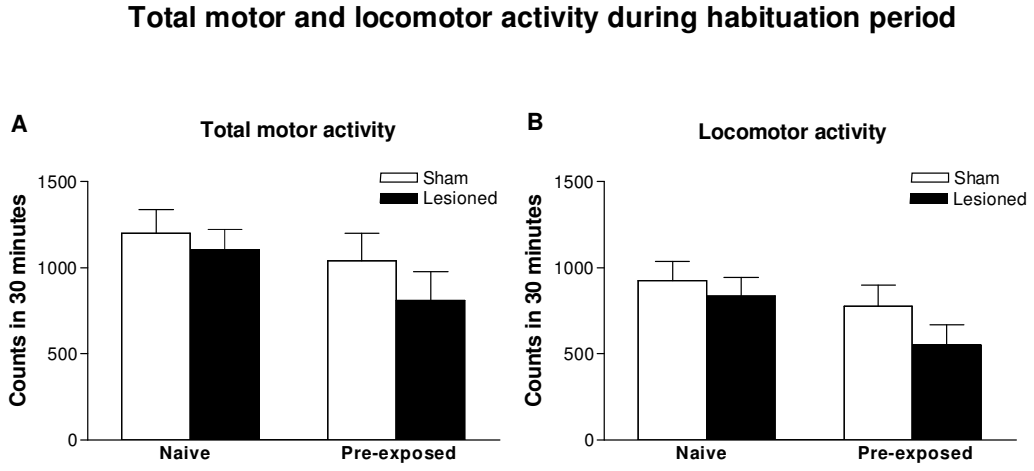


Figure C.4 Total motor (panel A) and locomotor activity (panel B) during the 30-minutes habituation period of sham (clear bars) and lesioned (filled bars) animals, naive (left) and pre-exposed (right) to chocolate. Results are expressed as mean±SEM photocell counts in 30 minutes (sham naïve: n=10; lesioned naïve: n=8; sham pre-exposed: n=7, lesioned pre-exposed: n=5).

In figure C.5 is represented the time course of total motor (panel A) and locomotive activity (panel B) after chocolate in sham naive, sham pre-exposed, lesioned naive and lesioned pre-exposed animals. Two-way ANOVA revealed a significant effect of time [$F_{(6,156)}$=7.71; $p<0.001$ for total motor and $F_{(6,156)}$=5.42; $p<0.001$ for locomotor activity] but no differences between the groups were revealed. Figure C.6 represents the overall total motor (panel A) and locomotor activity (panel B) during the 30 minute observation period after the chocolate

treatment. Unpaired t-test didn't reveal any statistical differences between the groups.

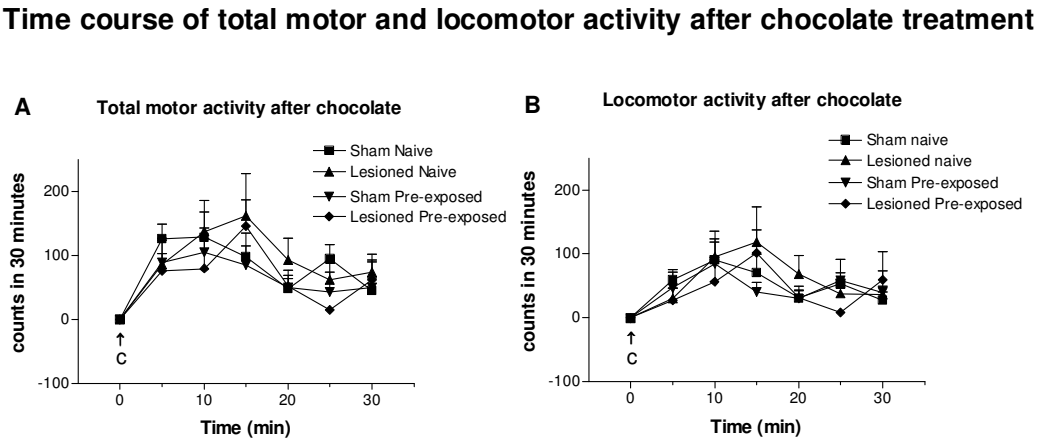


Figure C.5 Time course of total motor (panel A) and locomotor activity (panel B) during the 30-minutes observation period after chocolate treatment (1ml/5min, i.o.) of sham naive (squares, n=10), lesioned naive (triangles, n=8), sham pre-exposed (inverted triangles, n=7) and lesioned pre-exposed animals (rhombs, n=5) in 5 minute intervals. Results are expressed as mean±SEM photocell counts in 30 minutes after chocolate treatment. The arrow indicates the start of chocolate infusion (*C*, 1ml/5 min).

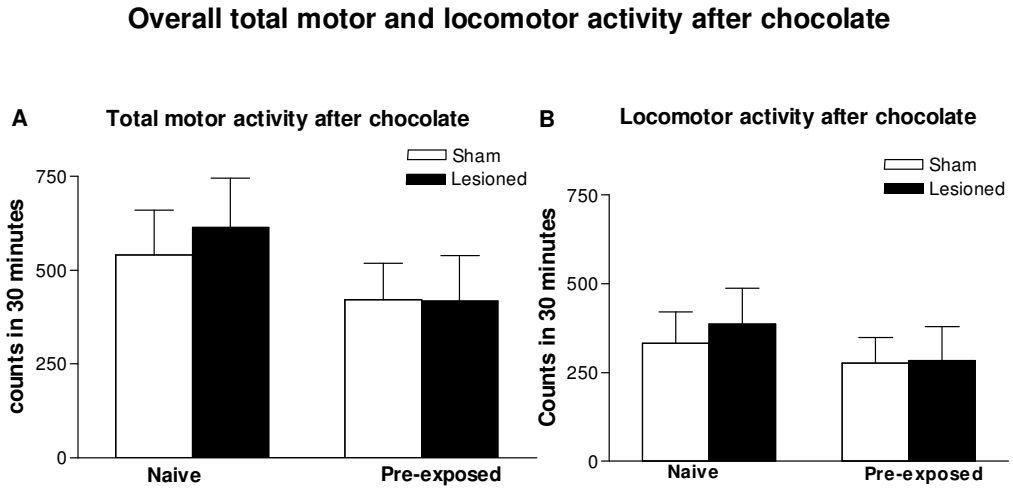


Figure C.6 Overall total motor (panel A) and locomotor activity (panel B) after chocolate treatment during the 30-minutes observation period of sham (clear bars) and lesioned (filled bars) animals, naive (left) and pre-exposed (right) to chocolate. Results are expressed as mean±SEM photocell counts in 30 minutes (sham naïve: n=10; lesioned naïve: n=8; sham pre-exposed: n=7, lesioned pre-exposed: n=5).

C.3 Lesion Evaluation

C.3.1 Concentration of neurotransmitters in the mPFC

Table C.1 shows the NA, DA and 5-HT tissue levels in the mPFC in sham and 6-OHDA lesioned rats and the subsequent percentage of depletion. Six-hydroxydopamine lesions produced a significant decrease in DA concentration (68% of depletion, unpaired *t*-test) but didn't produce a statistically significant effect on NA and 5-HT levels (14 and 10% depletion respectively) due to the protective effect of DMI pretreatment.

Table C.1 Tissue levels (pg/mg wet tissue weight) and percentage of depletion of noradrenaline, dopamine and serotonin in the mPFC of 6-OHDA and sham operated rats.

Group	**Noradrenaline**	**Dopamine**	**Serotonin**
Sham	132,8 ± 12,71 (N=18)	52,88 ± 7,88 (N=18)	193,6 ± 20,05 (N=18)
6-OHDA	113,9 ± 11,24 (N=20)	17,13 ± 4,32 (N=20)	175,2 ± 17,93 (N=20)
% depletion	14%	**68%***	10%

Means±SEM; * p<0.001 compared to sham operated animals (unpaired *t*-test)

C.3.2 Immunohistochemistry Studies – Images

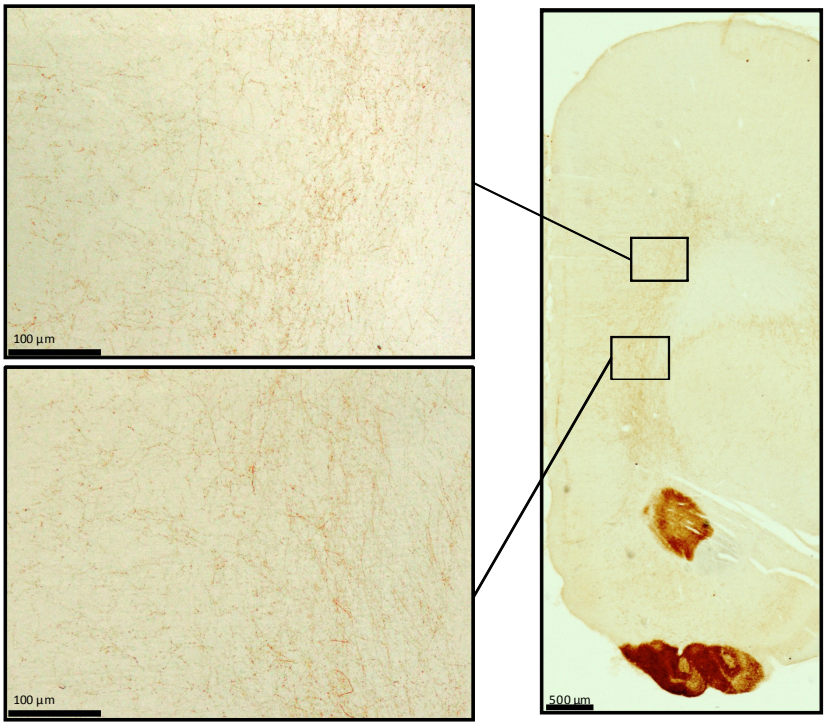


Image C.1 TH reactivity in infralimbic (lower rectangle) and prelimbic (upper rectangle) of a sham operated rat

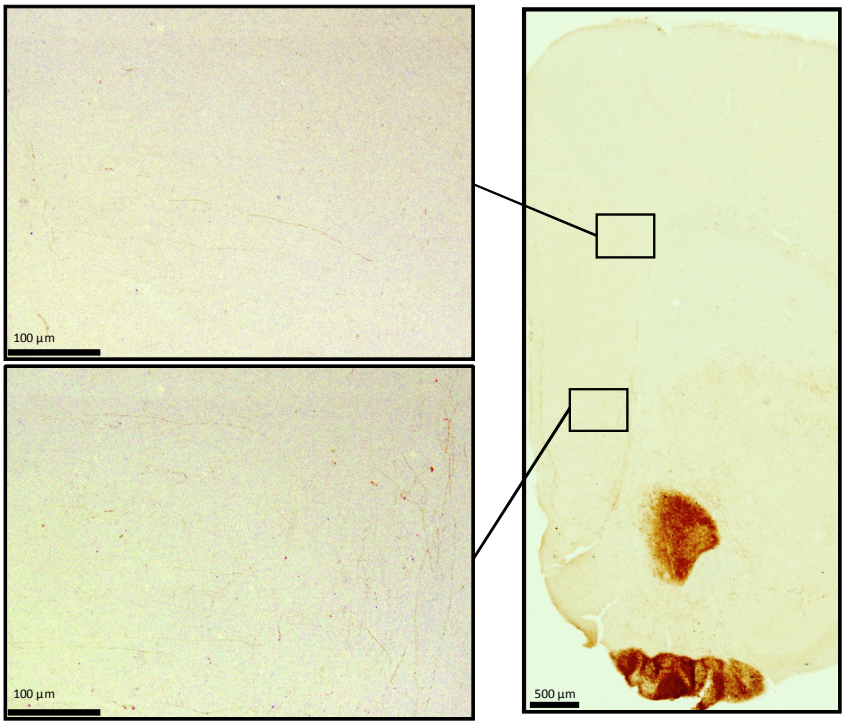


Image C.2 TH reactivity in infralimbic (lower rectangle) and prelimbic (upper rectangle) of a 6-OHDA lesioned rat

D. Discussion

The main findings of the present study are that 6-OHDA lesions of the mPFC alter NAc DA responsiveness to gustatory stimuli. While in the NAc shell of naive subjects the lesion did not produce any effect, in the NAc core resulted in a higher and delayed increase of DA in response to chocolate. On the contrary, in chocolate-pre-exposed subjects the lesion did not have any effect on NAc core DA responsiveness to chocolate but it abolished one-trial habituation of NAc shell DA response to taste. Therefore, the first exposure to chocolate induced a higher increase of DA in the core and a normal increase in the shell; the second exposure abolished the habituation of NAc shell DA and prevented the increase of DA in the NAc core observed after the first exposure to the taste stimulus. It appears as if the lesion abolished habituation in the shell while induced it in the core.

The studies described in the present thesis used a protocol of 6-OHDA lesion of the mPFC as reported by Beyer and Stekette (**1999**). Quantification of the mPFC tissue content of NA, DA and 5-HT showed that despite the fact that there was a reduction of all neurotransmitter levels in the lesioned group with respect to the sham operated animals (14, 68 and 10% for NA, DA and 5-HT, respectively) this reduction was statistically significant only in the case of DA. That indicates that treatment with DMI, which blocks the NAT, successfully protected NAergic terminals from 6-OHDA toxicity. Unlike Beyer & Stekette (**1999**) we did not observe a statistically significant reduction in 5-HT levels. The fact that there was a high reduction in DA levels without significant lesions of NA and 5-HT indicated that the results obtained are due to the disruption of the DAergic transmission in the mPFC.

Furthermore, the images from the immunohistochemistry studies (images C.1 and C.2) and the fact that no differences were observed between lesioned and sham operated animals on the basal DA levels in the microdialysis experiments indicates that the lesion was restricted to the mPFC and didn't affect subcortical DA, particularly in the NAc. This was expected because the coordinates used provide a sufficient distance from the NAc (**Beyer & Stekette, 1999**). Previous studies in rats and monkeys report increased in DA levels and DA turnover in subcortical areas as

NAc and striatum (**Carter & Pycock, 1978, 1980; Pycock et al., 1980; Martin-Iverson et al., 1986; Lecesse & Lyness, 1987; Haroutunian et al., 1988; Kolachana et al., 1995; Kurachi et al., 1995; Roberts et al., 1994; Sokolowski & Salamone, 1994**) or no effect (**Joyce et al., 1983; Oades et al., 1986; Schenk et al., 1991; Jones & Robbins, 1992; Rosin et al., 1992; Bubser, 1994; McGregor et al., 1996**). In our case, as already mentioned, no differences were observed in the basal levels of DA in the NAc shell and core between the sham and 6-OHDA lesioned animals. In agreement with this, no differences were observed in TH density between the groups (images C.1 and C.2). These discrepancies can be explained by experimental differences such as the dose of 6-OHDA utilized, the number of injections within the mPFC, the speed of infusion of the toxin and the coordinates.

As in the case of basal DA levels, the data in the literature regarding basal levels of motor activity are inconsistent. Thus, there have been reported increases (**Carter & Pycock, 1980; Espejo & Miñano, 2001**), no effects (**Oades et al., 1986; Beyer & Steketee, 1999; Wanchoo et al., 2010**) or even decreases (**Espejo, 1997**) in basal locomotor activity after 6-OHDA lesions in the mPFC. In our case, we did not observe any difference in total motor or locomotor activity between sham and lesioned animals during the 30 min habituation period. These levels of activity reflect the basal motor activity levels. In addition we did not observe any differences in motor activity after exposure to chocolate. Despite the fact that there was a tendency for total motor and locomotor activity augmentations in different time frames between sham and lesioned groups after chocolate treatment, the statistical analysis did not reveal any significant effects. These findings are in contrast with pharmacological studies that report increased levels of locomotion in the lesioned animals (**Beyer & Steketee, 1999; Wanchoo et al., 2010**). This could be explained by the fact that drugs of abuse and natural reinforcing stimuli as food, despite the fact that share some common neural substrates, they also have major differences in terms of intensity and duration of the effects but also regarding the locus of action with the neural substrates of basal activity.

Regarding the taste reactivity test results, the findings of this study are in line with previous ones from our laboratory (**De Luca et al., 2011a; De Luca et al., 2011b**)

but also with the general notion that pleasure, sensory hedonia or "liking" is independent of DAergic transmission (**Berridge & Robinson, 1998; Bassareo et al., 2002; Di Chiara & Bassareo, 2007**). Thus, hedonic reactions were increased in all groups to the same extent, independently of the lesion, pre-exposure or increases of DA in NAc shell or core.

Previous studies from this laboratory (**Bassareo et al., 2002**) have shown that adaptive mechanisms (habituation) in NAc shell DA response to highly salient, unfamiliar taste stimuli take place in a single-trial mode to both palatable and aversive tastes. Regarding the mPFC and the NAc core no single trial habituation is observed. Recently, we observed that sensitization to morphine can alter the response of different terminal areas of the mesocorticolimbic DA system (**De Luca et al., 2011a**). In the NAc core of rats naive to the taste, morphine sensitization was associated to an increased DAergic response to the taste stimulus and to reciprocal changes in the adaptive effects of taste pre-exposure on NAc shell and mPFC DA; in particular, we observed that morphine sensitization abolished habituation in the NAc shell DA and induced it in the mPFC. In the present study, we also showed that 6-OHDA lesions of the mPFC can influence the DAergic response of subcortical areas to gustatory stimuli and induce changes including the habituation phenomenon. These findings are in agreement with the observations of Beyer & Steketee (**1999**) who showed that mPFC 6-OHDA lesions induced sensitization of behavioral and neurochemical responses to cocaine. Despite the similarities between the changes obtained in our previous and in the current study, the experimental manipulations were quite different. The exact mechanisms underlying the induction of these phenomena under each condition as well as the neural substrate reflecting the one-trial habituation under normal conditions remain to be elucidated.

As already mentioned (see part A, section A.4), mPFC exerts an inhibitory influence on the cortical pyramidal projecting neurons (**Ferron et al., 1984; Sesack et al., 1995**) and this might be the mechanism by which it affects the stimulation of DA transmission in subcortical areas as NAc induced by drug or by conventional rewards. In agreement with this, we observed that ablation of mPFC DA disinhibited NAc core DA and abolished the habituation phenomenon in the NAc shell of naive and pre-

exposed animals respectively. This is in line with the previous studies showing that 6-OHDA lesions of the mPFC can modify certain aspects of subcortical functioning. Thus, it has been shown that these lesions can produce changes in DAergic responding of subcortical areas such as that of NAc DA (**King et al., 1997; Deutch et al., 1990**), TH activity (**Rosin et al., 1992**) and DAergic transmission in NAc induced by pharmacological challenges (**Banks & Gratton, 1995; Thompson & Moss, 1995; Beyer & Steketee, 1999**).

The results of the studies provide evidence of an inhibitory role of cortical DA on subcortical DA. To our knowledge only one previous study has investigated the changes in DAergic transmission in the NAc induced by natural stimuli in rats lesioned by 6-OHDA in the mPFC (**Mitchell & Gratton, 1992**). However, in this study no distinction was made between NAc shell and core. However, King et al. (**1997**) demonstrated that in rats lesioned with 6-OHDA in the mPFC basal levels of DA were increased and the stimulatory effect of stress was increased in the NAc shell but not in the core. Moreover, mPFC lesions potentiated the increase of DA induced by amphetamine in the NAc shell but reduced that in the NAc core. These results suggest the existence of differences in the DA responsiveness among the NAc compartments after 6-OHDA lesions of the mPFC which might explain the discrepancies existing in the literature on the effect of 6-OHDA lesions of the mPFC on DA in the NAc (see section A.4). The present study sheds light on this issue by investigating the DAergic responses of NAc shell and core separately. However, unlike King and his collegues (**1997**), we did not observe differences in the basal levels of DA in the shell and core of lesioned animals. We observed instead, clearcut differences in chocolate-induced increase in DA levels between the shell and core in response to the 1st and to the 2nd exposure to the taste stimulus.

A further difference between the present study and that of Mitchell and Gratton (**1992**), is the fact that 6-OHDA lesions were unilateral. This "partial" DAergic depletion could have particular effects on behavior or DAergic transmission. In fact, it has been observed that 6-OHDA lesions of the left brain hemisphere have higher impact than those on the right hemisphere, increasing voluntary ethanol consumption (**Nielsen et al., 1999**) and DA utilization in the NAc after footshock

(**Carlson et al., 1996**). In the experiments presented here, rats underwent two bilateral microinjections of 6-OHDA which induced a massive DAergic terminal loss as shown by the loss of tissue DA and of TH.

One possible explanation for the present microdialysis results could include as already mentioned the disinhibition of the pyramidal projecting neurons to subcortical areas. The mPFC sends glutamatergic projections to subcortical areas including the NAc and the VTA. Removing the inhibitory effect of DA in the mPFC could result in excitation of the projecting neurons to both cell bodies and axon terminals in VTA and NAc respectively. Moreover, indirect connections with other brain areas as the PPTg which has a direct excitatory connection with the VTA could influence the NAc DAergic neurotransmission (for further discussion see also **Tzschentke, 2001**). Further studies are needed in order to clarify the level of interaction between cortical and subcortical regions.

The disruption of the DAergic inhibitory control of the mPFC could potentiate stress-induced psychotic symptoms in schizophrenic patients (**Weinberger, 1987**), could lead to increased vulnerability to the addictive effects of drugs (**Deminière et al., 1989; Piazza et al., 1991**) or might result in a general alteration of DAergic responses to "pharmacological or environmental challenges of the system" that in turn could lead to increased efficacy of conventional or drug reinforcers (**McGregor et al., 1996**). Apart from producing increased responses in NAc core, it was also observed that the lesions interfere with the adaptive mechanisms on NAc shell DA, thus showing that dopamine in the mPFC plays an important role on these adaptive mechanisms. Future studies might reveal certain aspects of behavior more prominently affected by the lesions. These might include abnormal motivational learning with direct implications in compulsive disorders, substance abuse and binge eating. In addition, taking in consideration the important role of mPFC DA in higher order cognitive functions as working memory (**Sawaguchi & Goldman-Rakic, 1991**), behavioral flexibility (**Bubser & Schmidt, 1990; Lanser et al., 2001**) instrumental responses (**Naneix et al., 2009**) and anxiety (**Espejo, 1997**)(see also section A.3.2) the disruptions of DAergic transmission in this area might result in cognitive disturbances that could interact with motivational mechanisms and related aspects of behavior.

Loss of DA in the mPFC cmight result in an increased response of subcortical DA which in turn might abnormally facilitate incentive arousal and learning (**De Luca et al., 2011a**).

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