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Stress and Decision-Making: Structure and Molecular Correlates

Beatriz Limpo de Faria Cardoso Ribeiro

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Resumo

A tomada de decisão é um processo com o qual os indivíduos se deparam diariamente e que envolve a selecção e execução de acções, tendo em conta a avaliação dos resultados que delas possam advir. A sua optimização, com base no contexto em que o indivíduo se encontra, confere uma importante vantagem para o mesmo, uma vez que lhe permite responder de forma eficiente à mudança de situação. Perturbações destes processos podem reduzir a capacidade de avaliação do meio, o que pode diminuir os benefícios que a tomada de decisão possa proporcionar. A tendência para determinado tipo de comportamento está intimamente ligada às características de cada indivíduo, o que justifica a existência de comportamentos tão díspares. Assim, é possível distinguir indivíduos com preferência por comportamentos arriscados, de outros com inclinação para opções que não envolvam risco. As recompensas podem ser definidas como objectos ou acções com capacidade de gerar um comportamento específico e a aprendizagem desse mesmo comportamento. Representam resultados positivos produzidos por determinada decisão e provocam emoções positivas e prazer. Deste modo, é activado um processo de aprendizagem que permite ao indivíduo gostar dessa recompensa, detectar indícios que lhe permitam calcular a sua disponibilidade e avaliar os meios de a obter, de modo que possa seleccionar a opção que mais se adequa às suas necessidades.

A tomada de decisão é mediada por diversos neurotransmissores, nomeadamente catecolaminas – dopamina, norepinefrina e epinefrina – e serotonina (5-HT), bem como pelos seus metabolitos – ácido 5-hidroxi-indolacético (5-HIAA), ácido 3,4-dihidroxifenilacético (DOPAC) e ácido homovanílico (HVA), em diferentes áreas cerebrais, como o *núcleo accumbens* (NAcc), o hipotálamo (Hipp), o estriado dorsal (DS) e os córtex pré-frontal (PFC) e orbitofrontal (OFC).

A exposição dos indivíduos ao stresse crónico afecta os seus comportamentos, podendo por em causa a capacidade de tomar de decisões. Assim, utilizou-se o rato como modelo animal para estudar o efeito do stresse crónico na tomada de decisão. Para isso os animais foram submetidos a um protocolo de stresse crónico – Chronic Unpredictable Stress (CUS) – sendo depois avaliado o seu desempenho numa tarefa no qual o risco e o ganho podem ser manipulados de forma independentes, o Minho Gambling Task (MGT). Trata-se de uma tarefa em que os animais podem optar entre escolhas seguras (que garantem sempre uma recompensa baixa) e escolhas de risco (em que os animais podem garantir recompensas elevadas ou não receber qualquer recompensa). Este teste é composto por três protocolos diferentes: neutro, seguro e risco. O MGT é realizado na 5-Hole Box, que como o nome indica, é uma caixa que possui num dos lados 5 buracos aos quais estão associados luzes; na parede oposta encontra-se um sexto buraco, o dispensador, por onde são entregues as recompensas, *pellets* de açúcar. Assim, no protocolo neutro é atribuído a um orifício sem iluminação uma

resposta segura a que corresponde uma recompensa com 100% de probabilidade, e a quatro orifícios iluminados quatro respostas arriscadas a que correspondem quatro recompensas com 25% de probabilidade (4Risco:1Seguro). Nos protocolos de risco e seguro as probabilidades de receber recompensas são de 8Risco:1Seguro e de 4Risco:2Seguro, respectivamente.

Numa primeira experiência, após indução de stresse crónico e da realização do MGT, procedeu-se à análise estereológica das sub-regiões do NAcc, core e shell, e verificou-se que animais com maior volume do core escolhem preferencialmente opções seguras no protocolo neutro. Foi também observado que esta característica predominava em animais stressados, o que parece indicar que o stresse induz hipertrofia desta sub-região afectando, deste modo, a tomada de decisão e direccionando o comportamento para opções com menor recompensa.

Numa segunda experiência, os animais foram divididos em quatro grupos, dois dos quais foram utilizados como controlos (CRT), enquanto os outros foram submetidos a CUS. De seguida, foi avaliada a capacidade de tomada de decisão destes animais no MGT. Nesta fase da experiência um grupo CTR e um grupo CUS foram intraperitonealmente injectados com um agonista dos receptores D2/D3 da DA – CTR+Quin e CUS+Quin; enquanto os outros grupos foram injectados com solução salina – CTR+Vehic e CUS+Vehic.

Para avaliar a avaliar o efeito dos níveis dos neurotransmissores e seus metabolitos na tomada de decisão decidimos quantificar estas moléculas em diferentes regiões – NAcc core e shell, Hipp, DS, PFC e OFC – utilizando a cromatografia líquida de alta resolução com detecção electroquímica (HPLC-EC). Verificou-se que a concentração de 5-HT e 5-HIAA do Hipp apresenta ema correlação significativa com a percentagem de respostas seguras dadas pelos animais no protocolo de risco. Assim, uma vez que esta região está envolvida na previsibilidade e na avaliação de recompensas futuras e que níveis elevados destes neurotransmissores foram já descritos como indutores de comportamento impulsivos, este resultados parecem demonstrar que um aumento destas moléculas torna os animais mais impacientes, impedindo-os de esperar por opções mais rentáveis.

Foi também possível observar que animais com preferência por comportamentos seguros possuem maior *turnover* da 5-HT no PFC, o que significa que nesta região maior concentração deste neurotransmissor é metabolizada. Estando esta estrutura cerebral envolvida na associação de estímulos a recompensas, estes resultados parecem indicar que o aumento da degradação da 5-HT, diminui a capacidade dos animais detectarem alterações no valor das recompensas.

O DS desempenha um papel importante na motivação e na avaliação da probabilidade de receber recompensas, como tal, a diminuição da degradação da DA nesta região associada ao aumento de selecção de opções seguras, parece indicar que maiores concentrações deste neurotransmissor direccionam o comportamento para opções com maiores ganhos, que no protocolo de risco implica a escolha de opções arriscadas.

Ao nível molecular, avaliámos a expressão de alguns genes envolvidos na tomada de decisão, nomeadamente diferentes receptores da DA D1 (*Drd1a*), D2 (*Drd2*) e D3 (*Drd3*), o receptor canabinóide do tipo 1 (*Cnr1*), o factor neurotrófico derivado do cérebro (*Bdnf*) e o seu receptor do tipo tirosina cinase B (*NtrK2*), em regiões do cérebro envolvidas na tomada de decisão, procurando correlações com o desempenho de modelos animais no MGT, e avaliando o efeito do stresse. Para isso recorreu-se ao *quantitative real time polymerase chain reaction* (qRT-PCR).

Apenas se observou a existência de correlações no PFC no protocolo de risco. Verificou-se que o aumento da expressão de *Bdnf* e *NtrK2* estão relacionadas com o aumento de escolhas seguras. Estes genes foram já descritos como estando envolvidos no aumento dos níveis de 5-HT, induzindo comportamentos impulsivos. Assim, o aumento da sua expressão no PFC parece explicar a preferência por comportamentos seguros, apesar de menos vantajoso, no protocolo de risco. No que diz respeito ao gene *Cnr1*, verificou-se que o aumento da sua expressão no PFC induz comportamentos impulsivos no protocolo de risco, ou seja a selecção de opções seguras.

Neste trabalho foi possível verificar que apesar da concentração de alguns neurotransmissores e da expressão de alguns genes estar relacionada com comportamentos preferencialmente seguros, não se verificou a tendência de um grupo para um tipo específico de comportamento. Na realidade, verificou-se que em cada grupo os animais apresentaram comportamento muito variados, o que parece indicar que apesar do tratamento ao qual foram submetidos, cada indivíduo tem uma predisposição particular para seleccionar opções arriscadas.

Este trabalho teve também como objectivo caracterizar as alterações no sistema dopaminérgico em diferentes regiões envolvidas na tomada de decisão provocadas pela exposição a stresse crónico. Para além disso, pretendeu-se verificar se a administração de agonistas da dopamina, o Quinpirole, reverte as alterações induzidas pelo stresse crónico na tomada de decisão. No entanto, os nossos resultados não nos permitiram verificar a reversão do efeito do stresse na tomada de decisão.

Palavras-chave: Tomada de decisão; Stresse crónico; Minho Gambling Task, Quinpirole

Abstract

Decision-making refers to the process of forming preferences, selecting and executing actions, and evaluating outcomes. The optimization of this process, based on the environment that surrounds the subject, is important for the subject himself. Decision-making is mediated by neurotransmitters, such as catecholamines – dopamine (DA), norepinephrine (NE) and epinephrine (Epi) – and serotonin (5-HT), as well as by their metabolites – 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA), in specific brain areas, such as both core and shell subregions of the *nucleus accumbens* (NAcc), the hippocampus (Hipp), the dorsal striatum (DS), the prefrontal cortex (PFC)and the orbitofrontal cortex (OFC).

Stress exposure impairs behavior and can influence the decision-making process. Using a decision-making test, Minho Gambling Task (MGT), it was observed that an increase in safe responses is associated with an increase in NAcc core, but not in the shell, in a neutral protocol of this task. The increase in this structure volume was mainly found in stresses animals.

Several correlations were observed in the risk variant of MGT. It was also observed that the concentration of 5-HT and 5-HIAA, in the Hipp, presented a correlation with the risk-aversive behaviors. On the other hand, DA turnover, in the DS, had an inverse association with sort of behavior. Regarding the PFC, we verify that an increase in 5-HT turnover and enhance in the expression of the genes: brain-derived neurotrophic factor (*Bdnf*) and neurotrophic tyrosine kinase, receptor, type 2 (*NtrK2*) and cannabinoid receptor 1 (*Cnr1*) with the increase of safe behaviors.

The present study pretended to characterize changes in the dopaminergic system in different brain regions after chronic stress exposure and verify if dopamine agonists, e.g. Quinpirole, revert the changes in decision-making. However, regarding this topic, our results did not allow any conclusion.

Key words: Decision-making; Chronic stress; Minho Gambling Task; Quinpirole

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List of Abbreviations

ACTH	Adrenocorticotropic hormone
ANS	Autonomous nervous system
BDNF	Brain-derived neurotrophic factor
Bdnf	Brain-derived neurotrophic factor gene
CB_1	Cannabinoid receptor 1
Cnr_1	Cannabinoid receptor 1 gene
COMT	Catechol-o-methyltransferase
CRH	Corticotrophin-releasing hormone
C_t	Cycle threshold
CTR	Control
CUS	Chronic unpredictable stress
DA	Dopamine
DEPC	Diethylpyrocarbonate
DOPAC	3,4-dihydroxyphenylacetic acid
Drd1a	Dopamine D1 receptor gene
Drd2	Dopamine D2 receptor gene
Drd3	Dopamine D3 receptor gene
DS	Dorsal striatum
ECs	Endocannabinoids
Epi	Epinephrine
Fw	Forward
GR	Glucocorticoid receptors
5-HIAA	5-Hydroxyindoleacetic acid
Hipp	Hippocampus
HPA	Hypothalamus-pituitary-adrenal
HPLC-EC	High performance liquid chromatography with electrochemical detection
Hprt	Hypoxanthine guanine phosphoribosyl transferase
5-HT	Serotonin
HVA	Homovanillic acid
IGT	Iowa Gambling Task
L-DOPA	3,4-dihydroxyphenylalanine
MAO	Monoamine oxidase
MGT	Minho Gambling Task
mPFC	Medial prefrontal cortex
MR	Mineralocorticoid receptors
NAcc	*Nucleus accumbens*
NE	Norepinephrine
NSB	Nonspecific binding
Ntrk2	Tyrosine receptor kinase B gene
OFC	Orbitofrontal cortex
PFA	Paraformaldehyde

PFC	Prefrontal cortex
PNS	Parasympathetic nervous systems
qRT-PCR	Quantitative real time polymerase chain reaction
Quin	Quinpirole
RGT	Rat Gambling Task
RIA	Radioimmunoassay
RT	Room temperature
Rw	Reverse
SNS	Sympathetic nervous systems
Trk B	Tyrosine receptor kinase B
Vehic	Vehicle
VTA	Ventral tegmental area
WCST	Winconsin Card Sorting Test

Chapter 1

Introduction

Chapter 1 – Introduction

1.1. Decision-making

Rewards can be defined as objects or events that lead to a specific behavior, generate learning of such behavior, represent positive outcomes of a particular decision and hold positive emotions and hedonic feelings (pleasure) (Schultz, 2010). Thus, they will activate the learning processes that allow liking that reward, that detect signals that calculate its availability and actions that lead to its consumption and evaluation, so that it can be selected among the different options available (Arias-Carríon and Pöppel, 2007). The feeling of reward is essential for animal survival, since it supports basic processes such as eating, drinking and reproduction. Reward is also involved in processes that are not directly linked with the protection and the promulgation of the biological heritage of the species, such as money, art and all sort of mental events (Schultz, 2010).

Human and animal behaviors can be divided, according to the way subjects respond to the surrounding environment, in Pavlovian, habitual and goal-directed responses (Rangel *et al.*, 2008). Pavlovian responses, also known as classical conditioning (Schultz, 2006), have been suggested by the Russian behavioral physiologist Ivan Pavlov, one hundred years ago. Pavlov proposed that some responses can be associated with stimuli that initially were ineffective (Kandel *et al.*, 2000). In this sort of behavior a particular environment provides cues that predict the delivery of specific outcomes. Similarly, aversive stimuli that can lead to punishment induce avoidance behaviors(Rangel *et al.*, 2008). Thus, Pavlovian behavior includes information about the value of the reward and about the possible differences of potential outcomes (Schultz, 2010). On the other hand, habitual responses result from a slow learning process, through repeated training in a stable situation. In this sort of behavior individuals learn that a specific action should be taken in a particular environment. However, when the environment changes there is an action generalization (Rangel *et al.*, 2008), which means that subjects maintain the behavior they had in the previous situation.

In goal-directed behavior actions are selected according to their consequences (Balleine *et al.*, 2007). The major difference between this behavior and habit responses is the way subjects respond to environment changes; while habitual responses are the same in different situations, goal-directed behaviors are adjusted to novel circumstances (Rangel *et al.*, 2008). Moreover, in this type of behavior individuals can influence the environment and establish the amount of reward (Schultz, 2006).

Everyday individuals face situations in which they have to select options in other to receive specific outcomes. This ability to choose one option among several alternatives based on its consequences and on individual needs at the time of the decision, is known as decision-making (Balleine, 2007). The optimization of this process is extremely important since it allows an efficient answer when circumstances change (Dias-Ferreira *et al.*, 2009).

Decision-making processes can be divided in four important steps. Primarily, the situation is identified (Doya, 2008). This step involves the evaluation of internal states, external states, and potential direction of the option chosen (Rangel *et al.*, 2008). To illustrate this step a practical scenario can be used: thirst. In this situation thirst level (internal state) leads to searching available drinks (external states) and choose a hot or a cold drink (potential option). Then, the option is evaluated enhancing both profits and punishments each option can provide. Based on the previous step, subjects select the most appropriate option according to their needs. Finally, the option selected is evaluated based on its consequences (Doya, 2008); thus, this step involves a learning process, which allows the individual to make better decisions in the future (Rangel *et al.*, 2008). Therefore, decision-making is a fusion of several complex cognitive processes that combine the relation between actions and consequences with their value or utility (Balleine, 2007).

Several factors can influence subject decisions, such as needs and desires; effort, risk and time needed to obtain a specific outcome; and probability of receiving it. Moreover, decision-making can be affected by subject knowledge of the surrounding environment and by its own unexpected changes (Doya, 2008). Human decisions are made based on the potential profit they may provide and on the probability of receiving it. Once this evaluation is made humans tend to choose the bigger reward (Schultz, 2006). Furthermore, human studies revealed that when decision-makers have a poor knowledge of the risk factor they tend to avoid that choice (Camerer, 1992).

1.2. Neurotransmitters

Decision-making is a complex process that involves the communication of neurons from several brain regions. However, for several years the mode how neurons communicate with each other was a delicate and complex question that divided opinions. Nowadays, it is known that neuronal communication can be either electrical or chemical; however, chemical-mediated transmission is the major mode by which information is spread in the brain. The molecules responsible for this transmission are called neurotransmitters (Squire *et al.*, 2008). Several criteria were proposed in order to classify these molecules. Thus, neurotransmitters are “molecules synthesized by and released in presynaptic neurons in adequate amounts to transmit information to postsynaptic cells by interacting with their specific receptors”. The administration of transmitter agonists in a dose-dependent manner induces the same effect in

the postsynaptic cleft. Moreover, there should be mechanisms by which neurotransmitters become inactive, such as uptake or enzymatic inactivation (Kandel *et al.*, 2000).

Brain transmitters can be divided in classical, nonclassical and unconventional neurotransmitters. Classical neurotransmitters are considered to be molecules such as acetylcholine, biogenic amines and amino acids (Squire *et al.*, 2008). The term "biogenic amines" has been used to name some monoamines, including catecholamines and serotonin (5-HT) (Kandel *et al.*, 2000). These molecules are produced in neuronal axons, stored in small vesicles, and can be re-uptaken by the cell that produces and releases them, the presynaptic neuron (Squire *et al.*, 2008). Classic transmitters can be quickly replaced (Purves *et al.*, 2004).

Peptides are nonclassical transmitters synthesized in the cell body, transported to the terminal nerve and replaced quite slowly (Purves *et al.*, 2004). Thus, in situations in which neuronal communication needs to be done rapidly classical neurotransmitters are used instead of peptides, since the synthesis of these molecules is time consuming. Another characteristic that distinguishes classical from nonclassical transmitters is the storage. Peptides are stored in large vesicles (Squire *et al.*, 2008).

The development of new technologies allowed the detection of molecules that despite its very low concentrations, act as neurotransmitters. Some gases, such as nitric oxide, carbon monoxide and hydrogen sulfide, and other molecules, such as growth factors, and endocannabinoids (ECs) are examples of unconventional neurotransmitters. Since they can cross the lipid bilayer of the membrane, by diffusion, they are not stored, which makes them an exception to the definition of neurotransmitter (Kandel *et al.*, 2000; Squire *et al.*, 2008).

This work is going to give special attention to some classical neurotransmitters, namely catecholamines, which includes dopamine (DA), norepinephrine (NE; also named noradrenaline) and epinephrine (Epi; also known as adrenaline); and 5-HT (Kvetnansky *et al.*, 2009).

Classical neurotransmitters have a characteristic life cycle that begins with the entrance of the precursor amino acid in the presynaptic neuron (Squire *et al.*, 2008). Catecholamines are synthesized from tyrosine, while 5-HT is produced from tryptophan. After its conversion in the neurotransmitter these molecules can be either metabolized or stored in vesicles (Kvetnansky *et al.*, 2009; Simon *et al.*, 2009). In the presence of the appropriate stimuli these structures can fuse with the cell membrane and the neurotransmitters are released to the synaptic cleft. Once outside the cell these molecules can have different destinations. They can bind to postsynaptic specific receptors or to autoreceptors that control transmitters synthesis, release and neuronal firing rates; they can also be accumulated in the presynaptic cell or in glia (Purves *et al.*, 2004). Some transmitters diffuse from the synaptic cleft thus ending their action (Squire *et al.*, 2008) (Figure 1).

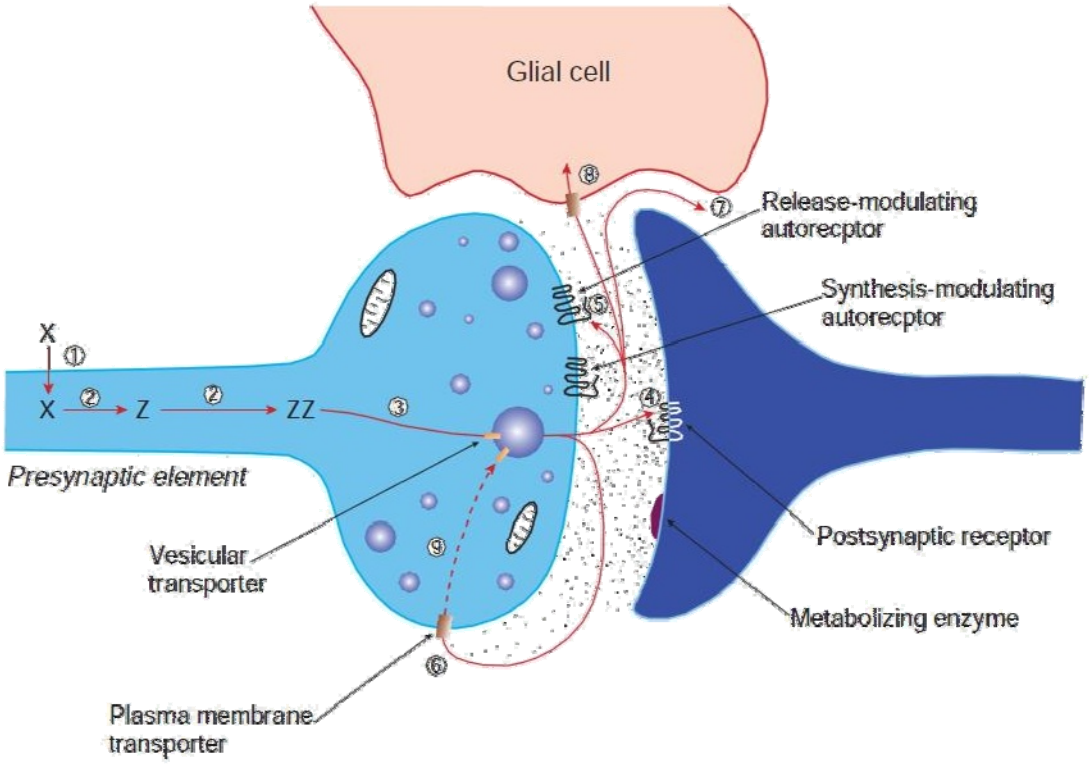

Figure 1 - Catecholamine cycle. The neurotransmitters precursors enter in the presynaptic terminal (1), are converted in the neurotransmitters (2) and are stored in vesicles (3). When released, they can bind to the postsynaptic cell (4) or to autoreceptors (5) or they can re-uptaked by the presynaptic cell (6). Some transmitters diffuse from the synaptic cleft (7) and others are accumulated in glia cells. Metabolic inactivation can also occur (9) (Squire et al., 2008).

Catecholamines are molecules composed by a catechol ring and by an amine group, that result from the sequential action of different enzymes (Kvetnansky *et al.*, 2009). Indeed, the L-tyrosine stereoisomer is transformed in L-dihydroxyphenylalanine (L-DOPA), which is then metabolized in DA. After that, this neurotransmitter is converted in NE and finally in Epi (Squire *et al.*, 2008).

These neurotransmitters can be inactivated through the action of enzymes such as aldehyde dehydrogenase, catechol-*O*-methyltransferase (COMT) and monoamine oxidase (MAO) (Squire *et al.*, 2008). MAO, together with aldehyde dehydrogenase, converts DA in 3,4-dihydroxyphenylacetic acid (DOPAC) (von Bohlen und Halbach and Dermietzel, 2006), which by the action of COMT is degraded in homovanillic acid (HVA) (Konstandi, 2000; Miyazaki and Asanuma, 2008).

Regarding 5-HT synthesis, it also involves the conversion of its precursor amino acid, in this case tryptophan. Moreover, like catecholamines, 5-HT can be degraded by MAO, producing 5-hydroxyindole acid aldehyde, which is then oxidized in 5-hydroxyindoleacetic acid (5-HIAA) (Konstandi, 2000; Squire *et al.*, 2008).

1.2.1. Dopamine

Dopaminergic neurons, cells responsible for DA synthesis, have small populations in the brain; however, they deeply influence brain functions (Björklund and Dunnett, 2007). Actually, DA is thought to be the major monoamine transmitter in the brain (Kandel *et al.*, 2000). This neurotransmitter plays a key role in several brain functions such as motivation, motor function, reinforcement, reward, decision-making, learning, memory and emotional behaviors (Goto *et al.*, 2007; Iversen *et al.*, 2010).

DA is extremely important in decision-making modulation, since its action controls several factors involved in this behavioral process, such as motivation level, reward value, cost/benefit (receiving immediate small reward or latter bigger rewards) and reinforcement (Iversen *et al.*, 2010; Onge *et al.*, 2010; Onge *et al.*, 2011). It has been suggested that both DA increasing and decreasing may have the same impact in behavior modulation (Goto *et al.*, 2007). However, this neurotransmitter influence in brain functions may not be constant. In fact, DA appears to be essential at the beginning of learning process; but after training, this process seems to become DA-independent (Wickens *et al.*, 2007).

Dopaminergic neurons have two different modes of releasing their transmitters. Under basal conditions (tonic) DA levels in the synaptic cleft depend on a slow and irregular firing of some dopaminergic neurons, while other neurons can be inactive. On the other hand, phasic DA burst firing leads to a massive DA release in the synaptic cleft (Taepavarapruk *et al.*, 2000). Dopaminergic firing patterns change according to the expected probability of receiving reward and with the reward value (Arias-Carríon and Pöppel, 2007; Onge and Floresco, 2009). These cells have a higher firing rate when the probability of obtaining a rewards it unknown or when the value of the reward received is higher than expected (Fiorillo *et al.*, 2003; Schultz, 2006). If the same reward is received over and over again, which mean the reward is received as predicted, dopaminergic neurons will not be activated (Schultz, 2010). Moreover, if the outcome is worse than expected neuron activity decreases (Schultz *et al.*, 1997). These neurons also respond to error prediction, which can indicate DA involvement in signaling events in which probability of receiving rewards is not expected. Thus, this neurotransmitter may be involved in learning the probability of future rewards (Onge and Floresco, 2009). Several studies revealed that individuals prefer small immediate reward to larger delayed rewards, which indicates that reward value decreases with time. So, dopaminergic neurons do not appear to distinguish delayed from small rewards. Although these neurons activation is mainly caused by rewards, it can also be induced by aversive events (Schultz, 2010).

Motivation leads to searching for a reward previously received or to the cues that lead to that specific reward. It is thought that DA plays an important role in stimulus-reward association. This connection is known as “cognitive value system”, which is responsible for directing actions (Iversen and Iversen, 2007). Once this association is made, it remains strong until reward devaluation occurs by the absence of appropriate stimulus. Stimuli devaluation

can result from repeated absence of reward or smaller rewards delivery (Arias-Carríon and Pöppel, 2007).

Dopamine pathways

The brain location where DA is released is intimately related with the role it is going to play (Ikemoto, 2010). Dopaminergic neurons are spread in different brain regions, but can be found mainly in the *substantia nigra*, ventral tegmental area (VTA) and hypothalamus (Björklund and Hökfelt, 2005). DA is released from these brain areas and distributed in other regions. Several dopaminergic pathways have been described according to DA release location and its action site, namely, nigrostriatal, mesocortical, mesolimbic and tuberoinfundibular pathways (Stahl, 2008) (Figure 2)

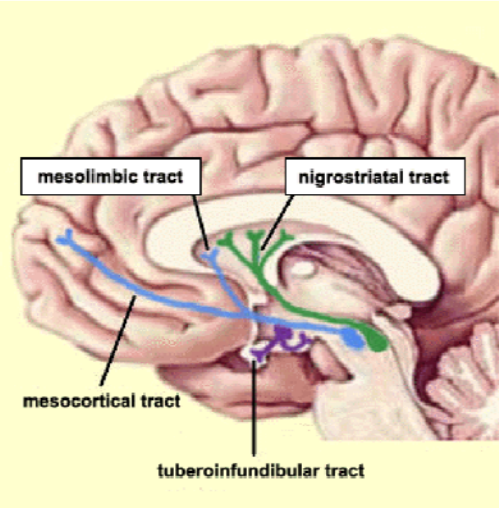

Figure 2 - Dopamine pathways: mesolimbic and mesocortical are highlighted in blue, nigrostriatal in green and tuberoinfundibular in purple (Seneff, 2009).

Neurons from *substantia nigra* project to the dorsal striatum (DS), in a system known as nigrostriatal dopaminergic pathway, which regulates motor functions (Stahl, 2008), such as initiation of movements and postural reflexes (Yao *et al.*, 2008).

In both mesolimbic and mesocortical systems, DA is produced by VTA neurons (Arias-Carríon and Pöppel, 2007). In mesolimbic pathway, VTA projects to the ventral striatum, which includes the *nucleus accumbens* (NAcc). As reviewed by Ikemoto, different studies involving drug administration and electrical stimulation demonstrated the involvement of mesolimbic pathway in reward properties of both natural and chemical rewards and in motivation (Björklund and Hökfelt, 2005; Ikemoto, 2010; Stahl, 2008). Thus, this dopaminergic system is usually known to regulate biological drives (Kandel *et al.*, 2000). On the other hand, in the mesocortical pathway VTA projects mostly to the prefrontal cortex (PFC) (Yao *et al.*, 2008), modulating executive functions, emotions, learning and memory (Björklund and Hökfelt, 2005; Stahl, 2008). Because of the overlap between mesolimbic and mesocortical systems, they are

usually referred as the mesocorticolimbic system (Wise, 2004). Ultimately, the tuberoinfundibular pathway, in which dopaminergic projections from the hypothalamus are sent to the anterior pituitary, is involved in prolactin production inhibition (Stahl, 2008).

Dopamine receptors

DA action is highly dependent on its binding to specific receptors located in both pre- and postsynaptic neurons. These receptors, like those from other catecholamines, are G-protein coupled receptors, which consist in a single polypeptide with seven membrane-spanning segments (Squire *et al.*, 2008; Yao *et al.*, 2008). Five subtypes of mammal DA receptors were described – D1, D2, D3, D4 and D5 (Iversen *et al.*, 2010). They were divided in two families, according to their amino-acid sequence similarity, functional and pharmacological characteristics, in D1-like and D2-like receptors (Björklund and Hökfelt, 2005). The first group includes D1 and D5 receptors and interacts with G_s proteins (stimulatory G-proteins) activating adenylate cyclase. On the other hand, D2-like receptors, which include D2, D3 and D4 receptors, activate G_i/G_o (inhibitory G-proteins/"other" G-proteins), which inhibit adenylate cyclase (Squire *et al.*, 2008).

The D1 receptor is expressed mainly in the striatum which includes the NAcc, DS and *substantia nigra*, but can also be found in the cortex, hippocampus (Hipp) and amygdala (Levey *et al.*, 1993). These receptors has been associated with drugs abuse, motor and cognitive function (Iversen *et al.*, 2010). Furthermore, it has been found that D1 receptors located in the NAcc mediate behavior when both reward or non-reward stimuli are presented (Floresco, 2007). Although, D2 receptors are mainly located in the NAcc, they are also present in the PFC, amygdala, VTA, *substantia nigra* and Hipp (Iversen *et al.*, 2010). These receptors are directly involved in motor function and motivation during task performance (Floresco, 2007; Taepavarapruk *et al.*, 2000). D3 receptors, also found in the NAcc, have been involved in emotional, motivational and endocrine functions (Iversen *et al.*, 2010).

Studies have shown that rats with systemic DA receptors blockage reduce their preference to wait longer or work harder for larger rewards (Cardinal *et al.*, 2000). In 2009, Onge and Floresco suggested that D1 and D2 systemic activation induces preference for large rewards, while D3 appears to exert the opposite effect (Onge and Floresco, 2009). Recently, it has been demonstrated the contribution of D1 and D2 receptors in the medial PFC (mPFC) in a risk-based decision making. This study revealed that D1 receptors blockage in the mPFC decreases large/risky options, while D2 receptors blockage has the opposite effect and impaired probabilistic discounting. Moreover, D2 receptor activation in this brain region impairs decision-making (Onge *et al.*, 2011). Thus, in PFC, D1 and D2 receptors act together playing an important role in behavior flexibility mediation (Iversen *et al.*, 2010).

Another work reported the involvement of D2/D3 receptors in impulsivity; it demonstrated that animals with high natural impulsivity increase and decrease their impulsivity when receive

a D2/D3 antagonist in the NAcc shell and core, respectively. However, systemic administration of the same antagonist did not alter impulsive behavior (Besson *et al.*, 2009).

Studies showed that D4 receptor is localized in the NAcc, in rats (Svingos *et al.*, 2000), and in the cerebral cortex in primates. In humans, this receptor has been found in abundance in different brain structures, such as the PFC, Hipp, and in smaller amounts in the basal ganglia. D4 receptors are involved in cognition, emotion, reasoning and perception. D5 receptors are expressed in the NAcc, *substantia nigra*, cerebral cortex, hypothalamus, striatum, olfactory tubercle and Hipp. These receptors are thought to influence mood, addiction and locomotion (Iversen *et al.*, 2010; Neve, 2010).

It has also been demonstrated that DA receptors from the PFC modulate set-shifting. Actually, the blockage and activation of D2 and D4 receptors, respectively, impairs shifting behavior; while D4 receptor blockage improves this behavior (Floresco *et al.*, 2006b). Moreover, D1 receptor blockages in the NAcc core impair individual capacity to maintain novel strategies (Iversen *et al.*, 2010).

1.2.2. Norepinephrine and epinephrine

As already mentioned, NE is a catecholamine synthesized from DA. This neurotransmitter is implicated in sleep and wakefulness, attention, and feeding behavior (Purves *et al.*, 2004). Furthermore, NE is also involved in decision-making, acting in the orbitofrontal cortex (OFC), where it induces preference for risky and novel options (Doya, 2008). Moreover, it has been demonstrated that individuals with decreased NE levels have more difficulty to evaluate the magnitude of different losses (Rogers *et al.*, 2004).

Another catecholamine, Epi, is present in the brain in smaller quantities (Purves *et al.*, 2004). However, Epi is highly released in response to stress (Kvetnansky *et al.*, 2009). This topic will be addressed latter.

1.2.3. Serotonin

5-HT is another classical neurotransmitter, composed by an indole and an amine group. Despite its important functions in the brain, only 1% is stored in this part of the body (Squire *et al.*, 2008). 5-HT modulates mood states, such as food intake, anxiety, impulsive violence and depression (Kandel *et al.*, 2000). Moreover, this transmitter influences decision-making, since under a predictable environment, 5-HT is involved in the capacity to calculate the probability of receiving long-delayed rewards, an ability that involves both the PFC and the DS (Clarke *et al.*, 2004). Furthermore, studies observed low levels of the 5-HT metabolite, 5-HIAA, in individuals with preference for risky options (Mehlman *et al.*, 1994).

1.2.4. Endocannabinoids

ECs are a family of lipid messengers synthesized from lipid precursors present in neuronal membranes (Gelman and Fricker, 2010). These molecules are highly hydrophobic, which allows them to pass through plasma membranes. Since ECs cannot be stored, they are considered to be unconventional neurotransmitters (Squire *et al.*, 2008). ECs system regulates emotional responses, feeding behavior, mood, reward, pleasure, motivation, learning and memory (Lupica and Riegel, 2005; Lutz, 2009; Squire *et al.*, 2008). These neurotransmitters act retrogradely; they are released from the postsynaptic neuron and activate cannabinoid receptors type 1 (CB_1) found in presynaptic neurons, inhibiting the release of other neurotransmitters (Piomelli, 2003). CB_1 cannabinoid receptors are G-protein coupled receptors highly expressed in the brain, but can be mainly found in some regions such as VTA, Hipp and basal ganglia (Herkenham *et al.*, 1990). As reviewed by Pattij, *et al.*, these receptors have been found on presynaptic terminals of neurons involved on the synthesis of several neurotransmitters, such as NE, 5-HT, and others, like (GABA; inhibitory neurotransmitter) and glutamate (excitatory neurotransmitter). However, although they have not yet been detected in dopaminergic neurons; their activation seems to indirectly induce DA transmission, in the NAcc and PFC, through inhibition of dopaminergic neurons, in the VTA and PFC, by GABAergic neurons action (Pattij *et al.*, 2008). Thus, ECs action in the VTA and NAcc promotes the release of DA in this last brain region, activating striatal D2 receptors (Squire *et al.*, 2008; Yin and Lovinger, 2005). The expression of CB_1 receptors in brain regions involved in the mesolimbic dopaminergic pathway suggests that these receptors may have an important role in reward and motivation (Herkenham *et al.*, 1990). However, the way how decision-making is influenced by ECs is not known yet. CB_1 receptors are known to modulate addictive behaviors; the activation of these receptors is involved in drug-seeking; while their blockage decreases the likelihood of a relapse (Cossu *et al.*, 2001; Piomelli, 2003). It has also been demonstrated that ECs/CB_1 linkage is essential for habit formation, in fact CB_1 blockage impairs this process (Hilário, 2007). Moreover, it has been demonstrated that the blockage of these receptors provokes anxiety (von Bohlen und Halbach and Dermietzel, 2006).

1.3. Other molecules involved in decision-making

The brain-derived neurotrophic factor (BDNF) is a protein involved in neurogenesis regulation, cell survival and synaptic efficacy (McEwen, 2010; Purves *et al.*, 2004; Southwick *et al.*, 2005). It has been demonstrated that levels of this molecule in the brain have an important impact on neuronal communication, since they regulate the development of neuronal structures; BDNF increases the dendritic complexity of neurons by increasing total dendritic length (Squire *et al.*, 2008). BDNF is highly expressed in regions such as the PFC, DS, VTA, *substantia nigra* and Hipp. When released, BDNF acts on tyrosine receptor kinase B (Trk B)

receptors extensively expressed in the nervous system (von Bohlen und Halbach and Dermietzel, 2006). The activation of Trk B receptors in the mesolimbic system is extremely important in reward/aversive information processing. In fact, BDNF binding to Trk B receptors is crucial for creation of proper memories of potential rewards and dangerous situations, and for the establishment of associations with negative emotional stimuli (Gasic *et al.*, 2009; Nestler and Carlezon, 2006; Schoenbaum *et al.*, 2007). It was demonstrated that BDNF has an important role in social behavior. Actually, individuals with social withdrawal behavior have high levels of BDNF in the NAcc; however, when BDNF gene is deleted this behavior is reverted (Iversen *et al.*, 2010). The activation of the Trk B receptor by BDNF has also been involved in memory, learning and appetitive behavior (Nestler and Carlezon, 2006; Yamada *et al.*, 2001).

1.4. Brain regions involved in decision-making

Since decision-making is a complex and flexible process, in which an individual needs to learn about possible options and how to achieve them, beyond action adjustment to new situations, it is not surprising that several brain regions, and their extensive and diverse connections, influence this behavior. The brain areas with major influence in this process are located in the frontal cortical region of the brain. These regions project to other cortical and subcortical areas, such as the basal ganglia (Squire *et al.*, 2008).

1.4.1. *Nucleus accumbens*

The NAcc, the main constituent of the ventral striatum, which is part of the basal ganglia is involved in several brain functions, including motivation, motor function, reinforcement and reward (Iversen *et al.*, 2010).This brain region is located in a position that allows it to receive limbic inputs and to send output to motor regions, for this reason it can be considered as a "limbic-motor interface" (Mogenson *et al.*, 1980).

The NAcc is considered to have two main subregions according to their histochemical markers, inputs and outputs; a medial shell subregion and a more lateral core (Floresco, 2007). The NAcc core is involved in voluntary motor function (Björklund and Hökfelt, 2005) and environment evaluation, having been implicated in individual sensitization to both reward, aversive stimuli, conditioned reinforcers (Iversen and Iversen, 2007) and behavioral shifting (Floresco *et al.*, 2006a), which allows individuals to achieve and preserve new behavioral strategies. Actually, subjects with lesions on this brain region show preference for small certain rewards over large uncertain rewards (Cardinal and Howes, 2005).

Studies in rodents implicated the NAcc shell in anticipatory responses (Chang *et al.*, 1994), goal-directed behavior, behavioral sensitization and affective states alteration (Iversen *et al.*,

2010). Moreover, this subregion seems to plays an important role in initiating and maintaining the rewarding action of drugs of abuse (Björklund and Hökfelt, 2005).

The NAcc has been implicated in both reward novelty and predictability. However different researches have demonstrated different results with respect to which of NAcc subregions is involved in these factors modulation (Björklund and Hökfelt, 2005; Iversen and Iversen, 2007).

DA release in the NAcc seems to facilitate choice in the presence of several options with different cost, beyond being essential to overcome large costs (Day *et al.*, 2010). Differences in the firing of NAcc DA neurons induced by specific stimuli increase the probability of the subject to perform the learned reward-seeking response proper for those stimuli (Nicola *et al.*, 2005). Thus, individual adaptation to world changes is highly influenced by both DA inputs and outputs to the NAcc, since they synchronize goal-direct reinforcement and procedural learning (Iversen and Iversen, 2007). Lesions in this area, and in other dopaminergic brain regions, induce behavioral shifting toward small, certain rewards over larger, uncertain rewards (Cardinal and Howes, 2005). Moreover, it has been demonstrated that individuals with dysfunctions of the NAcc may present several depression symptoms, such as social withdrawal, anhedonia and even sex drive (Nestler and Carlezon, 2006).

1.4.2. Prefrontal cortex

DA is involved in important brain functions mediated by the PFC, such as working memory, attentional process and behavioral flexibility (Floresco *et al.*, 2006b). In addition to these functions PFC has also an important role in probabilistic discounting (Onge *et al.*, 2010), motivation (Kandel *et al.*, 2000) and motor coordination (Squire *et al.*, 2008).The mPFC integrates information about changing reward probabilities and gives a new value to the reward, which can aid in decision-making (Onge *et al.*, 2011). It has also been described as important for shifting behavior, association between stimulus and both reward and error detection and cost/benefit valuation (Block *et al.*, 2006; Narayanan and Laubach, 2008; Onge *et al.*, 2011).

1.4.3. Orbitofrontal cortex

The OFC is a PFC area implicated in emotional processes; actually this brain region is involved in attributing “affective value” to people, objects or even situations (Squire *et al.*, 2008). The OFC also influences adaptive response (Takahashi *et al.*, 2009), by encoding reward (Schultz, 2000; Schultz, 2010) and controlling behavior selection and execution (Schoenbaum *et al.*, 1999). Actually, OFC neurons can discriminate between rewards according to animal preference (Schultz, 2000).

Studies revealed that OFC neurons become more rapidly active when subjects receive expected big rewards than when they are threatened by punishments (Squire *et al.*, 2008; Thorpe *et al.*, 1983). This brain area is involved in learning processes in which neutral stimuli can indicate the value of a future reward (Holland, 2004). Moreover, when the outcome is worse than expected, this brain region facilitates the storage of new information allowing a rapid learning, followed by behavior modification, according to environmental changes (Takahashi *et al.*, 2009). Furthermore, the OFC seems to support exploratory and risk options (Doya, 2008). Indeed, studies revealed that changes in DA action in this area lead to alteration in risk decision-making evaluation (Bechara *et al.*, 1999; Clark *et al.*, 2007; Pais-Vieira *et al.*, 2007).

1.4.4. Dorsal striatum

The DS is a component of the basal ganglia that has an important role in affective stimuli detection, its predictability and in reward or punishment value (Apicella *et al.*, 1991; Delgado *et al.*, 2003; Schultz, 2000).

This region can be divided in dorsomedial or associative striatum and dorsolateral or sensorimotor striatum. The first subregion appears to be involved in the early phases of visuomotor learning and in rapid association between action and reward. On the other hand, the sensorimotor striatum influences the acquisition of habitual behavior (Yin *et al.*, 2009). Thus, this brain structure seems to influence behaviors by coding incentive affective properties, building responses according to stimuli magnitude, which underlines the impact of this region in motivated behavior (Delgado *et al.*, 2003).

1.4.5. Hippocampus

The Hipp, an extremely sensitive brain structure (McEwen *et al.*, 2011), mediates both formation and storage (Kandel *et al.*, 2000) of spatial and nonspatial memories (Squire *et al.*, 2008). Indeed, this brain area is involved in declarative, episodic, spatial and contextual learning and memory (McEwen, 2000). In addition, it has been demonstrated that the Hipp influences spatial exploration (Gruber *et al.*, 2009), development and maintenance of a cognitive map of space (Kandel *et al.*, 2000). Moreover, the Hipp has an important role in perceptual and cognitive phases of emotional processes (Ikemoto, 2010; Kandel *et al.*, 2000).

It has been proposed that the Hipp may play an important function in selection and maintenance of memory events before reward (Björklund and Hökfelt, 2005). Thus, the formation of long term memories related to daily life may have strong impact in decision-making allowing subjects to choose the better options according to their knowledge of previous situations (Kandel *et al.*, 2000).

1.5. Damages in dopaminergic pathways

In the past few years, there has been increased evidence that several DA mesocorticolimbic system components are associated with decision-making, risk evaluation and action-reward connection (Onge *et al.*, 2010). It has been demonstrated that damages in dopaminergic systems can induce several diseases.

Parkinson's disease is the major neurodegenerative disorder associated with DA cells. In this disease dopaminergic neurons of the *substantia nigra* of the ventral midbrain are progressively lost, which leads to DA depletion in the striatum, and, thus, to the motor symptoms that characterize this disease (Cools, 2006). This disease also affects other dopaminergic pathways; however, neurodegeneration does not occur simultaneously in all dopaminergic systems. It begins in dorsal structures and progresses to more ventral regions. Thus, after nigrostriatal pathway, mesocortical pathway neurons are also degenerated, which explains executive functions impairment; followed by mesolimbic pathway, which is only affected in latter stages of the disease. Nowadays, neuron degeneration cannot be controlled, so treatments available are only symptomatic (Dauer and Przedborski, 2003). Thus, in order to normalize DA levels, Parkinson's disease patients are submitted to DA replacement therapies (Robert *et al.*, 2009). L-DOPA is the most common therapeutic agent in this disease. After administration, this molecule crosses the blood-brain barrier and is converted in DA, diminishing the motor symptoms of the disease. Beyond L-DOPA, many other DA agonists acting in different dopaminergic receptors can be used (Squire *et al.*, 2008). Nevertheless, these therapies have a downside; they highly increase DA levels in the mesolimbic pathway, impairing the ability to calculate the probability of receiving a reward and to learn how to act in order to obtain it (Cools, 2006; Robert *et al.*, 2009). It has been demonstrated that DA replacement therapies, mainly those that activate D2/D3 receptor, lead to temporary pathological gambling behaviors. These behaviors can disappear with simultaneous intake of DA antagonist or when treatment ends (Clark *et al.*, 2007; Imamura *et al.*, 2006; Seedat *et al.*, 2000).

Schizophrenia is a mental illness considered to be one of the most severe psychiatric conditions (Iversen *et al.*, 2010). These disease's symptoms can be divided in two categories: positive and negative, that result from alteration or increase and loss or decreasing of cognitive and emotional functions, respectively. Positive symptoms include hallucinations, delusions and bizarre behaviors. On the other hand, negative symptoms consist of disturbances in social interaction characterized by anhedonia, withdrawal, poverty of speech, attention impairment, deficits in motivation, inability to develop goal-directed behavior or to express emotions (Squire *et al.*, 2008). Several theories have been proposed to explain the set of symptoms that characterizes this disease. One of the hypothesis proposed that schizophrenia may be induced by a deregulation of DA neurotransmission. Thus, negative

symptoms are thought to be due to a decrease of DA levels in the mesocortical DA pathway; while positive symptoms are thought to result from an increase of DA action in mesolimbic DA projections, mainly by excessive activation of D2 receptors (Iversen *et al.*, 2010). However, it has also been proposed that schizophrenic patients mesolimbic pathway may present a hyperactivation of a pool of neurons, leading to positive symptoms; whereas other set of neurons are hypoactivated, inducing negative symptoms, namely dysfunctions of reward mechanism (Stahl, 2008)

1.6. Stress and decision-making

In the past decades, life-style in modern societies has become agitated promoting both internal and external events that can induce rupture of both physical and psychological homeostasis (de Kloet *et al.*, 2005). Disequilibrium in this state results from the presence of threatening stimuli or from stimuli perceived to be so, known as stress, (Chrousos, 2009), and leads to changes in both present or future behaviors (Joëls and Baram, 2009). Thus, the brain detects these stimuli and determines the better behavioral and physiological response, so that environment adaptation can occur and individual health be maintained (McEwen, 2000; McEwen *et al.*, 2011).

Stress responses can be influenced by several stress factors and individual characteristics. Stress duration and type (physical or psychological) and the way it is presented to subjects have different effects in individuals. Similarly, subject age, sex and genetic background also influence both the magnitude and the response pattern. This response is regulated by a combination of several stress mediators, according to stress characteristics (Joëls and Baram, 2009).

Stress responses are controlled by both the hypothalamus-pituitary-adrenal (HPA) axis and the autonomous nervous system (ANS) (McEwen, 2000). The ANS is the component of nervous system responsible for monitoring body physiology, being involved in respiration, circulation, blood chemistry, digestion, reproduction and immune response (Squire *et al.*, 2008). It can be divided in sympathetic (SNS) and parasympathetic nervous systems (PNS) (Purves *et al.*, 2004). The first system adjusts glandular, metabolic and vascular functions to threatening situations, leading to catabolic increase and emergency responses, and is triggered by stress (Kandel *et al.*, 2000). On the other hand, PNS activation promotes anabolism and protective responses that regulate and limit the reduction of energy spending, slow the heart and heat loss (Squire *et al.*, 2008), thus preserving body resources and restoring homeostasis (Kandel *et al.*, 2000).

Furthermore, there is an important connection between the HPA axis and the SNS, since the HPA axis plays an in important role in the activity of the enzyme responsible for Epi synthesis. This catecholamine, the main neurotransmitter of the SNS in mammals, is released under global and metabolic threats (Kvetnansky *et al.*, 2009; Squire *et al.*, 2008).

Under stress, some brain circuits are activated. In fact, stress induces the release of corticotrophin-releasing hormone (CRH) and vasopressin, two neuropeptides involved in the behavioral and metabolic response to stress, by the hypothalamus, which regulate the HPA axis (de Kloet *et al.*, 2005). The increase of CRH levels promotes the secretion of adrenocorticotropic hormone (ACTH) in the anterior pituitary, which stimulates the release of corticosteroids in the adrenal cortex, cortisol in humans and corticosterone in rats (Squire *et al.*, 2008).

Corticosteroids act through both mineralocorticoid (MR) and glucocorticoid receptors (GR). These stress hormones have high affinity for MR, which means that at a basal state most MR are occupied (Joëls and Baram, 2009). These receptors are thought to be involved in corticosteroid levels variation in the circadian cycle, and in both stress process evaluation and beginning (de Kloet *et al.*, 2005; Raone *et al.*, 2007). On the other hand, corticosteroids have a lower affinity for GR; therefore, under basal conditions only a small fraction of these receptors are occupied, becoming less available after stress hormones increase in the circulation. These receptors are implicated in stress response regulation. MR and GR have different brain distribution: while the first group is mainly located in the Hipp, the last one, although highly expressed in the Hipp, is widespread in the brain (Joëls and Baram, 2009; Raone *et al.*, 2007).

The Hipp was the first region documented to be affected by stress hormones (McEwen *et al.*, 2011). However, it has been demonstrated that chronic stress is also responsible for structural alterations in other brain regions such as the PFC (Cerqueira *et al.*, 2007) or the striatum (Dias-Ferreira *et al.*, 2009). It is important to notice that although stress can affect functioning of the Hipp, this structure has an important role in stress response, mainly in the adjustment to repeated stressful stimuli (Kvetnansky *et al.*, 2009). Together with the PFC, the Hipp exerts a negative feedback on corticosteroid release by the adrenal glands. Thus, a high amount of GRs in this brain region leads to a low basal level and to a tight control of corticosteroid's release when subjects are under stress and vice-versa (Raone *et al.*, 2007; Squire *et al.*, 2008).

Circulation allows corticosteroids to achieve every organs and, thus, coordinate stress response and recovery (de Kloet *et al.*, 2005). The increase of stress hormones in circulation in response to stress leads to adaptive behaviors, that include an increase of fear, state of vigilance and focused attention, and, in humans, anxiety and worrying (Chrousos, 2009; McEwen, 2000; Squire *et al.*, 2008). Therefore, an adequate response to stress should be rapidly activated when needed and terminated when the condition is accomplished (de Kloet *et al.*, 2005). Insufficient or excessive responses to stress can lead to physiological and behavioral disorders (Chrousos, 2009; McEwen *et al.*, 2011).

As already mentioned, stress can induce different responses according to its own characteristics. Under acute stress huge amounts of catecholamines, mainly Epi and NE, are released from the adrenal medulla and sympathetic nerve terminals into the blood

(Kvetnansky *et al.*, 2009). Thus, brain perception of threatening stimuli leads to a release of both catecholamines and corticosteroids in the blood, so that all the body can act according to the threatening stimuli (de Kloet *et al.*, 2005). When the threat no longer exists, hormone and neurotransmitters levels return to baseline (Joëls and Baram, 2009). Once more, these hormones are released in order to achieve an homeostatic state; however, when their release exceeds the individual needs, they can induce widespread damage (McEwen, 2000). However, in chronic stress conditions, baseline levels are never achieved, which means that corticosteroids levels in the blood are kept elevated, leading to changes in the expression of some genes, neuronal structure and synaptic transmission (de Kloet *et al.*, 2005; Joëls and Baram, 2009).

Taking into account the involvement of the systems mentioned above there is no surprise that stress can affect growth, reproductive, cardiorespiratory, metabolic and immune systems beyond the impairment of cognitive and executive functions, sleep and mood, also affecting decision making. In fact, it has been reported that chronic stress can lead people to choose worse options (Chrousos, 2009; McEwen *et al.*, 2011).

1.7. Test used to evaluate decision-making

Several tests have been developed in order to better understand the different brain structures involved in decision-making processes. Iowa Gambling Task (IGT) and Winconsin Card Sorting Test (WCST) are two tests used to study human decision-making. IGT is a task that simulates daily life decision-making in which subjects have to maximize their monetary gain (Rivalan *et al.*, 2009). At the beginning of this task subjects are not informed about the rules concerning gains and losses. They have to make a series of card choices that lead to small gains with small penalties, or big gains with big penalties (Basar *et al.*, 2010; Clark, 2010). This task activates several cognitive processes and has an important emotional component, since limbic system is also involved (Robert *et al.*, 2009). On the other hand, the WCST consists in cards with different geometric figures that differ in their color (green, blue, red and yellow), number (1 to 4) and shape (star, circle, triangle and cross). Subjects have to find out how to organize the cards. When the task is successfully executed the rules change and the individual has to reorganize the cards according to new rules (Dehaene and Changeux, 1991).

In order to study rodent decision-making a rat version of IGT, the Rat Gambling Task (RGT), was developed (Rivalan *et al.*, 2011). However, results from this test are not easy to analyze, since study variables are not independent. In RGT, like in IGT, big rewards are associated to big punishments. Thus, our laboratory developed a new test in which risk and gain can be independently manipulated, the Minho Gambling Task (MGT). In this task rats can choose between safe options, which ensure the delivery of a small reward, and risky options, in which,

animals may or may not receive bigger rewards. Importantly, both probability and size of the reward can be independently manipulated.

Previous works developed in our laboratory has shown that stressed animals became resistant to reward value change, maintaining their previous behavior. Moreover, animals submitted to chronic stress have less risky behaviors, when compared to non stressed control animals (Morgado *et al.*, 2011).

1.8. Main goals

The main goal of this thesis is to understand the dopaminergic modulation in decision-making processes and how it is altered upon chronic stress exposure. Specifically, we aim to:

1. Correlate levels of catecholamines and 5-HT, as well as their metabolites, in brain regions involved in decision-making with animal performance in MGT, in the presence or absence of chronic stress.

2. Correlate the expression of some genes, namely, D1 (*Drd1a*), D2 (*Drd2*) and D3 (*Drd3*) DA receptors, BDNF (*Bdnf*), Trk B (*NtrK2*) and CB1 (*Cnr1*) in brain regions involved in decision-making with animal performance in MGT, in the presence or absence of chronic stress.

3. Analyze the effect of Quinpirole, a D2/D3 receptor agonist, in stressed animals, in a decision-making task.

4. Correlate NAcc core and shell volume and cells number with animal performance in MGT, in the presence or absence of chronic stress.

Chapter 2

Material and Methods

Chapter 2 – Material and Methods

This thesis is based on the study of decision-making behavior in animal models. The choice of an animal model is an important step that should take into account the sort of experience to be achieved. An animal model ought to reproduce as close as possible the condition in study. It should be commercially available in several inbred strains, so that results can be validated. It is very important that researchers can easily manipulate these animals. Moreover, an animal model should be robust and have a lifetime sufficiently large so that results can be meaningful (Chow *et al.*, 2007; Paxinos and Watson, 1998).

In this work we used male Wistar Han rats (Charles River Laboratories, Barcelona, Spain), with 10 weeks at the beginning of the experiment. Animals were housed in pairs under animal facility conditions: room temperature (RT) of 22°C, 12 hours lights/12 hours dark cycle, light on at 8:00 AM, *ad libitum* access to food and water. All the procedures were conducted in accordance with European Union Directive 86/609/EEC and National Institutes of Health guidelines on animal care and experimentation.

2.1. Behavior procedures

In other to assess decision-making behavior, animals were submitted to MGT in the 5-Hole Box (TSE Systems, Germany) whose functioning was controlled by TSE Operant Behavior software (TSE Systems, Germany). The apparatus consists of a box in which one of the walls is slightly curved, with 5 holes each of them with a light. The opposite wall has a sixth hole connected to an external *pellet* dispenser (Bari *et al.*, 2008).

In MGT rats can choose between safe and risky options. In safe options, which are linked to a hole without light associated, animals receive one sucrose *pellet* (BioServ, USA) with 100% of probability. On the other hand, risky options are connected with holes with associated light. When animals choose these options, they have 25% probability to receive 4 *pellets* (4Risk:1Safe or neutral protocol – 4R:1S). In order to select options, animals have to do a nose-poke in one of the holes and then collect a food *pellet* from the food dispenser. This test ends when animals perform 100 trials or after 30 minutes (Figure 3).

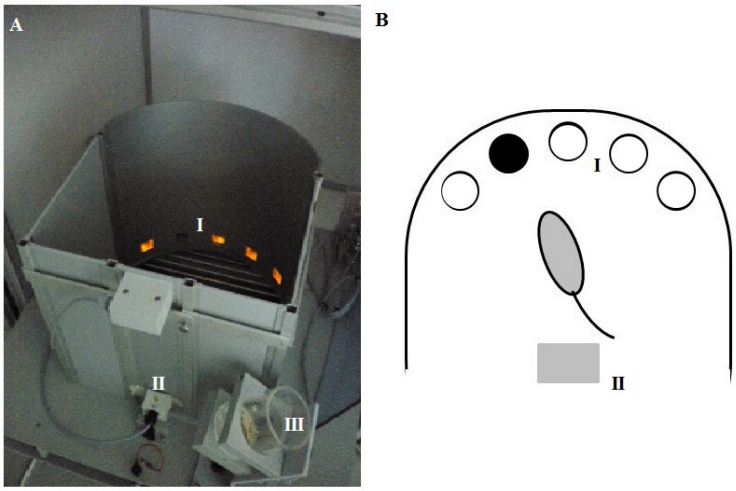

Figure 3 - Decision-making can be evaluated using the MGT performed in the 5-Hole Box. Animals select their option by performing a nose-poke in the apertures (I) and collect the rewards (*pellets*) on an aperture on the opposite wall (II) delivered by the *pellet* dispenser (III).

2.2. Chronic unpredictable stress

To demonstrate the effects of chronic-stress in decision-making, rats were submitted to chronic unpredictable stress (CUS), for 28 days, as mentioned before. CUS consists in animal exposure to stressful situations presented randomly, once per day. Thus, animals were exposed to the aversive stimulus described in Table 1. The type of stimulus and time of presentation were random. (Cerqueira *et al.*, 2007). In this stress protocol, corticosteroids levels are usually maintained elevated, unlike other stress protocols that cause habituation (Sousa *et al.*, 1997).

Table 1 - Stress stimuli description

Stressful situations	Stress duration	Stress description
Restraint	1 hour	Animals were placed, in pairs, in plastic boxes
Overcrowding	1 hour	Groups of animals, of 4 to 6 rats, were placed in the same cage
Shaking	30 minutes	Groups of animals, of 4 to 6 rats, are placed in plastic bags and shaked
Hot drier	15-20 minutes	Animal cage covers were removed and animals were submitted to drier heat
Cold water	1 hour	Groups of animals, of 4 to 6 rats, were placed in high buckets with 5 cm of water (18°C)

2.3. Experiment 1

In the first experiment 8 rats were submitted to 20 days of MGT learning in the 5-Hole Box. Then animals were randomly divided in stress and control (CTR) groups (4 animals per group), and submitted to CUS and gentle handling, respectively, for 28 days. In order to better

understand rats' behavioral flexibility, they were submitted to three different variants of the MGT: the neutral (described above), risk and safe protocols. In the risk protocol, the number of rewards received in the risk options doubles (8R:1S); while in the safe protocol, safe options lead to a gain of 2 *pellets* (4R:2S). Each of these variants was daily performed for 13 days with 3 days break between protocols (Fig.). During learning and MGT variants, animals were fed only one hour per day.

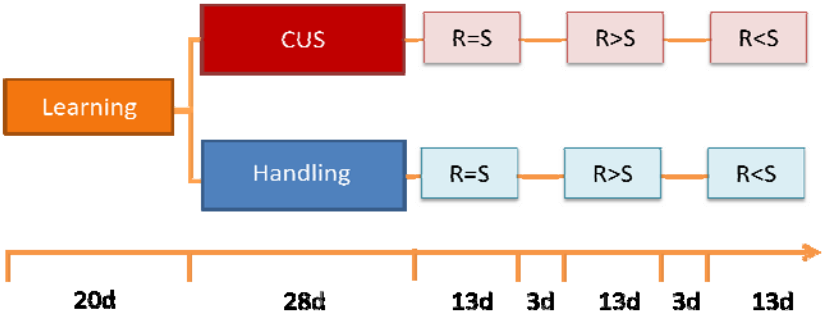

Figure 4 - Experiment 1 protocol description.

At the end of MGT protocol animals were sacrificed, in order to determine the volume and cell number of different brain regions. Thus, rats were anesthetized intraperitoneally with 500 µL sodium pentobarbital and submitted to a transcardial perfusion with 4% paraformaldehyde (PFA). Then brains were removed and fixated in PFA for 4 weeks, which allows brain structures to be preserved (Hughes and Mehmet, 2003). After embedding of the brains in glycolmethacrylate (Tecnovit 7100; Heraeus Kulzer, Germany) they were cut in 30 µm sections, placed on a noncoated glass slide, stained with Giemsa, and mounted with Entellan New (Merck, Germany).

The NAcc core and shell were analyzed in order to determine their volume and total cell number. Sections containing this structure were randomly selected, starting at a random position and with predetermined uniform intervals (West *et al.*, 1991). Thus, every three sections were used to outline the NAcc core and shell areas according to Paxinos and Watson atlas (Paxinos and Watson, 1998) description using the StereoInvestigator® software (Microbrightfield, USA) and a camera (DXC-390; Sony, Japan) attached to a motorized microscope (Axioplan 2; Carl Zeiss, Germany). Volumes were determined by the Cavalieri's principle. Briefly, the volume is estimated by point counting inside the region of interest, using a virtual equally spaced grid which is randomly superimposed onto every selected section (West and Gundersen, 1991).

Cell numbers were estimated using the optical fractionators stereology method. (West *et al.*, 1991). For this, a virtual three-dimensional box (30 x 30 x 15 µm) was placed in each point of the grid used for volume estimation, on sections previously used to outline the NAcc. Neurons whose nucleus was focused within the grid box were counted. It was possible to distinguish neurons from other brain cells, since their nucleus is bigger (Peinado *et al.*, 1997).

2.4. Experiment 2

The second experiment was slightly different, once MGT learning lasted 15 days and each of task variants was performed for 7 days. Moreover, in other to characterize changes in dopaminergic system, each group was subdivided in two subgroups that were intraperitoneally injected with a D2/D3 dopamine receptor agonist, Quinpirole (Quin; 0.15 mg/kg, Sigma-Aldrich, Germany), or with saline 30 minutes before MGT (Figure 5). Thus, 14 animals were used as CTR and 16 animals were submitted to CUS. Seven CTR and 8 CUS rats were injected with Quinpirole (CTR+Quin, CUS+Quin), while the same amount of animals was injected with a vehicle (Vehic) (CTR+ Vehic, CUS+Vehic), saline solution. Like in the experiment 1, during learning and MGT variants, animals were fed only one hour per day.

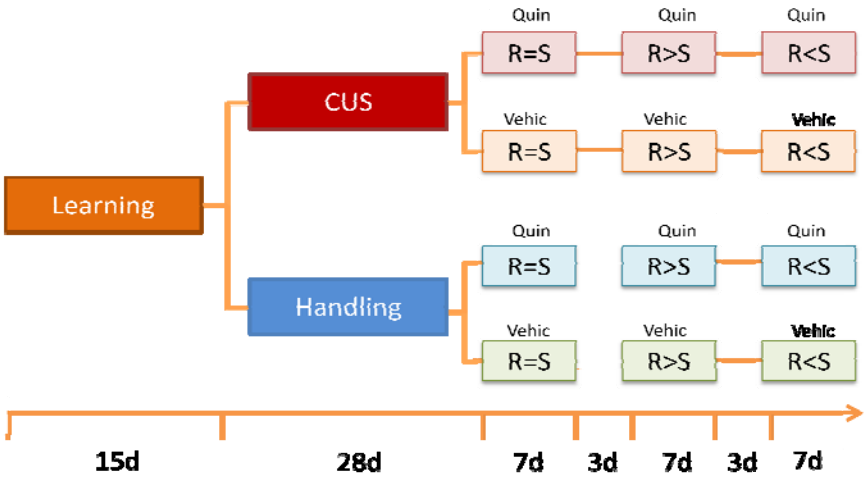

Figure 5 - Experiment 2 protocol description. Quin and Vehic indicate animals groups injected with Quinpirole and saline, respectively.

As in the first experiment, animals were sacrificed after MGT protocol. However, after intraperitoneal anesthesia with 500 µL sodium pentobarbital, animals were decapitated, heads were placed in liquid nitrogen for a few seconds and brains were quickly removed. The NAcc core and shell, Hipp, PFC, DS and OFC were dissected, using a microscope, according to Paxinos and Watson (Paxinos and Watson, 1998) atlas description. The Hipp, PFC, DS and OFC of each animal were divided in two and stored in two different *eppendorfs* so that they could be analyzed by both high performance liquid chromatography with electrochemical detection (HPLC-EC) and quantitative real time polymerase chain reaction (qRT-PCR). The NAcc core and shell were just analyzed by HPLC-EC. Samples were frozen at -80°C until HPLC-EC and qRT-PCR analysis.

2.4.1. Corticosterone levels measurement in animals' serum

Stress induces the activation of the HPA axis, which leads to corticosterone release by adrenal glands (Squire *et al.*, 2008). As mentioned before, the secretion of this hormone is regulated by a complex negative feedback involving the HPA axis, adrenals and autonomous nervous systems. In order to assess this stress hormone levels in serum, blood form all animals was collected right after sacrifice (in the morning) and corticosterone levels were measured by radioimmunoassay (RIA), using ImmuChem^{TM} Corticosterone-^{125}I Kit (MP Biomedicals, LLC, USA). RIA is a sensitive assay used to measure the concentration of antigen molecules, such as hormones, using radioactive markers. These radioactive markers allow the identification and extension of antigen-antibody reaction.

In this test, a specific amount of antibody binds to a hormone (H*) labeled with a radioisotope. The addition of a non-label hormone (H) leads to a decreasing fraction H* bound to antibody. Upon H and H* separation, the radioactivity of one of these fractions or both H and H* are assessed. The results of this evaluation are used to construct a standard curve against which samples are measured.

Thus, blood was collected after animals sacrifice and centrifuged for 10 minutes at 13000 rpm. The supernatant (serum) was, then stored at -80°C until corticosterone analysis. Briefly, rat serum samples were diluted (1:200) with steroid diluents, adding 2.5 µL of serum to 500 µL of diluents. Then, 100 µL of each sample were added to the respective tube in duplicate. After this, nonspecific binding (NSB) tubes (triplicate), total tubes (duplicate) and blanks (triplicate) were arranged according to Table 2; followed by the preparation of corticosterone calibrators and controls 1 and 2. These calibrators contained 0, 25, 50, 100, 250, 500 and 1000 ng/mL of corticosterone. For each tube (triplicates), 100 µL of the respective calibrator were pipetted. To arrange the control tubes it was necessary to reconstitute them with 2.0 mL of distilled water and let them incubate at temperature for 30 minutes. Following the reconstitution, 100 µL were pipetted to the respective tubes (triplicate). Then, 200 µL of Corticosterone ^{125}I was added to all of the tubes (except total tubes), followed immediately by the addition of 200 µL of anti-corticosterone (with the exception of the NSB and total tubes). Tubes were then vortexed and incubated for 2 hours at RT. After this incubation, 500 µL of precipitant solution was added to all tubes (except totals) and all tubes were vortexed thoroughly. All the tubes of the assay (except total tubes) were centrifuged at 2500 rpm for 15 minutes and the supernatant aspired afterwards. Finally, the precipitate was counted in an automatic gamma counter (Perkin Elmer 1470, United Kingdom).

Table 2 - Main steps for the RIA according to the instructions of the kit manufacture

Description	Steroid diluents (µL)	Calibrator/diluted serum samples (µL)	CpB* 125 I (µL)	Anti CpB*		Precipitation Solution (µL)	
NSB	300	0	200	0	Vortex and incubation for 2h at RT	500	Centrifugation, decantation and counting
Total	0	200	0	0		0	
Blank	100	0	200	200		500	
25 ng/mL	0	100	200	200		500	
50 ng/mL	0	100	200	200		500	
100 ng/mL	0	100	200	200		500	
250 ng/mL	0	100	200	200		500	
500 ng/mL	0	100	200	200		500	
1000 ng/mL	0	100	200	200		500	
Control 1	0	100	200	200		500	
Control 2	0	100	200	200		500	
Unknow serum	0	100	200	200		500	
…	0	100	200	200		500	

*Corticosterone

2.4.2. Neurotransmitters measurement by High Performance Liquid Chromatography with Electrochemical Detection

5-HT, its metabolite 5-HIAA, DA, its metabolites DOPAC and HVA, Epi and NE were measured using the HPLC-EC.

Brain regions were incubated at -20°C overnight in percloric acid (0.2M) solution, according to the Table 3.

Table 3 - Percloric acid volume added to each brain region sample

Brain region	Percloric acid Volume (µL)
Hipp	400
NAcc shell	200
PFC	160
DS	
OFC	
NAcc core	

Tissues were sonicated in cold and centrifuged at 5000 rpm for 3 minutes in cold (4°C). The *pellets* obtained were stored at -80°C for protein quantification by the Bradford Method. Supernatant was filtered, centrifuged at 10000 rpm for 8 minutes in cold (4°C) and assayed by HPLC-EC using a Gilson instrument (Gilson, Inc., USA), fitted with an analytical column (Supelco Supelcosil LC-18-3 M; 7.5 cmx4.6 mm; flow rate: 1.0-1.5 mL/min; Supelco, USA). Samples injection in HPLC system was performed using a mobile phase of 0.7 M aqueous monobasic

potassium phosphate, with pH 3.0 adjusted with phosphoric acid, in 222 mg/L of 1-heptanesulfonic acid, 40 mg/L of Na-EDTA and 10% methanol.

The total proteins amount of each brain region was quantified by the Bradford Method. This method allows the detection and quantification of soluble protein through the addition of acidic dye, Coomassie® Brilliant Blue G-250, followed by absorbance measurement. Several dilutions of bovine serum albumin (BSA, 0.1 mg/mL) were used to construct a calibration curve, since this protein presents a similar color to the proteins being assayed.

Regarding the *pellets* previously obtained, they were sonicated and centrifuged, at 3000 rpm for 5 minutes in cold (4°C), after the addiction of phosphate buffer (0.2 M). The volume of phosphate buffer added to each sample is equal to the volume of percloric acid described in the Table 3. Bradford solution (Biorad) was diluted in distilled water (1:4) and distributed in a 96 wells plaque, 200 µL per well. Then 5µL of each sample was added and quantified at a wavelength of 570 nm using a Model 680 Microplate Reader (Biorad). According to Bradford solution manufacturer, samples absorbance should be measured at 595 nm; however the equipment available does not allow analyzing samples at this wavelength. Thus, since the acidic dye present in this solution has maximum absorbance between 465 and 595 nm when bound to protein, samples were measured at 570 nm.

2.4.3. Gene expression measurements by Quantitative Real Time Polymerase Chain Reaction

Quantitative real time PCR is a technique used to quantify gene expression that combines gene amplification and detection in a single step. This can be achieved using fluorescent chemistries that allow the correlation between PCR products and fluorescence intensity (Wong and Medrano, 2005). This sort of reaction is evaluated using the PCR cycle in which the gene amplification is detected for the first time. Gene amplification detection occurs when fluorescence intensity becomes greater than background fluorescence; the value that represents this scenario is known as cycle threshold (C_t). Thus, samples with greater DNA have grater fluorescence intensity, and so smaller C_t (Heid *et al.*, 1996).

Real-time PCR is a very accurate technique, easy to perform, that allows reproducible quantification of gene copies. It allows fast and high throughput assays; having a large dynamic range approaching 1.000.000-fold starting target molecules determination. This technique does not need a post-PCR samples handling, which diminishes the probability of samples contamination (Heid *et al.*, 1996). This method is highly sensitive, allowing the detection of similar mRNA and can be performed using small amounts of RNA template (Wong and Medrano, 2005).

To standardize gene expression an internal control, usually referred as housekeeping gene, must be used. This gene has to present a constant expression level in tissues and cells under

investigation and should not vary in response to experimental treatment (Vandesompele *et al.*, 2002). Thus hypoxanthine guanine phosphoribosyl transferase (*Hprt*; accession number from GenBank: NM_012583) gene was used as internal standard for normalization.

Tissue samples were homogenized in 750 µL Trizol® Reagent (Invitrogen, USA) with 20G needles. After 5 minutes of incubation at RT, 200 µL of chloroform were added and the eppendorfs were shaked vigorously for 15 seconds. Samples were then incubated at RT for 2 to 3 minutes and centrifuged at 8000 rpm for 15 minutes at 4°C. The upper phase was removed to a new eppendorf to which was added 0.5 µL of glycogen and 500 µL of isopropanol. After shaking the tubes for 15 seconds followed by 10 minutes of incubation at RT, the samples were centrifuged at 9000 rpm for 10 minutes at 4°C. In the next step, the *pellet* was washed with 150 µL of 75% ethanol and centrifuged at 5000 rpm for 10 minutes at 4°C. The samples *pellet* was dissolved in 10 µL diethylpyrocarbonate-treated water (DEPC-treated water) and the RNA quantified in the ND-1000 Spectrophotometer (NanoDrop Technologies). Finally the samples were stored at -80°C.

RNA samples were converted in cDNA using the iScript™ cDNA Synthesis Kit (Biorad, USA) according to the recommendations of the manufacturer. Initially, RNA samples were diluted using nuclease-free water, staying each sample with 2 µg of RNA. Then, 15 µL of each sample was distributed to the respective tube and 4 µL of 5x iScript reaction mix and 1 µL of iScript reverse transcriptase were added. It is important to notice that all this process was developed in cold. After, homogenization samples were incubated according to Table 4 and stored at -20°C until analysis.

Table 4 - cDNA synthesis reaction protocol

Time (minutes)	Temperature (°C)
5	25
30	42
5	85
∞	4

Levels of expression of *Bdnf*, *Ntrk2*, *Cnr$_1$*, *Drd1a*, *Drd2* and *Drd3* were determined by qRT-PCR on a CFX96™ Real-Time System combined with a C1000™ Thermal Cycler (Biorad), using QuantiTect® Syber® Green PCR Kit (Quiagen), accordingly to the recommendations of the manufacturer. This kit includes a fluorescent dye that intercalates with the double-stranded DNA molecules emitting a fluorescent signal on binding, allowing the analysis of the different genes. It also comprises a normalization dye that permits the normalization of the fluorescence. Thus, samples were distributed in a 96-well PCR plate to which PCR reagents were added according to Table 5. *Primers* forward and reverse sequence (5´-3´) and their annealing temperature are described in the Table 6.

Table 5 - Reaction mix used in PCR reaction

Components	Volume (µL)
QuantiTect SYBR Green PCR Master Mix: HotStarTaq® DNA Polymerase QuantiTect SYBR Green PCR Buffer dNTP mix, including dUTP SYBR Green I ROX™ passive reference dye 5 mM MgCl2	5
Forward *primer* (10 µM)	0.5
Reverse *primer* (10 µM)	0.5
RNase-Free Water	3
Sample cDNA	1
Total	10

Table 6 - *Primers* characteristics

Gene	Fw and Rw Sequence (5´-3´)	Annealing Temperature (°C)
Hprt	Fw: gcagactttgctttccttgg	59
	Rw: tccactttcgctgatgacac	
Drd1a	Fw: tccttcaagagggagacgaa	60
	Rw: ccacacaaacacatcgaagg	
Drd2	Fw: cattgtctgggtcctgtcct	60
	Rw: gaccagcagagtgacgatga	
Drd3	Fw: ggggtgactgtcctggtcta	60
	Rw: tggcccttattgaaaactgc	
Ntrk2	Fw: caagctgacgagtttgtcca	59
	Rw: gagccacatgatgtcacagg	
BDNF	Fw: gcggcagataaaaagactgc	59
	Rw: gcagccttccttcgtgtaac	
CB_1	Fw: aggagcaaggacctgagaca	60
	Rw: taacggtgctcttgatgcag	

Fw – Forward; **Rw -** Reverse

During PCR reaction samples were submitted to a denaturation step in which temperature is maintained at 95 °C for 15 minutes. Then, samples were amplified and quantified in 40 cycles. In this step temperature varies, 94 °C for 15 seconds, 50-60 °C for 30 seconds and 72 °C for 30 seconds. After this, temperature reaches 65 °C and increases 0.5 °C per second until 95 °C. At the end samples are cooled at 4 °C.

2.5. Statistical analysis

Data were analyzed using IBM SPSS Statistics (Statistical Package for Social Sciences) version 19.0 software (SPSS Inc., USA). Differences between groups were analyzed using the one-way analysis of variance (ANOVA). ANOVA is a parametric test (it is assumed that samples have a normal distribution) that allows comparison of more than two groups. Results were analyzed post-hoc with a non-parametric test, the Tukey's honest significant difference (HSD)

test. Tukey is a one-step multiple comparisons test that assumes that there is an equal variance between results. Correlations between animal behavior and gene expression and neurotransmitters amount was analyzed using the Pearson's product-moment correlation coefficient. This method allows the evaluation of linear relationship between two variables. Differences were considered to be significant if $p<0.05$, but in multiple comparisons these threshold was corrected using the Šidák correction (Chernick and Friis, 2003).

Chapter 3

Results

Chapter 3 – Results

The MGT is a task designed to evaluate the animals' ability to select the most profitable options among safe (small, certain) and risk (larger, but uncertain) choices. This test includes three different protocols: neutral, safe and risk. Results will be presented using an association between the parameter under analysis and the average of the percentage of safe choices per animal and sort of MGT protocol.

3.1. Experiment 1

3.1.1. Volumes and neuronal number

Stereologic analysis provides an information about structural alterations in the NAcc and the sort of behavior those alterations have in individual behavior.

The volume and the total number of cells of both NAcc core and shell were compared with the proportion of safe responses in the different behavior MGT protocols (neutral, risk and safe). A significant correlation was found between the percentage of safe choices and the total volume of NAcc core (p=0.0093; R^2=0.7712), but only in the neutral protocol (Figure 6). It was observed that, in general, animals submitted to CUS (n=4) had higher NAcc volume than CTR (n=3), and an elevated percentage of safe responses. There was no association between the NAcc shell volume and total cell number in any protocol.

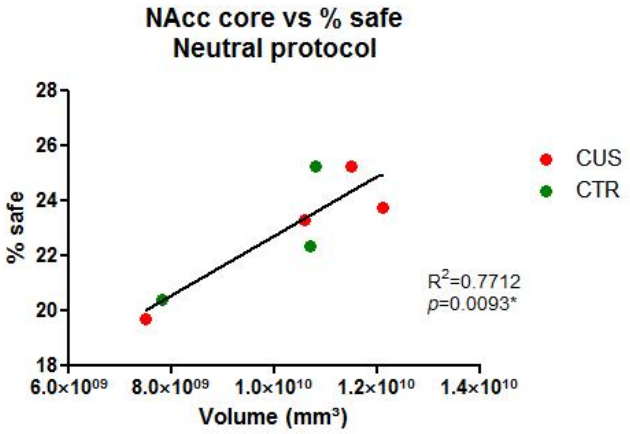

Figure 6 - An increase in NAcc core was associated with a greater amount of safe choices.

3.2. Experiment 2

3.2.1. Biometric parameters and hormonal determinations

The efficacy of CUS protocol can be detected by the evaluation of body weight variation and the increase of corticosterone levels in animals' plasma. All groups presented a similar trend in their weights with an initial increase followed by a slight decreased and stabilization. Between the second and the fifth week, animals were submitted to CUS or to handling according to their groups. Although the weight of the two groups submitted to stress had a slightly smaller increase when compared to handled animals the difference is not significant. From the fifth week onward a decrease o body weight was observed in all groups, and resulted from the food restriction procedures implemented throughout the testing period. Throughout weeks it was possible to observe that control groups weight was maintained higher than those from stresses groups (Figure 7).

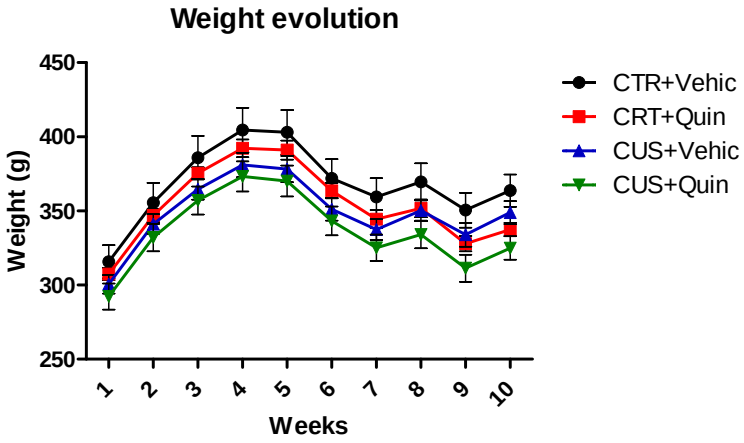

Figure 7 - Animals submitted to CUS present a smaller increase in their body weight, when compared to CTR groups.

Regarding the evaluation of stress protocol efficacy, the exposure to CUS did not increase serum corticosterone levels. In fact, control animals presented a higher level of this hormone (data not shown). Moreover, there was no correlation between corticosterone levels and percentage of safe choice in any protocol.

3.2.2. Behavior

MGT revealed behavior disparity. The Figure 8 illustrates the average of each individual behavior on the different MGT protocols. All groups presented animals with preference for safe and risk behaviors, which means there was no standard group behavior. An animal from the CUS+Quin group become sick and had to be excluded from the trial.

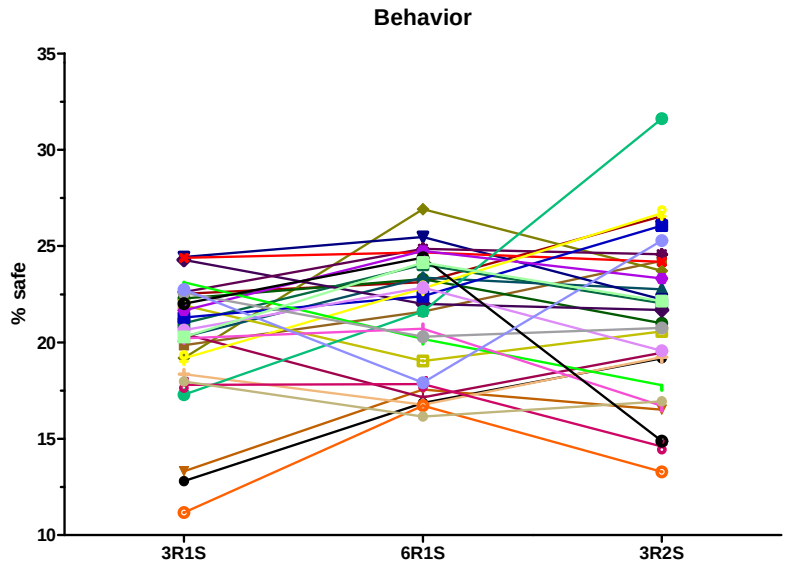

Figure 8 - During MGT behavioral differences were observed independently of group assignment.

3.2.3. Neurochemical correlates

In order to understand the effect of levels of neurotransmitters and their metabolites - NE, Epi, DA, DOPAC, 5-HIAA, 5-HT and HVA - in decision-making, we decided to quantify the amount of these molecules in the NAcc shell and core, PFC, OFC, Hipp and DS. There was no correlation between these molecules concentrations in the different brain regions, and the percentage of safe option in neutral and safe protocols. However, it was observed some significant associations in the risk protocol. In the Hipp, levels of 5-HT and its metabolite 5-HIAA presented a significant correlation with the percentage of safe choices (p=0.019, R^2=0.300; p=0.002, R^2=0.447) (Figure 9). Due to problems in the processing of samples from the Hipp which resulted in the loss of some batches, final sample sizes for this region were reduced (n=6, CTR+Vehic; n=4, CTR+Quin; n=5, CUS+Vehic; n=3, CUS+Quin).

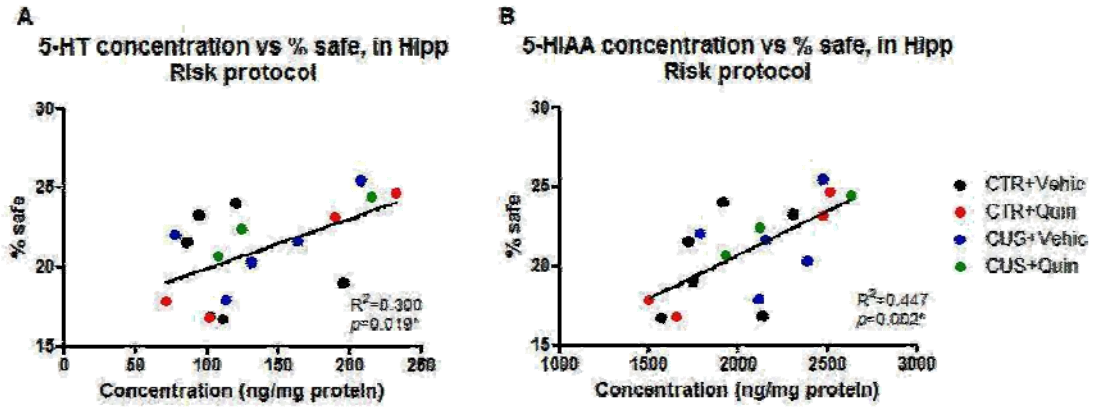

Figure 9 - An increase in 5-HT (A) and 5-HIAA (B) was associated with risk-aversive behavior, in the risk protocol.

The PFC presented an association between 5-HT turnover and the proportion of safe choices (p=0.006, R^2=0.245) (Figure 10).

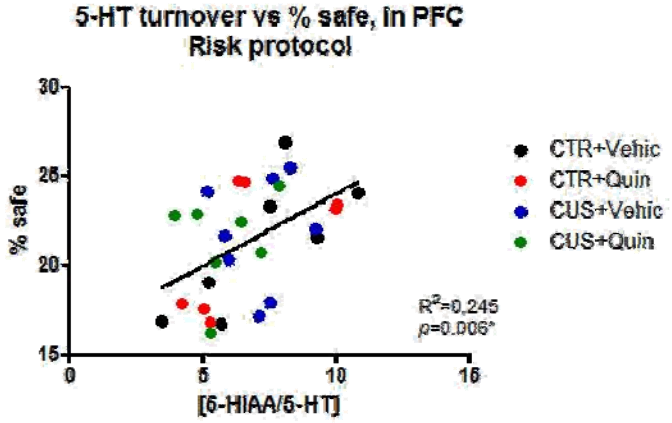

Figure 10 - An increase in 5-HT turnover was observed in the PFC, in the risk protocol; showing that enhanced 5-HT degradation is correlated with an increase of safe responses.

In the DS, DA turnover was inversely correlated with the percentage of secure options (p=0.008, R^2=0.244) (Figure 11).

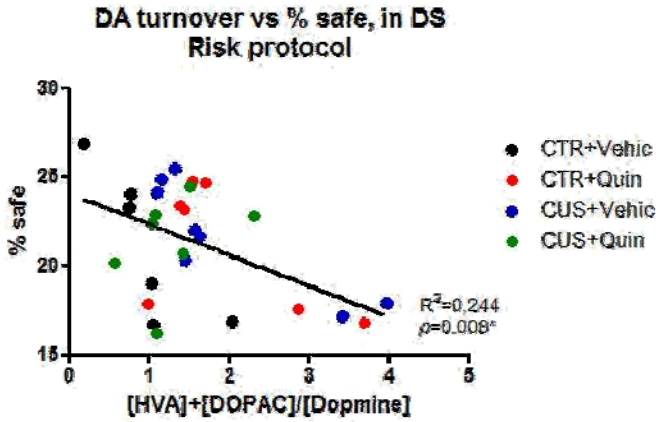

Figure 11 - In the DS, DA degradation diminished with the increase of safe options, in the risk protocol.

3.2.4. Molecular correlates

At molecular level, the expression of several genes involved in decision-making was determined. Genes like *Bdnf*, *Ntrk2*, *Cnr*$_1$, *Drd1a*, *Drd2* and *Drd3* were assessed in the PFC, OFC, Hipp and DS. The levels of these genes expression did not presented any correlation with the proportion of safe options, in both neutral and safe protocol.

In the risk protocol, the PFC was the only brain region in which the percentage of safe choices had significant correlations with gene expression. It was observed an association with

the expression of *Bdnf*, Ntr*k2* and *Cnr*$_1$ (p=0.026, R^2=0.171; p=0.006, R^2=0.249; p=0.007, R^2=0.241) (Figure 12)

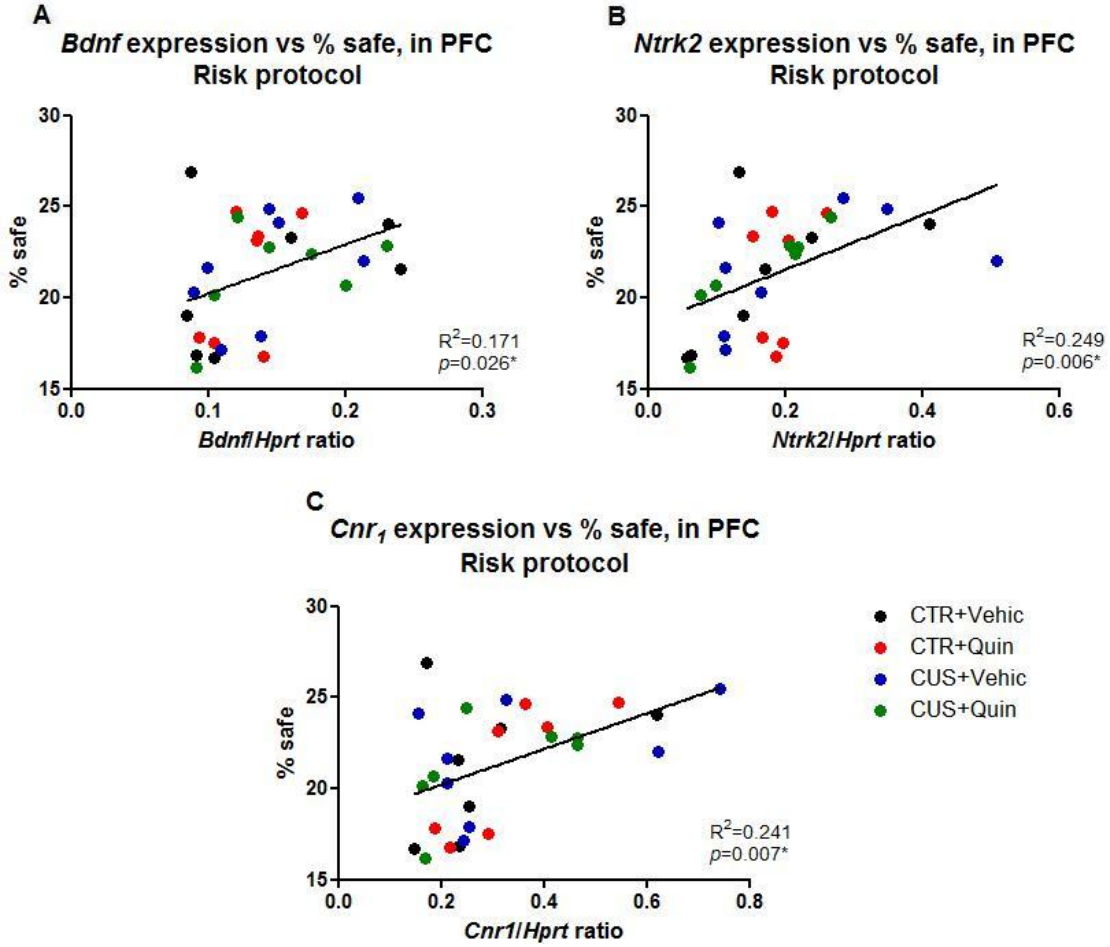

Figure 12 - In the PFC was observed that animals that had risk-aversive behaviors present higher expression of the genes *Bdnf* (A), *Ntrk2* (B), *Cnr*$_1$ (C), in the risk protocol.

Chapter 4

Discussion

Chapter 4 – Discussion

Decision-making is a complex behavior that includes several processes, which embrace the selection of the most appropriate option, according to individual needs. Thus, it is important to develop the ability to get the maximum reward and take the minimum risk (Knutson and Bossaerts, 2007). Subjects with disturbances in these processes may become unable to evaluate the environment that surrounds them, developing impulsive behaviors and decreasing the profits they can achieve with their actions (Moeller *et al.*, 2001; Pattij and Vanderschuren, 2008). However, it should be taken into account that the tendency to make certain sort of decisions is closely related to the characteristic of each subject. Thus, not all individuals have the same predisposition to choose risky options. Some individuals, risk-aversive subjects, prefer options with low or none risk associated, while others, in a similar situation, have the opposite behavior (Schultz, 2010).

Previous studies in our laboratory showed that animals not submitted to stress do not present preference for risk or safe options in the neutral protocol of the MGT. In other words, animals tend to give 20% of safe responses and 80% risky ones, this being proportional to the number of holes attributed to each sort of behavior (four holes for risk options and one hole for safe responses). Importantly, this condition allows us to study the effects of different manipulations on the animals risk preference. Indeed, percentages of safe options higher than 20% correspond to safe behaviors; while smaller percentages indicate the preference for risk behaviors (Morgado *et al.*, 2011).

4.1. Experiment 1

In this experiment we observed a significant association between NAcc core volume and the percentage of safe choices. We also verified that animals that presented a higher proportion of safe choices were mainly animals submitted to CUS. As previously described, NAcc is a target of chronic stress, which induces hypertrophy of this structure (Dias-Ferreira *et al.*, 2009). It is already known that the core region of the NAcc is a structure sensitive to rewards probability, and that damages in this brain region make animals risk-aversive (Basar *et al.*, 2010; Cardinal and Howes, 2005). Surprisingly, we showed that increased NAcc core volumes, particularly in animals' submitted to chronic stress, correlate with a risk-aversive behavior. However, it is important to notice that the number of animals used in this experiment was reduced, thus it is necessary to develop further works.

4.2. Experiment 2

The data obtained in this experiment demonstrated that overtime all groups presented a similar weight pattern. However, it would be expected that, after stress, animals submitted to CUS presented a significantly smaller body weight increase when compared to CTR groups (Cerqueira *et al.*, 2007). At the end of the stress period (5th week), animals performance in the MGT was evaluated, which implies food deprivation, and might explain the decrease in their body weights. Moreover, during MGT, animals were injected, which is a stress factor that may also have influenced the general body weight decrease.

Regarding the evaluation of corticosterone levels in serum, we decided not to take into account these hormone values, because of the way how blood samples were collected. Actually, blood samples were collected during animal killing; thus, blood was obtained only after animals were anesthetized. Moreover, samples were not collected all at the same time, but at different time points during the morning sacrifice. Given the well described diurnal variation of corticosterone levels, blood collection should have been done at two different time points: one in the morning between 8:00 and 9:00 A.M., at the beginning of rat inactive period; and another at the end of the day, at the beginning of rat active period, when they are wakening; so that circadian cycle oscillations could be taken into account. This cycle lasts about 24 hours, in constant environment, and presents some hormonal oscillations, that include high corticosterone levels in the evening in rats (Squire *et al.*, 2008).

In this experiment we evaluated the performance of animals submitted to four different treatments – CTR+Vehic, CTR+Quin, CUS+Vehic, CUS+Qui – in the different protocols of the MGT. Animals presented a very heterogeneous behavior, independent of the group to which they belonged. In all groups we found animals whose actions were mainly risky and animals that prefer safer options. These different behaviors obtained from animals submitted to the same group treatment seem to indicate that animals, like humans, may also have a tendency to develop behaviors with specific characteristics. One explanation to the risk each individual is willing take may be based on the value it attributes to reinforcers and how it discounts them as their value increases or decreases (Ho *et al.*, 1999). As this might also be related to neurochemical differences in the brain, we decided to analyze the catecholamine content in the key areas involved in decision making.

Goal-directed decision-making involves the need to predict and evaluate the value of future outcomes. In order to increase the probability to obtain the highest gains, it is important to plan, evaluating the options available, predicting rewards and selecting the action, processes that are modulated by the Hipp. This structure has also been described to play an important role in the evaluation of potential future circumstances (Johnson *et al.*, 2007; Squire *et al.*, 2008). Moreover, the Hipp is sensitive to delay delivery of rewards. In fact, studies revealed that animals with lesions in the Hipp become more impulsive preferring immediate small rewards over large delayed outcomes (Cheung and Cardinal, 2005; McHugh

et al., 2008). We observed that animals which had risk-aversive behaviors in situations where risky options were favorable (risk protocol) had higher amounts of 5-HT and its metabolite, 5-HIAA, in this brain structure. These results are consistent with previous studies that had associated low levels of 5-HT and its metabolite, 5-HIAA, with risk taking (Cardinal, 2006). It has been demonstrated that individuals with elevated levels of these neurotransmitters prefer immediate small rewards over larger but delayed reward, which is also supported by the idea of 5-HT involvement in impulsive behaviors (Pattij and Vanderschuren, 2008; Wogar *et al.*, 1993). However, the effect of 5-HT in impulsivity is a controversial issue. Several studies have demonstrated the opposite effect of this neurotransmitter in this sort of behavior (Homberg *et al.*, 2007). This may be due to the existence of fourteen different receptors subtypes each belonging to one of seven receptors families (Barnes and Sharp, 1999). Nevertheless, the increase of safe options preference in these animals may be involved in their inability to wait for more profitable rewards; which may be explained by the increase of 5-HT and 5-HIAA in these animals.

In attention testing, the number of premature nose-pokes is used has a parameter of impulsivity. The number of impulsive nose-pokes, in this sort of tests, has been associated with 5-HIAA/5-HT ratio, which means animals with higher 5-HT turnover have more impulsive behaviors (Puumala and Sirvio, 1998). In order to better adapt to new circumstances individuals have to be able to develop an adequate shifting behavior. To do so, individuals have to detect alteration in the stimulus and examine how this new stimulus may benefit them; these processes are modulated by the PFC (Block *et al.*, 2006). Moreover this brain structure allows the creation of a linkage between stimuli and outcomes (Narayanan and Laubach, 2008). Thus, it is not surprising that animals with higher 5-HIAA/5-HT ratio, in the PFC, preferred mainly safe options in the risk protocol. These results seem to indicate that an increase in 5-HT degradation diminished animals' capacity to detect alterations in the value of rewards, preventing them to adapt their behaviors to the new situation and choose the more profitable option.

The dorsal region of the striatum is involved in motivated behavior, supporting the development of probabilistic classification learning (Squire *et al.*, 2008), which leads to a rapid association between actions and rewards (Yin *et al.*, 2009). Moreover, DS codes incentive affective properties, which also helps to build responses according to stimuli magnitude in the flexible goal-directed actions (Delgado *et al.*, 2003; Johnson *et al.*, 2007; Schultz, 2006).

In the DS, higher number of safe responses was associated with smaller DA turnover, which means that less DA is being degraded. A decrease in DA consumption is associated with enhanced risk-aversive behaviors; which is supported by other studies. In fact, it has been demonstrated that in tasks in which animals' effort to obtain reward is measured, an increase of Da levels is accompanied by an increase of effort animal is willing to take, and thus by an increase number of risky options (Assadi *et al.*, 2009). Thus, it seem that an increase in DA

degradation may help the evaluation of reward value, which directs animals behavior to the option with more gains, in the case of the risk protocol, the risky options.

As mentioned before, the PFC has an essential role in the evaluation of the information about changing reward probabilities, allowing individuals to adapt to their behaviors according to the situation in which they are inserted (Onge *et al.*, 2011). On the other hand, BDNF is an important protein, whose action modulates several processes that include neurogenesis, cell survival, connectivity and synaptic efficacy, being involved in the release of several neurotransmitters, namely 5-HT (Oades *et al.*, 2008; Southwick *et al.*, 2005). Increased levels of this neurotrophic factor increase 5-HT neurotransmission, which potentiates impulsivity (Lyons *et al.*, 1999). Moreover, BDNF binding to its receptor, Trk B, is involved in memory, learning and appetitive behavior (Nestler and Carlezon, 2006; Yamada *et al.*, 2001). Thus, the increase of safe choices in animals with higher expression of BDNF, in the PFC, is consistent with the results previously presented that show a correlation between 5-HT and the percentage of safe choices. These data seem to indicate that an increase in the expression of BDNF and its receptors, Trk B, may lead to an increase in 5-HT, which leads to more impulsive behaviors and explain the preference of these animals for more safe, although less profitable options, in the risk protocol.

Regarding the involvement of CB_1 receptors in risk behaviors, it has been demonstrated that the blockage of CB_1 receptors reduces impulsive behaviors (Pattij *et al.*, 2007; Vinod and Hungund, 2006). Our results are in agreement with this study, since animals that expressed reduced levels of this receptor, in the PFC, demonstrated preference for risky options in the risk protocol.

Chapter 5

Conclusions

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Lesions in the NAcc core impair the capacity to change behavior, diminishing individual ability to adapt to new situations and making their behavior risk-aversive (Cardinal and Howes, 2005; Floresco *et al.*, 2006a). As reviewed by Cardinal, damages in this brain structure have also been described to impair rats performance in a task in which they have to choose between immediate uncertain rewards and delayed certain rewards (Cardinal, 2006). In our experiment we further show that NAcc volume is directly correlated with risk-aversion. This might explain why chronic stress exposure, which has been shown to induce hypertrophy of the NAcc core (Dias-Ferreira *et al.*, 2009), makes animals more risk-aversive. In addition to this finding, we also showed that, in risk protocol, risk aversion was directly correlated with the levels of 5-HT and 5-HIAA in the Hipp, with the 5-HT turnover in the PFC and inversely correlated with DA turnover in the DS. Moreover, our results show that the same sort of behavior, in the risk protocol, has connection with the expression of *Bdnf*, *NtrK2* and *Cnr1* in the PFC.

These results demonstrate that although some neurotransmitters concentration and genes expression present correlation with decision-making behaviors in certain brain regions, we do not observe a characteristic group behavior. In fact, in each group animals' present different behaviors, this may indicate that, although the treatment to which they were submitted, individuals do not have the same predisposition to choose risky options.

This study expected to describe changes in dopaminergic system in different brain regions caused by chronic stress. We also intent to verify if the administration of dopamine agonists, e.g. Quinpirole, revert the effect of chronic stress in decision-making; however, our result were not conclusive.

References

Apicella, P., Ljungberg, T., Scarnati, E., Schultz, W., 1991. Responses to reward in monkey dorsal and ventral striatum. Experimental Brain Research. 85**,** 491-500.

Arias-Carríon, Ó., Pöppel, E., 2007. Dopamine, learning, and reward-seeking behavior. Acta Neurobiologiae Experimentalis. 67**,** 481-488.

Assadi, S.M., Yücel, M., Pantelis, C., 2009. Dopamine modulates neural networks involved in effort-based decision-making. Neuroscience & Biobehavioral Reviews. 33**,** 383-393.

Balleine, B.W., 2007. The Neural Basis of Choice and Decision Making. Journal of Neuroscience. 27**,** 8159-8160.

Balleine, B.W., Delgado, M.R., Hikosaka, O., 2007. The Role of the Dorsal Striatum in Reward and Decision-Making. Journal of Neuroscience. 27**,** 8161-8165.

Bari, A., Dalley, J.W., Robbins, T.W., 2008. The application of the 5-choice serial reaction time task for the assessment of visual attentional processes and impulse control in rats. Nature Protocols. 3**,** 759-767.

Barnes, N.M., Sharp, T., 1999. A review of central 5-HT receptors and their function. Neuropharmacology. 38**,** 1083–1152.

Basar, K., Sesia, T., Groenewegen, H., Steinbusch, H.W.M., Visser-Vandewalle, V., Temel, Y., 2010. Nucleus accumbens and impulsivity. Progress in Neurobiology. 92**,** 533-557.

Bechara, A., Damasio, H., Damasio, A.R., Lee, G.P., 1999. Different Contributions of the Human Amygdala and Ventromedial Prefrontal Cortex to Decision-Making. The Journal of Neuroscience. 19**,** 5473–5481.

Besson, M., Belin, D., McNamara, R., Theobald, D.E.H., Castel, A., Beckett, V.L., Crittenden, B.M., Newman, A.H., Everitt, B.J., Robbins, T.W., Dalley, J.W., 2009. Dissociable Control of Impulsivity in Rats by Dopamine D2/3 Receptors in the Core and Shell Subregions of the Nucleus Accumbens. Neuropsychopharmacology. 35**,** 560-569.

Björklund, A., Hökfelt, T., 2005. Handbook of Chemical Neuroanatomy, Vol. Volume 21 - Dopamine, Elsevier, San Diego, U.S.A.

Björklund, A., Dunnett, S.B., 2007. Dopamine neuron systems in the brain: an update. Trends in Neurosciences. 30**,** 194-202.

Block, A.E., Dhanji, H., Thompson-Tardif, S.F., Floresco, S.B., 2006. Thalamic-Prefrontal Cortical-Ventral Striatal Circuitry Mediates Dissociable Components of Strategy Set Shifting. Cerebral Cortex. 17**,** 1625-1636.

Camerer, C., 1992. Recent Developments in Modeling Preferences: Uncertainty and Ambiguity. Journal of Risk and Uncertainty. 5**,** 325-370.

Cardinal, R., 2006. Neural systems implicated in delayed and probabilistic reinforcement. Neural Networks. 19**,** 1277-1301.

Cardinal, R.N., Robbins, T.W., Everitt, B.J., 2000. The effects of d -amphetamine, chlordiazepoxide, a-flupenthixol and behavioural manipulations on choice of signalled and unsignalled delayed reinforcement in rats. Psychopharmacology. 152**,** 362-375.

Cardinal, R.N., Howes, N.J., 2005. Effects of lesions of the nucleus accumbens core on choice between small certain rewards and large uncertain rewards in rats. BMC Neuroscience. 6**,** 37.

Cerqueira, J.J., Mailliet, F., Almeida, O.F.X., Jay, T.M., Sousa, N., 2007. The Prefrontal Cortex as a Key Target of the Maladaptive Response to Stress. Journal of Neuroscience. 27**,** 2781-2787.

Chang, J.-Y., Sawyer, S.F., Lee, R.-S., Woodward, D.J., 1994. Electrophysiological and Pharmacological Evidence for the Role of the Nucleus Accumbens in Cocaine Self-Administration in Freely Moving Rats. The Journal of Neuroscience. 74**,** 1224-1244.

Chernick, M.R., Friis, R.H., 2003. Introductory Biostatistics for the Health Sciences - Modern Applications Including Bootstrap, Vol., Wiley-Interscience, Canada.

Cheung, T.H.C., Cardinal, R.N., 2005. Hippocampal lesions facilitate instrumental learning with delayed reinforcement but induce impulsive choice in rat. BMC Neuroscience. 6**,** 36.

Chow, P.K.H., Ng, R.T.H., Ogden, B.E., 2007. Using animal models in biomedical research: A primer for the investigator, Vol., World Scientific Publishing, Singapore.

Chrousos, G.P., 2009. Stress and disorders of the stress system. Nature Reviews Endocrinology. 5**,** 374-381.

Clark, L., Bechara, A., Damasio, H., Aitken, M.R.F., Sahakian, B.J., Robbins, T.W., 2007. Differential effects of insular and ventromedial prefrontal cortex lesions on risky decision-making. Brain. 131**,** 1311-1322.

Clark, L., 2010. Decision-making during gambling: an integration of cognitive and psychobiological approaches. Philosophical Transactions of The Royal Society Biological Sciences. 365**,** 316-330.

Clarke, H.F., Dalley, J.W., Crofts, H.S., Robbins, T.W., Roberts, A.C., 2004. Cognitive inflexibility after prefrontal serotonin depletion. Science. 304**,** 878–880.

Cools, R., 2006. Dopaminergic modulation of cognitive function-implications for l-DOPA treatment in Parkinson's disease. Neuroscience & Biobehavioral Reviews. 30**,** 1-23.

Cossu, G., Ledent, C., Fattore, L., Imperato, A., Böhme, G.A., Parmentier, M., Fratta, W., 2001. Cannabinoid CB1 receptor knockout mice fail to self-administer morphine but not other drugs of abuse. Behavioural Brain Research. 118**,** 61–65.

Dauer, W., Przedborski, S., 2003. Parkinson’s Disease: Mechanisms and Models. Neuron. 39**,** 889–909.

Day, J.J., Jones, J.L., Wightman, R.M., Carelli, R.M., 2010. Phasic Nucleus Accumbens Dopamine Release Encodes Effort- and Delay-Related Costs. Biological Psychiatry. 68**,** 306-309.

de Kloet, E.R., Jöels, M., Holsboer, F., 2005. Stress and the brain: From adaptation to disease. Nature Reviews Neuroscience. 6**,** 463-475.

Dehaene, S., Changeux, J.-P., 1991. The Wisconsin Card Sorting Test: Theoretical Analysis and Modeling in a Neuronal Network. Cerebral Cortex. l**,** 62-79.

Delgado, M.R., Locke, H.M., Stenger, V.A., Fiez, J.A., 2003. Dorsal striatum responses to reward and punishment: Effects of valence and magnitude manipulations. Cognitive, Affective, & Behavioral Neuroscience. 3**,** 27-38.

Dias-Ferreira, E., Sousa, J.C., Melo, I., Morgado, P., Mesquita, A.R., Cerqueira, J.J., Costa, R.M., Sousa, N., 2009. Chronic Stress Causes Frontostriatal Reorganization and Affects Decision-Making. Science. 325**,** 621-625.

Doya, K., 2008. Modulators of decision making. Nature Neuroscience. 11**,** 410-416.

Fiorillo, C.D., Tobler, P.N., W., S., 2003. Discrete coding of reward probability and uncertainty by dopamine neurons. Science. 299**,** 1898–902.

Floresco, S.B., Ghods-Sharifi, S., Vexelman, C., Magyar, O., 2006a. Dissociable Roles for the Nucleus Accumbens Core and Shell in Regulating Set Shifting. Journal of Neuroscience. 26**,** 2449-2457.

Floresco, S.B., Magyar, O., Ghods-Sharifi, S., Vexelman, C., Tse, M.T.L., 2006b. Multiple Dopamine Receptor Subtypes in the Medial Prefrontal Cortex of the Rat Regulate Set-Shifting. Neuropsychopharmacology. 31**,** 297-309.

Floresco, S.B., 2007. Dopaminergic regulation of limbic-striatal interplay. Journal of Psychiatry Neuroscience. 32**,** 400-11.

Gasic, G.P., Smoller, J.W., Perlis, R.H., Sun, M., Lee, S., Kim, B.W., Lee, M.J., Holt, D.J., Blood, A.J., Makris, N., Kennedy, D.K., Hoge, R.D., Calhoun, J., Fava, M., Gusella, J.F., Breiter, H.C., 2009. BDNF, Relative Preference, and Reward Circuitry Responses to Emotional Communication. Am J Med Genet Part B. 150B**,** 762–781.

Gelman, J.S., Fricker, L.D., 2010. Hemopressin and Other Bioactive Peptides from Cytosolic Proteins: Are These Non-Classical Neuropeptides? The AAPS Journal. 12**,** 279-289.

Goto, Y., Otani, S., Grace, A., 2007. The Yin and Yang of dopamine release: a new perspective. Neuropharmacology. 53**,** 583-587.

Gruber, A.J., Hussain, R.J., O'Donnell, P., 2009. The Nucleus Accumbens: A Switchboard for Goal-Directed Behaviors. PloS ONE. 4**,** e5062.

Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M., 1996. Real time quantitative PCR. Genome Research. 6**,** 986-994.

Herkenham, M., Lynn, A.B., Little, M.D., Johnson, M.R., Melvin, L.S., De Costa, B.R., Riceo, K.C., 1990. Cannabinoid receptor localization in brain. Proceedings of the National Academy of Sciences USA. 87**,** 1932-1936.

Hilário, M.R.F., 2007. Endocannabinoid signaling is critical for habit formation. Frontiers in Integrative Neuroscience. 1.

Ho, M.Y., Mobini, S., Chiang, T.J., Bradshaw, C.M., Szabadi, E., 1999. Theory and method in the quantitative analysis of "impulsive choice" behaviour: Implications for psychopharmacology. Psychopharmacology. 146**,** 362-372.

Holland, P., 2004. Amygdala–frontal interactions and reward expectancy. Current Opinion in Neurobiology. 14**,** 148-155.

Homberg, J.R., Pattij, T., Janssen, M.C.W., Ronken, E., de Boer, S.F., Schoffelmeer, A.N.M., Cuppen, E., 2007. Serotonin transporter deficiency in rats improves inhibitory control but not behavioural flexibility. European Journal of Neuroscience. 26**,** 2066–2073.

Hughes, D., Mehmet, H., 2003. Cell proliferation & Apoptosis, Vol., BIOS Scientific Publishers, Oxford, UK.

Ikemoto, S., 2010. Brain reward circuitry beyond the mesolimbic dopamine system: A neurobiological theory. Neuroscience & Biobehavioral Reviews. 35**,** 129-150.

Imamura, A., Uitti, R.J., Wszolek, Z.K., 2006. Dopamine agonist therapy for Parkinson disease and pathological gambling. Parkinsonism & Related Disorders. 12**,** 506-508.

Iversen, L.L., Iversen, S.D., Dunnett, S.B., Björklund, A., 2010. Dopamine Handbook, Vol., Oxford University Press, New York, USA.

Iversen, S.D., Iversen, L.L., 2007. Dopamine: 50 years in perspective. Trends in Neurosciences. 30**,** 188-193.

Joëls, M., Baram, T.Z., 2009. The neuro-symphony of stress. Nature Reviews Neuroscience. 10**,** 459-466.

Johnson, A., van der Meer, M.A.A., Redish, A.D., 2007. Integrating hippocampus and striatum in decision-making. Current Opinion in Neurobiology. 17**,** 692-697.

Kandel, E.R., Schwartz, J.H., Jessel, T.M., 2000. Principles Of Neural Science, Vol., McGraw-Hill, New York, U.S.A.

Knutson, B., Bossaerts, P., 2007. Neural Antecedents of Financial Decisions. Journal of Neuroscience. 27**,** 8174-8177.

Konstandi, M., 2000. Noradrenaline, Dopamine, Serotonin: Different Effects of Psychological Stress on Brain Biogenic Amines in Mice and Rats. Pharmacological Research. 41**,** 341-346.

Kvetnansky, R., Sabban, E.L., Palkovits, M., 2009. Catecholaminergic Systems in Stress: Structural and Molecular Genetic Approaches. Physiological Reviews. 89**,** 535-606.

Levey, A.I., Hersch, S.M., Rye, D.B., Sunaharat, R.K., Niznik, H.B., Kitts, C.A., Price, D.L., Maggio, R., Brann, M.R., CILIAX, B.J., 1993. Localization of D1 and D2 dopamine receptors in brain with subtype-specific antibodies. Proceedings of the National Academy of Sciences USA. 90**,** 8861-8865.

Lupica, C.R., Riegel, A.C., 2005. Endocannabinoid release from midbrain dopamine neurons: A potential substrate for cannabinoid receptor antagonist treatment of addiction. Neuropharmacology. 48**,** 1105-1116.

Lutz, B., 2009. Endocannabinoid signals in the control of emotion. Current Opinion in Pharmacology. 9**,** 46–52.

Lyons, W.E., Mamounas, L.A., Ricaurte, G.A., Coppola, V., Reid, S.W., Bora, S.H., Wihler, C., Koliatsos, V.E., Tessarollo, L., 1999. Brain-derived neurotrophic factor-deficient mice develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities. PNAS. 96**,** 15239–15244.

McEwen, B.S., 2000. The neurobiology of stress: from serenpidity to clinical relevance. Brain Research Interactive. 886**,** 172-189.

McEwen, B.S., 2010. Stress, sex, and neural adaptation to a changing environment: mechanisms of neuronal remodeling. Annals of the New York Academy of Sciences. 1204**,** 38-59.

McEwen, B.S., Eiland, L., Hunter, R.G., Miller, M.M., 2011. Stress and anxiety: Structural plasticity and epigenetic regulation as a consequence of stress. Neuropharmacology.

McHugh, S.B., Campbell, T.G., Taylor, A.M., Rawlings, J.N.P., Bannerman, D.M., 2008. A Role for Dorsal and Ventral Hippocampus in Inter-Temporal Choice Cost – Benefit Decision Making. Behavioral Neuroscience. 122**,** 1-8.

Mehlman, P.T., Higley, J.D., Faucher, I., Lilly, A.A., Taub, D.M., Vickers, J., Suomi, S.J., Linnoila, M., 1994. Low CSF 5-HIAA concentrations and severe aggression and impaired impulse control in nonhuman primates. American Journal of Psychiatry. 151**,** 1485-1491.

Miyazaki, I., Asanuma, M., 2008. Dopaminergic Neuron-Specific Oxidative Stress Caused by Dopamine Itself. Acta Medica Okayama. 62**,** 141-150.

Moeller, F.G., Barratt, E.S., Dougherty, D.M., Schmitz, J.M., Swann, A.C., 2001. Psychiatric Aspects of Impulsivity. Am J Psychiatry. 158**,** 1783–1793.

Mogenson, G.J., Jones, D.L., Yim, C.Y., 1980. From motivation to action: functional interface between the limbic system and the motor system. Progress in Neurobiology. 14**,** 69-97.

Morgado, P., Ribeiro, B., Almeida, A. A., Sousa, N., Cerqueira, J. J., 2011. Decision-making in a rat gambling task is modulated by D2/D3 receptors. Society for Neuroscience Abstracts available online at www.abstractsonline.com

Narayanan, N.S., Laubach, M., 2008. Neuronal Correlates of Post-Error Slowing in the Rat Dorsomedial Prefrontal Cortex. Journal of Neurophysiology. 100**,** 520-525.

Nestler, E.J., Carlezon, W.A., 2006. The Mesolimbic Dopamine Reward Circuit in Depression. Biological Psychiatry. 59**,** 1151-1159.

Neve, K.A., 2010. The Dopamine Receptors, Vol., Humana Press, Portland, U.S.A.

Nicola, S.M., Taha, S.A., Kim, S.W., Fields, H.L., 2005. Nucleus accumbens dopamine release is necessary and sufficient to promote the behavioral response to reward-predictive cues. Neuroscience. 135**,** 1025-1033.

Oades, R.D., Lasky-Su, J., Christiansen, H., Faraone, S.V., Sonuga-Barke, E.J.S., Banaschewski, T., Chen, W., Anney, R.J.L., Buitelaar, J.K., Ebstein, R.P., Franke, B., Gill, M., Miranda, A., Roeyers, H., Rothenberger, A., Sergeant, J.A., Steinhausen, H.-C., Taylor, E.A., Thompson, M., Asherson, P., 2008. The influence of serotonin- and other genes on impulsive behavioral aggression and cognitive impulsivity in children with attention-deficit/hyperactivity disorder (ADHD): Findings from a family-based association test (FBAT) analysis. Behavioral and Brain Functions. 4**,** 48.

Onge, J.R.S., Floresco, S.B., 2009. Dopaminergic Modulation of Risk-Based Decision Making. Neuropsychopharmacology. 34**,** 681-697.

Onge, J.R.S., Chiu, Y.C., Floresco, S.B., 2010. Differential effects of dopaminergic manipulations on risky choice. Psychopharmacology. 211**,** 209-221.

Onge, J.R.S., Abhari, H., Floresco, S.B., 2011. Dissociable Contributions by Prefrontal D1 and D2 Receptors to Risk-Based Decision Making. The Journal of Neuroscience. 31**,** 8625-8633.

Pais-Vieira, M., Lima, D., Galhardo, V., 2007. Orbitofrontal cortex lesions disrupt risk assessment in a novel serial decision-making task for rats. Neuroscience. 145**,** 225-231.

Pattij, T., Janssen, M.C.W., Schepers, I., González-Cuevas, G., Vries, T.J., Schoffelmeer, A.N.M., 2007. Effects of the cannabinoid CB1 receptor antagonist rimonabant on distinct measures of impulsive behavior in rats. Psychopharmacology. 193**,** 85-96.

Pattij, T., Vanderschuren, L., 2008. The neuropharmacology of impulsive behaviour. Trends in Pharmacological Sciences. 29**,** 192-199.

Pattij, T., Wiskerke, J., Schoffelmeer, A.N.M., 2008. Cannabinoid modulation of executive functions. European Journal of Pharmacology. 585**,** 458-463.

Paxinos, G., Watson, C., 1998. The Rat Brain in Stereotaxic Coordinates, Vol., Academic Press, San Diego, California, USA.

Peinado, M.A., Quesada, A., Pedrosa, J.A., Martinez, M., Eesteban, F.J., Del Moral, M.L., Peinado, J.M., 1997. Light Microscopic Quantification of Morphological Changes During Aging in Neurons and Glia of the Rat Parietal Cortex. The Anatomical Record 247**,** 420–425.

Piomelli, D., 2003. The molecular logic of endocannabinoid signalling. Nature Reviews Neuroscience. 4**,** 873-884.

Purves, D., Augustine, G.J., Fitzpatrick, D., Hall, W.C., Lamantia, A.-S., McNamara, J.O., Williams, S.M., 2004. Neuroscience, Vol., Sinauer Associates, Sunderland, Massachusetts U.S.A.

Puumala, T., Sirvio, J., 1998. Changes in activities of dopamine and serotonin systems in the frontal cortex underlie poor choice accuracy and impulsivity of rats in an attention task. Neuroscience. 83**,** 489–499.

Rangel, A., Camerer, C., Montague, P.R., 2008. A framework for studying the neurobiology of value-based decision making. Nature Reviews Neuroscience. 9**,** 545-556.

Raone, A., Cassanelli, A., Scheggi, S., Rauggi, R., Danielli, B., De Montis, M.G., 2007. Hypothalamus–pituitary–adrenal modifications consequent to chronic stress exposure in an experimental model of depression in rats. Neuroscience. 146**,** 1734-1742.

Rivalan, M., Ahmed, S.H., Dellu-Hagedorn, F., 2009. Risk-Prone Individuals Prefer the Wrong Options on a Rat Version of the Iowa Gambling Task. Biological Psychiatry. 66**,** 743-749.

Rivalan, M., Coutureau, E., Fitoussi, A., Dellu-Hagedorn, F., 2011. Inter-Individual Decision-Making Differences in the Effects of Cingulate, Orbitofrontal, and Prelimbic Cortex Lesions in a Rat Gambling Task. Frontiers in Behavioral Neuroscience. 5.

Robert, G., Drapier, D., Verin, M., Millet, B., Azulay, J.-P., Blin, O., 2009. Cognitive impulsivity in Parkinson's disease patients: Assessment and pathophysiology. Movement Disorders. NA-NA.

Rogers, R.D., Lancaster, M., Wakeley, J., Bhagwagar, Z., 2004. Effects of beta-adrenoceptor blockade on components of human decision-making. Psychopharmacology. 172**,** 157-164.

Schoenbaum, G., Chiba, A.A., Gallagher, M., 1999. Neural Encoding in Orbitofrontal Cortex and Basolateral Amygdala during Olfactory Discrimination Learning. The Journal of Neuroscience. 19**,** 1876–1884.

Schoenbaum, G., Stalnaker, T.A., Shaham, Y., 2007. A role for BDNF in cocaine reward and relapse. Nat Neurosci. 10**,** 935–936.

Schultz, W., Dayan, P., Montague, R.R., 1997. A neural substrate of prediction and reward. Science. 275**,** 1593–99.

Schultz, W., 2000. Multiple reward signals in the brain. Nature Reviews Neuroscience. 1**,** 199-207.

Schultz, W., 2006. Behavioral Theories and the Neurophysiology of Reward. Annual Review of Psychology. 57**,** 87-115.

Schultz, W., 2010. Dopamine signals for reward value and risk: basic and recent data. Behavioral and Brain Functions. 6**,** 24.

Seedat, S., Kesler, S., Niehaus, D.J.H., Stein, D.J., 2000. Pathological Gambling Behaviour: Emergence Secondary to Treatment of Parkinson's Disease with Dopaminergic Agents. Depression and Anxiety. 11**,** 185–186.

Seneff, S**.,** 2009. Is ADHD Caused by Insufficient Dietary Fat? Available online at http://people.csail.mit.edu/seneff/adhd_low_fat_diet.html

Simon, N.W., Gilbert, R.J., Mayse, J.D., Bizon, J.L., Setlow, B., 2009. Balancing Risk and Reward: A Rat Model of Risk Decision Making. Neuropsychopharmacology. 34**,** 2208-2217.

Sousa, N., Almeida, O., Holsboer, F., Paula-Barbosa, M., Madeira, M., 1997. Maintenance of hippocampal cell numbers in young and aged rats submitted to chronic unpredictable stress. Comparison with the effects of corticosterone treatment. Stress. 2**,** 237–249.

Southwick, S.M., Vythilingam, M., Charney, D.S., 2005. The Psychobiology of Depression and Resilience to Stress: Implications for Prevention and Treatment*. Annual Review of Clinical Psychology. 1**,** 255-291.

Squire, L.R., Bloom, F.E., Spitzer, N.C., Lac, S.d., Ghosh, A., Berg, D., 2008. Fundamental Neuroscience, Vol., Elsevier, Burlington, U.S.A.

Stahl, S.M., 2008. Stahl's Essential Psuchopharmacology - Neuroscientific Basis and Practical Applications, Vol., Cambrige University Press, New york, USA.

Svingos, A.L., Persiasamy, S., Pickel, V.M., 2000. Presynaptic Dopamine D4 Receptor Localization in the Rat Nucleus Accumbens Shell. Synapse. 36**,** 222–232.

Taepavarapruk, P., Floresco, S.B., Phillips, A.G., 2000. Hyperlocomotion and increased dopamine efflux in the rat nucleus accumbens evoked by electrical stimulation of the ventral subiculum: role of ionotropic glutamate and dopamine D1 receptors. Psychopharmacology. 151**,** 242–251.

Takahashi, Y.K., Roesch, M.R., Stalnaker, T.A., Haney, R.Z., Calu, D.J., Taylor, A.R., Burke, K.A., Schoenbaum, G., 2009. The Orbitofrontal Cortex and Ventral Tegmental Area Are Necessary for Learning from Unexpected Outcomes. Neuron. 62**,** 269-280.

Thorpe, S.J., Rolls, E.T., Maddison, S., 1983. The Orbitofrontal Cortex: Neuronal Activity in the Behaving Monkey. Experimental Brain Research 49**,** 93-115.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geomatric averaging of multiple internal control genes. Genome Biology. 3**,** Research 0034.1-0034.11.

Vinod, K.Y., Hungund, B.L., 2006. Role of the endocannabinoid system in depression and suicide. Trends in Pharmacological Sciences. 27**,** 539-545.

von Bohlen und Halbach, O., Dermietzel, R., 2006. Neurotransmitters and Neuromodulators - Handbook of Receptors and Biological Effects, Vol., Wiley-VCH, Weinheim, Germany.

West, M.J., Gundersen, H.J.G., 1991. Unbiased Stereological Estimation of the Number of Neurons in the Human Hippocampus. The Journal of Comparative Neurology. 296**,** 1-22.

West, M.J., Slomianka, L., Gundersen, H.J.G., 1991. Unbiased Stereological Estimation of the Total Number of Neurons in the Subdivisions of the Rat Hippocampus Using the Optical Fractionator. The Anatomical Record. 231**,** 482-497.

Wickens, J.R., Horvitz, J.C., Costa, R.M., Killcross, S., 2007. Dopaminergic Mechanisms in Actions and Habits. The Journal of Neuroscience. 27**,** 8181-8183.

Wise, R.A., 2004. Dopamine, learning and motivation. Nature Reviews Neuroscience. 5**,** 483-494.

Wogar, M.A., Bradshaw, C.M., Szabadi, E., 1993. Effect of lesions of the ascending 5-hydroxytryptaminergic pathways on choice between delayed reinforcers. Psychopharmacology. 11**,** 239-243

Wong, M.L., Medrano, J.F., 2005. Real-time PCR for mRNA quantitation. BioTechniques. 39**,** 75-85.

Yamada, K., Mizuno, M., Nabeshima, T., 2001. Role for brain-derived neurotrophic factor in learning and memory. Life Sciences. 70**,** 735–744.

Yao, W.-D., Spealman, R.D., Zhang, J., 2008. Dopaminergic Signaling in Dendritic Spines. Biochemical Pharmachology. 75**,** 2055-2069.

Yin, H.H., Lovinger, D.M., 2005. Frequency-specific and D2 receptor-mediated inhibition of glutamate release by retrograde endocannabinoid signaling. PNAS. 103**,** 8251– 8256.

Yin, H.H., Mulcare, S.P., Hilário, M.R.F., Clouse, E., Holloway, T., Davis, M.I., Hansson, A.C., Lovinger, D.M., Costa, R.M., 2009. Dynamic reorganization of striatal circuits during the acquisition and consolidation of a skill. Nature Neuroscience. 12**,** 333-341.