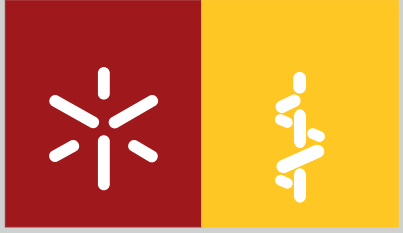

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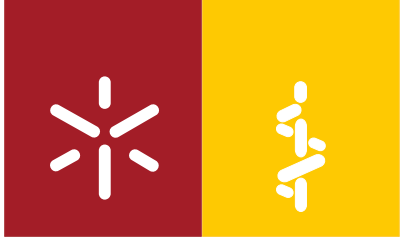

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The impact of stress in the risk-based decision-making processes: insights from the lab and the clinics.

Tese de Doutoramento em Medicina

Trabalho realizado sob orientação do
Professor Doutor João Cerqueira
e co-orientação do
Professor Doutor Nuno Sousa

setembro de 2013

Aos meus pais e irmã.

The things one feels absolutely certain about are never true.

Oscar Wilde

Acknowledgments

To my parents, Ana e José and my sister, Ana Cristina, for all the love, freedom and support.

To my supervisors, Professor João Cerqueira and Professor Nuno Sousa for all their support and interesting discussions.

To Professor João Cerqueira (and Sofia), for his intelligent advices and enthusiastic attitude; for all the time we spent together working on this project (and laughing about life); and for being my friend.

To Professor Nuno Sousa (and Bela, Rita e Hugo) for being my friend. I take his extraordinary character and his unattainable wisdom as a challenge to overcome my own possibilities.

To Professor Cecília Leão for believing in me and for all her support and affection.

To Professor Fernanda Marques for her friendship, for all the work she put into this project and for always being available when needed.

To Dr. Jorge Gonçalves for believing in me, for encouraging me to overcome difficulties, for teaching me the true essence of Psychiatry and for being my friend.

To Professor Joana Palha and Professor Margarida Correia Neves for being my friends and teaching me the importance of freedom.

To Professor José Miguel Pego (and Sara) for challenging me for Research, for his character and for his constant friendship.

To Professor Osborne Almeida for all the interesting discussions and insightful advices.

To Professor Hugo Almeida for all the help and support. To Professor João Sousa for his advices and for always keeping me down to earth. To Professor João Bessa, Dr. Vitor Hugo Pereira, Dr. Ana Salgueira and Dr. Mónica Gonçalves for all their support.

To Eng. Paulo Marques, Eng. José Miguel Soares, Professor Nadine Santos, Dr. Cristina Mota, Professor Ana João Rodrigues, Dr Ana Paula Silva, Dr. Luís Martins and Eng. Goretti Pinto for all their work and support.

To Dr. Bessa Peixoto for all his support, for understanding me and for believing in me.

To Dra. Filipa Pereira for her clever humor and for being my friend. To Dr. Daniela Freitas, Dr. Tiago Dias, Dr. Juliana Carvalho, Dr. Liliana Silva and all Psychiatry specialists and residents for their support, fellowship and friendship. To Enfermeira Dores for her wisdom. To all the nurses and administrative staff in the Psychiatry Department of Hospital de Braga for all their help and support.

To my students, namely to Miguel Borges Silva, Sofia Lopes, Daniel Machado, Tiago Fernandes, Beatriz Ribeiro, Miguel Mendes, Eduardo Domingues and Catarina Lima, for the challenging questions they raised.

To all researchers in the neurosciences group and to the technical staff at ICVS for all their support and teachings.

To the patients and healthy volunteers for their precious contribute for this work.

To all my friends for their warm words and huge hugs.

To Inês for her tenderness and support.

To Sónia and João Manuel for their faithful friendship.

To Marina for the character, the patience and the love.

To Cláudio for all his support and for teaching me the importance of the little things.

Braga, September 2013.

This work was supported by a grant from Fundação para a Ciência e Tecnologia: SFRH/SINTD/60129/2009.

Abstract

Decision-making is a routine in our daily life, constituting one of the most prominent differential features of each human being. Several psychiatric disorders, including obsessive compulsive spectrum disorders, schizophrenia and depression, present significant impairments of decision-making abilities. Decision-making requires complex cognitive processes, modulated by a variety of intrinsic and environmental elements, including stress. Indeed, the brain networks involved in decision-making, have been found to be targeted by chronic stress exposure.

In the present series of studies, we have thoroughly characterized how decision-making processes, namely pavlovian-to-instrumental transfer (PIT) processes and risk-based decision-making, can be influenced by chronic stress, detailing some neurochemical, neuroanatomical and neurophysiologic mechanisms underlying these changes and proposing therapeutic interventions to revert stress-induced impairments. We also explored the relationship between stress and features of obsessive compulsive disorder and analyzed risk-based decision-making in a cohort of patients with this psychiatric pathology.

We show that chronic stress transiently impairs PIT, reducing the ability of environmental cues to influence instrumental actions, and induces a risk-aversive behavior in a novel decision making task. Using *c-fos* labeling techniques we found that stress-induced risk-aversion was related with an overactivation of the orbitofrontal and insula cortices. Chronic stress also induced an hypertrophy of apical dendritic trees of layer II/III pyramidal neurons of the orbitofrontal cortex, an effect that was also observed in neurons activated during the decision-making task. Finally, we reveal that stress induces a hypodompaminergic status in the orbitofrontal cortex, characterized not only by decreased dopamine levels, but also by an increased expression of the D2 receptor, and show that stress-induced changes in risk-based behavior can be reverted by systemic administration of the D2/D3 agonist quinpirole. In a separate set of experiments, we found that obsessive compulsive patients displayed higher levels of perceived stress and cortisol, when compared with age and sex-matched healthy controls, and had difficulties in risk-based decision-making that correlated with decreased activity in the dorsal striatum when deciding, hypoactivation of the amygdala before making high-risk choices and increased activity in several areas of the (orbito)fronto-striato-thalamic circuit implicated in decision upon loosing.

In this thesis we show that chronic stress profoundly influences decisions, biasing behavior to risk-aversion, and impairing PIT. We further revealed that stress is also associated with symptoms in obsessive compulsive disorder patients, who present impairments in risk-based decision-making. We conclude by suggesting that decision-making deficits are key in obsessive compulsive disorders clinical presentation and might be used as diagnosis and/or prognosis markers and finally hypothesize that the neurochemical mechanisms and therapeutic approaches identified in the study of chronic stress effects can be translated to obsessive-compulsive spectrum disorders and challenge our current knowledge, paving the way for new treatments.

Resumo

A forma como tomamos decisões é uma das características mais diferenciadoras dos indivíduos. Alterações dos processos de tomada de decisão são frequentes em várias doenças psiquiátricas, incluindo as doenças do espectro obsessivo-compulsivo, a esquizofrenia e a depressão. A tomada de decisão envolve processos cognitivos complexos que são modulados por uma panóplia de elementos internos e externos dos indivíduos, incluindo o stresse. Sabe ainda que este último, sobretudo em situações de exposição prolongada, modula as áreas e as redes cerebrais que se sabe estarem implicadas nos processos de tomada de decisão.

Nos estudos apresentados nesta tese, caracterizamos a forma como os processos de tomada de decisão, nomeadamente os processos de transferência pavloviano-instrumental (PIT) e a decisão baseada no risco, podem ser influenciados pelo stresse crónico. Adicionalmente, detalhamos alguns dos mecanismos neuroquímicos, neuroanatómicos e neurofisiológicos subjacentes às alterações encontradas e propomos intervenções terapêuticas capazes de reverter as consequências negativas induzidas pelo stresse crónico nos processos de tomada de decisão. As relações entre o stresse e a doença obsessivo compulsiva foram também exploradas e analisámos os processos de tomada de decisão de risco num grupo de doentes com esta patologia.

Os nossos resultados demonstraram que o stresse crónico provoca alterações reversíveis no PIT, prejudicando a forma como as pistas ambientais influenciam as acções instrumentais. Verificámos também, numa nova tarefa de tomada de decisão de risco em roedores, que o stresse crónico induz um padrão de comportamento aversivo ao risco. A utilização de técnicas de marcação com *c-fos* permitiu demonstrar que a aversão ao risco está relacionada com uma hiperactivação dos córtices orbitofrontal e insular. Verificámos também que o stresse crónico induz uma hipertrofia das dendrites apicais dos neurónios piramidais das camadas II e III do córtex orbitofrontal, um efeito que também foi observado em neurónios activados durante a tarefa de tomada de decisão descrita. Concomitantemente, demonstrámos que o stresse crónico induz um estado hipodopaminérgico no córtex orbitofrontal, caracterizado tanto pela diminuição dos níveis de dopamina como pelo aumento da expressão do mRNA dos receptores de dopamina D2. Por último, demonstrámos que as alterações induzidas pelo stresse podem ser

revertidas pela administração sistémica do agonista selectivo dos receptores da dopamina D2/D3, quinpirole.

No contexto dos nossos trabalhos clínicos, demonstrámos que os doentes com perturbação obsessivo compulsiva apresentam níveis mais elevados de stresse percebido e de cortisol, quando comparados com voluntários saudáveis, emparelhados para sexo, idade e nível educacional. Verificámos também que apresentam dificuldades nos processos de tomada de decisão de risco que estão relacionadas com uma diminuição da actividade do estriado dorsal no momento da decisão, uma activação paradoxal da amígdala antes da tomada de decisões de risco e um aumento da actividade em várias áreas cerebrais do circuito (orbito)fronto-estriato-talâmico nas decisões que implicam perdas.

Em síntese, ao longo desta tese demonstrámos que o stresse crónico influencia profundamente os processos de tomada de decisão, prejudicando o PIT e induzindo comportamentos de aversão ao risco. Adicionalmente demonstrámos que o stresse está associado com sintomas da doença obsessivo-compulsiva, cujos pacientes apresentam défices nos mecanismos de tomada de decisão. No seu conjunto, estes dados permitem afirmar que os défices da tomada de decisão são fundamentais no fenótipo das doenças do espectro obsessivo-compulsivo e podem ser utilizados como ferramentas diagnósticas e/ou como marcadores do prognóstico. Por último, propomos que os mecanismos neuroquímicos e as estratégias terapêuticas identificados no estudo dos efeitos do stresse crónico podem ser extrapolados para as doenças do espectro obsessivo, desafiando o conhecimento actual acerca da doença e suportando novas abordagens para o desenvolvimento de tratamentos mais efectivos.

Abbreviation List

ACTH – Adrenocorticotropic Hormone

BLA – Basolateral amygdala

CeA – Central Nucleus of the Amygdala

Cont - Control

CS – Conditioned stimulus

CUS – Chronic Unpredictable Stress

HIPP - Hippocampus

HPA – Hypothalamic-Pituitary-Adrenal

IL – Infralimbic area

mPFC – media Prefrontal Cortex

OCD – Obssessive Compulsive Disorder

OFC – Orbitofrontal Cortex

PBS - phosphate-buffered solution

PFC – Prefrontal cortex

PL – Prelimbic area

PTSD – Posttraumatic stress disorder

SD – Standard Deviation

SEM – standard error of the mean

US – Unconditioned stimulus

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Chapter 1

Introduction

1. INTRODUCTION

1.1. General

During last decades, different fields of knowledge, including psychology, economics and neurosciences, have focused on decision making process, highlighting its broad impact and huge complexity and contributing to the raise of a new area devoted to the study of brain computations implicated on valued decisions. Making decisions based on the probability of future events is routine in everyday life; it occurs whenever individuals select an option from several alternatives, each one associated with a specific value. To manage its limited resources, living organisms have to make critical decisions that have survival value, which means that being a good decider has selective and evolutionary impact. Conversely, impaired/poor decision making can have catastrophic impact and constitutes an important feature of several neuropsychiatric disorders, such as schizophrenia, anxiety disorders, substance abuse disorders, obsessive compulsive disorder and pathological gambling.

Most times individuals have to decide knowing the precise outcomes of each option, but sometimes they have to bet unknowing the consequences of it. 'Uncertainty' refers to lack of knowledge of what outcome will follow a specific choice. Uncertain events can be categorized by the confidence in the probability assignment of each outcome: 'ambiguity' refers to situations when the outcomes cannot be specified and the variance of its occurrence is completely unknown and 'risk' refers to situations when the distribution (or probability) of each possible outcome is (at least partially) known. Interestingly, it is believed that ambiguity and risk processing are supported by distinct neural mechanisms, involving different brain regions (Huettel et al, 2006); whether value and probability shared common neuronal circuits/mechanisms is still an open question.

For comprehensive purposes, the process of decision making can be divided into five steps: first, the representation of present situation (or state); second, the assessment and valuation of available options culminating in formation of preference; third, the selection and execution of an action; fourth, the outcome evaluation and processing; and fifth, the learning phase, when a new value is reassigned to each option according to the experience of completed action-outcome

sequence. These steps are not rigid, as they often intermingle; however, they are highly integrated and, as a consequence, impairments in one can lead to a several disruption of decision making processes and promote maladaptive behaviors (Rangel, 2008).

1.2. A Computational Model of Decision-Making

In the next lines we summarize the critical steps to get to a decision (Figure 1). It should be highlighted that in many occasions some of these steps are not sequential, nor even mandatory.

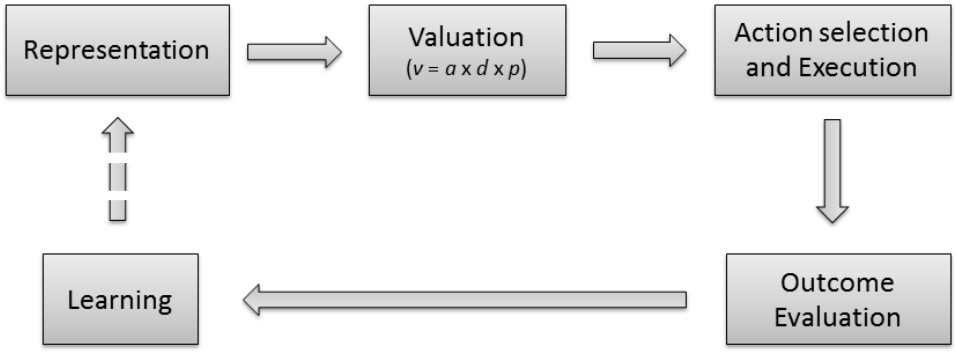

Figure 1. Schematic representation of decision-making processes (adapted from Rangel, 2008). Firstly individuals had a mental representation of each option available that is subsequently valuated concerning amount (a), delay (d) and probability (p). After action selection and execution, individuals can evaluate the outcome obtained which encompasses somewhat learning that can influence internal representations and further decisions.

Representation. When it is necessary to make a decision, individuals have to compose a mental imagery about the present and forthcoming situation, considering inner and outer states. Perception of condition and its variables are critical for subsequent steps and, thus, an erroneous perception can profoundly affect the decision-making process. Such impairments can result from mere sensory deficits or from more complex deficits as a result of poor processing of sensory inputs in the brain.

Valuation of Options. The formation of values is a critical step for decisions and involves cognitive and emotional processes that culminate with the assignment of a subjective value to each option. In general, the expected value (*v*) of an outcome is given by a function of reward amount (*a*), delay (*d*) and probability (*p*) (Doya, 2008). However, valuation has strong modulators such as type of uncertainity, cost and effort involved and social modulators that turn the process excessively complex to be translated into a simple equation. Additionally, human and animal behavioral observations lead to the establishment of three distinct valuating systems: a goal-directed learning system that associates an action to a specific outcome; a habit-based system that assign values to repeated actions; and a stimulus-triggered conditioning system (pavlovian) that associates stimulus to specific responses (Rangel, 2008).

Action Selection and Execution. During this phase, subjects select the most valuable option according to previously valuation of different possible outcomes, initiating, performing and completing an action. This step is highly modulated by motivation and arousal and is the phase implied in behavioral disruptions such as impulsive disorders or motivational deficits.

Outcome Evaluation. Subjective evaluation of action outcome is performed and somatic states induced by outcomes are coded. Values are attributed to outcome experiences.

Learning. Previous expected value is replaced by actual value and individuals learn to assign the more accurate value to actions, influencing future experiences. Neuronal networks, particularly the ones implicated in these steps, compute the difference between expected and actual value, that is, the degree of surprise that outcome elicited, which is called *predictive error*.

1.2.1. Variables influencing decision-making

The relevance of the different steps described above is influenced by several variables that include risk and uncertainty, cost and effort, motivation and social modulators.

Risk and uncertainty. In order to make good decisions, the decision-making systems have to estimate likelihood and value of different reward assigned to each option. As virtually all events involve some degree of uncertainty, comprehension of probabilistic computations is critical to understand the risk-based decision making.

Regarding uncertain events, Blaise Pascal proposed the notion of 'expected value', which account a combination of value (v) and probability (p) of different outcomes (v x p). According with this notion, if a decision is adaptive, subjects select the option that has the greater relationship between value and probability. If one has to decide between an option A that gives 100€ with 100% probability and an option B that rewards 300€ with 50% probability, according with Pascal principles, the subject would choose option B because it has higher expected value. However, if the gambler is a hungry homeless, choosing the option that surely warrants money to buy food would be the most appropriate option. Based on observations like that, Daniel Bernoulli recognized that choice depends on personal needs and feelings, which encompasses the subjective value, or utility, of goods (u) and introduced the notion of 'expected utility' (u x p) (Bernoulli, 1738). A behavior that deviates from simple linear evolution of this equation can be regarded as ´risk-averse' or 'risk-prone' behavior. Interestingly, in real life situations, our behavior often deviates from this model whether when we buy insurance or when we play lottery (in both situations, at the long-term, probability of obtain any gain is really low).

Additionally, the expected utility models are useful as a framework to understand decision under uncertainty but are subjected to frequent violations across a wide range of common situations (Platt and Huettel, 2008). Uncertainty is the main factor that accounts to that, not only because the probability of outcomes is commonly unknown in real life, but also due to limitations in human's capacity of estimate probabilities. These limitations are easily recognized in pathological gamblers and included overvalue winning outcomes and undervalue loosing outcomes, overrepresentation of rare events, overgeneralization based on sparse data and superstitiously believe on controlling game outcomes.

Cost and effort. Motivational theories often focus on the influence of incentive value of the goal or outcome and strength of reinforcement to explain behavior. However, making good decisions implicates estimate not only the value and the likelihood of each reward but also the costs and efforts implied in its obtaining. This valuation integrates the hedonic properties of a stimulus ("liking"), characteristics that remain constant besides changes in motivation or devaluation procedures, and the disposition to overcome costs in order to obtain a goal ("wanting") (Kurniawan et al, 2011). Animals (including humans) experience effort as a burden and tend to avoid effortful actions when reward magnitude is kept constant (Kool et al, 2010). Furthermore,

to reach a desired goal, animals are ready to expend efforts which encompass an accurate integration of costs and benefits of each action.

Despite it has been observed that rodents use a constant ratio to decide between two options with different variable amounts of effort and rewards (van den Bos et al, 2006), human studies have shown that probabilities are not always weighted by an absolute numerical value; in fact, they are rather weighted relatively to a reference point (Camerer et al, 2008). Additionally, animals are able to recognize changes in punishment and risks involved and there is correlational evidence suggesting that rats with a preference for large, risky reward in decision-making tasks also demonstrate preference for the large reward in the probability-discounting task (Simon et al, 2009).

Time-discount. Another relevant factor for decisions that had been extensively studied over recent years is the delay to get the outcome. Along with risk and effort, individuals usually include time lag to get the reward in the decision algorithm. In our daily life, there are many situations in which it is required a long delay to obtain the best outcome but, sometimes, we prefer get less immediately than wait for more. Preference for small rewards delivered immediately over larger rewards delivered after a delay is commonly known as delay discounting. Interestingly, this pattern of behavior is explored commercially with increased frequency. Higher rates of delay discounting resulting in a pattern of impulsive choice and are associated with attention deficit hyperactivity disorder (ADHD) (Barkley et al., 2001), addictive disorders (Kirby and Petry, 2004) and pathological gambling (Madden et al., 2009), disorders in which the ability to delay gratification is significantly impaired.

Social factors. Decisions are commonly made into social contexts and are frequently subjected to judgment by others; this is, namely in humans, an important modulator of decision-making behaviors. When deciding, individual desires and social expectations are carefully balanced, but the specific inputs and the relative value of social factors to the valuation network remain unknown. Some studies focused on game theory explored neurobiological correlates of reciprocal exchange, altruism and mutual cooperation which contribute to identify aberrant neural substrates underlying social abnormalities associated with some psychiatric disorders such as antisocial personality disorder, borderline personality disorder and schizophrenia (for review see Rilling et al., 2008).

1.3. Neuronal networks implicated in decision-making

Decision-making processes are mediated by parallel circuits linking cerebral cortex and the basal ganglia, which encode three distinct (and, somehow, conflicting) valuating systems: goal-directed, habit-based and pavlovian/conditioning systems.

Goal-directed system involves a lot of top-down processing of numerical and abstract concepts which encompasses consistency of choices, being modulated by variables related to cognition, attention and expertise. The medial pre-frontal cortex (mPFC), in particular the prelimbic region, and the dorsomedial striatum (caudate in humans) are key components of the associative network, the neural corticostriatal circuit regulating goal-directed choice (Balleine and O'Doherty, 2010). The dorsomedial striatum is central in this circuit, receiving inputs directly from association cortices, and projecting to areas known to participate in motor control, such as *substancia nigra* reticulata and mediodorsal thalamus. Other areas such as basolateral amygdala (BLA) and ventral tegmental area (VTA) were also found to influence this circuit. Importantly, the dorsomedial striatum is crucial either to learning and expressing goal-directed behaviors (Yin et al 2005), but also for the valuating system (Balleine, 2005). Interestingly, learning of goal-directed behaviors encompasses the assignment of predicted values for each possible outcome, a process dependent on integrity of prelimbic cortex which does not appear to be crucial to goal-directed action (Ostlund and Balleine, 2005). Prelimbic cortex was found to be one target of dopaminergic signal arising from VTA which encompasses the adaptation to reward contingencies in goal-directed learning (Naneix et al., 2009). Consequently, prelimbic cortex is crucial to the updating of the value when outcome changes, a specific feature of goal-directed behavior.

Additionally, other cortical and subcortical areas also play a role in goal-directed decision-making. Amongst these are the ventromedial prefrontal cortex (vmPFC) - including areas of mPFC and medial orbitofrontal cortex (mOFC) – that encodes the expected future reward attributable to chosen action (Gläscher et al, 2009) associated with action-outcome but not stimulus-response decision (Valentin et al, 2007). The anterior cingulated cortex (ACC) is another area involved in goal-directed decision by monitoring conflicts related to actions and balancing costs and benefits associated with different options. Its activation increases as a function of cognitive control

demanded by the task (Brown and Braver, 2005). In contrast, the orbitofrontal cortex (OFC) was found critically involved in goal-directed behaviors by holding on information on relationships between environmental patterns and somatic states induced by those patterns. Interacting reciprocally with the OFC, the BLA plays an important role on forming representations linking cues to outcome expectancies (Pickens et al, 2003) and promoting the assignment of incentive value to predicted outcome (Balleine et al, 2003), a function that seems to be mediated by local opioid receptors (Wassum et al, 2009). Interestingly, OFC seems to be essential to keep these representations updated and stored in memory (Pickens et al, 2003). Additionally, the BLA, by its known connections with hypothalamus, seems to be responsible for processing affective and motivational properties of outcomes. The BLA influences corticostriatal circuit through its direct projections to prelimbic cortex, dorsomedial striatum and dorsomedial thalamus and its indirect projections to ventral striatum via insular cortex. Moreover, the ventral striatum, namely the core part of nucleus accumbens, had been found crucial for instrumental performance, participating in translating motivation into actions (for revision see Balleine and O'Doherty, 2010).

With intensive training and repetition, control of actions can be transferred to the ***Habit-based system***. Habitual actions, involve an ordered, structured action sequence that can be quickly elicited by particular rewards (Graybiel, 2008), and can be neutral, desirable or undesirable. The sensorimotor network, a circuit that includes sensorimotor cortex and the dorsolateral striatum (putamen in humans) as key components, was found to be implicated in habit formation. It is known that the habitual stimulus-response learning is modulated by dopaminergic projections from *substancia nigra* and VTA into dorsolateral striatum, encoding the assignment of a specific value for each action and promoting the acquisition of a response to conditioning stimulus by striatal neurons (Aosaki et al, 1994). Furthermore, the mPFC was found to be of relevance for coordinating shifts between goal-directed actions and habits (Dias-Ferreira et al., 2009). This observations support the hypothesis of dynamic hierarchical interplay between goal-directed (associative) and habit based (sensoriomotor) networks and as well with pavlovian learning (limbic) circuit (for revision see Yin and Knowlton, 2006).

In ***Pavlovian conditioning system***, individuals learn to associate a particular cue/stimulus with a reward. This is an innate passive learning procedure, associated with a limited number of behaviors that include automatic behaviors such as preparing for eat when approaching a table with food or consummating the approach to the reward magazine when outcome is delivered in a

decision-making apparatus (Rangel et al, 2008). Neural basis of Pavlovian system includes responses to stimuli with specific spatial organization in dorsal periaqueductal grey (Keay et al, 2001) and is encoded in a neural network involving OFC, ventral striatum and BLA (Gottfriend et al., 2003; Ostlund and Balleine, 2007). As stated before, the OFC stores information on the relationships between environmental cues and somatic states induced by those; thus, it is crucial not only to action-outcome learning but also stimulus-response conditioning. Interestingly, lateral and central parts of OFC, receiving inputs from sensory areas, are involved in pavlovian valuing while medial parts participate in associative learning networks.

1.3.1. Neural networks implicated in risk and ambiguity

Humans are intrinsically averse to risk and, even more, to ambiguity. Even when risky options have a positive expected value, subjects preferred to take it safer, avoiding risky options (Platt and Huettell, 2008). Therefore, whenever decisions are preferably risky, this is likely to be viewed as inappropriate. Several studies have tried to understand how this mis-processment occurred at the neurobiological mechanisms. Previous studies have highlighted the role of distinct brain regions in these biased events. As examples, individuals with decision-making impairments that lead to increased risk displayed high insular activation when compared with controls, which is consistent with increased insular activation seen when healthy individuals choose higher-risk outcomes (Paulus et al, 2003). Moreover, there is an increased insular activation with higher risk outcomes, which might contribute to natural risk-aversive pattern of choice by its putative role in representing somatic states related with potential negative consequences of risk and losses (Paulus et al., 2003; Damasio 1996). Using also neuroimaging tools, a recent work examined the effects of different types of uncertainty on neural processes of decision-making; when compared with risk, ambiguous conditions produced higher activation on OFC, amygdala and dorsomedial prefrontal cortex, while the dorsal striatum (caudate nucleus) and precunneus cortex were less activated during risk condition (Hsu et al, 2005).

Another point of interest is the internal assessment of gains and losses during outcome evaluation. Usually, losses are valued about twice as large as equal-sized gains, which reflect a natural pattern of aversion to loss. In a functional magnetic resonance imaging (fMRI) study, Tom and colleagues (2007) observed that gains and losses promote changes in similar regions,

including striatum, ventral prefrontal cortex and anterior cingulate cortex, with putative gains enhancing activation and putative losses decreasing activation. However, decreased activity induced by losses on striatum and vmPFC was greater than increased activity induced by similar gains in such regions of interest. Additionally, the same study found an interesting correlation between behavioral and neural loss aversion in several regions such as ventral striatum and vmPFC. Interestingly, the valuation of efforts and delays seems to be processed in distinct brain circuits. The anterior cingulate cortex (Walton et al, 2003) and ventral striatum (Salamone et al, 1994) may be involved in choices encompassing barriers as effort, whereas ventral striatum is involved in choices involving delays as effort (Cardinal et al, 2001). Likewise, other functional imaging studies identified several regions of interest for delay discounting; in fact, while the dorsolateral prefrontal cortex, dorsal premotor cortex, parietal cortex and insula were found activated when high time-delay were expected (Tanaka, 2004; Tanaka, 2006), the ventral striatum, medial OFC, ACC and posterior cingulate cortex were found activated in situations related with immediate delivery of outcomes (McClure, 2004, 2007).

1.4. The role of dopamine in risky-based decision-making

There are certainly several neurotransmitters implicated in the process of decision-making. Yet, here we will focus on the role of dopamine, due to its critical relevance in the process of decision-making, particularly when risk is involved. It is important to highlight, though, that other neurotransmitters, namely other cathecolamines such as serotonin and norepinephrin, are also known to be determinant in these processes (for review, see Rogers, 2011).

Dopamine exerts a myriad of functions along mesolimbic, striatal and cortical pathways, playing a critical role in decision-making processes. The specificity of its contribution to decision making have been extensively studied nowadays. Dopamine signalling within the mesolimbic system is initially triggered by the receipt of reward but, after associative learning, it will be initiated by the cue that predicts the reward (Schulz 1997). Redgrave et al. (1999) proposed that dopamine signalizes stimulus salience, which includes the novelty and unpredictability of events, but recent theories highlight dopamine role in predicting rewards in Pavlovian, habit-based and goal-directed learning, updating the value of different options available (Costa et al., 2007). Interestingly, it has been found that phasic activity of dopaminergic along midbrain neurons is increased by delivery

of unexpected rewards (positive prediction errors) and decreased by omissions of expected outcomes (negative prediction errors) (Schulz, 2007), encoding discrepancies between received and predicted rewards.

Direct evidence of the role of dopaminergic agents on estimation of prediction errors on instrumental learning was provided by Pessiglione et al (2006). In this study, differential contribution of dopaminergic agonists (L-Dopa) and antagonists (haloperidol) was probed using a task involving both monetary gains and losses. Young healthy individuals treated with L-Dopa earned more money than ones treated with haloperidol and, importantly, this was related with the enhanced magnitude of positive and negative prediction errors among ventral striatum and putamen. Similar observations were provided by Menon et al (2007) measuring participants' prediction errors as BOLD responses within ventral striatum during an aversive conditioning procedure. As expected, BOLD activity was enhanced by amphetamine and abolished by haloperidol treatment. Dopamine is also proposed to mediate specific features of motivated behavior such as vigor control and effort, playing a role in overcoming "costs" of each choice (for review, see Kurniawan 2011). Experiments conducted by Salamone and colleagues (1994) clearly shown the role of dopamine in maintaining instrumental responses that require physical efforts (such as climbing a barrier), but not in keeping reward preference. Aside from a role in motivated behavior and prediction errors, dopamine is also required to flexibly initiate goal-directed behaviors. In a recent paper, Nicola (2010) shown that ability of rats in which dopamine was depleted in the nucleus accumbens to reinitiate an instrumental task is dependent on duration of inter-trial interval; in other words, dopamine depleted animals can keep an instrumental task but is unable to flexibly reinitiate it if engaged in another behaviors.

Altogether these results raised the question about which dopamine receptors sub-types might mediate these effects. Frank et al. (2004) proposed a model of action control by dopamine centered on basal ganglia, a set of subcortical nuclei comprising dorsal (putamen and caudate nucleus) and ventral striatum (synonymous with nucleus accumbens), globus pallidus, substancia nigra and subthalamic nucleus. Basal ganglia integrate a complex network, receiving information in input nuclei (striatum) from all cortical areas, especially the frontal cortex, and projecting to the thalamus, mainly via internal segment of globus pallidus and substancia nigra pars reticulata. These networks work with parallel loops and are known to play a critical role in almost all cognitive and motor functions. Information received in striatum is transmitted by two

different pathways: a direct pathway, that expresses dopamine receptors type 1 (D1) and promotes cell activity and long-term potentiation (LTP), facilitating the execution of responses identified in cortex ('Go Signals'); and an indirect pathway, that expresses dopamine receptors type 2 (D2) and promotes cell inhibition and long-term depression (LTD), suppressing responses ('No-Go Signals'). Thus, increased dopaminergic activity promoted by positive reinforcers facilitates response mediated by D1 receptors on direct pathway and inhibits activity within indirect pathway (by D2 receptors), whereas decrease in dopamine activity promotes the opposite pattern of responses. This theory was supported by observations showing that dopamine depleted non-treated Parkinson patients exhibit impairments on learning from positive outcomes, but enhanced learning from negative reinforcers (Frank et al., 2004). Additionally, treatment with small doses of pramipexole, a D2/D3 receptor agonist, impaired the acquisition of a biased response toward the most rewarded choice (Pizzagalli et al., 2008), which emphasizes the relevance of D2 receptors in learning from decision outcomes (for review, see Rogers 2011). Interestingly, healthy volunteers treated with pramipexole make riskier choices following high wins than ones taking placebo which can be related with a lower activation of ventral striatum after unexpected high wins in those individuals (Riba et al., 2008). Analogous hypoactivation of reward system is observed in pathological gamblers not suffering from neurological disorders (Reuter et al, 2005; Riba et al, 2008). .

Aside its relevance on striatum, dopamine also plays a critical role in PFC, by monitoring changes in reward probability and, consequently, in adjusting behaviors. While PFC D1 signaling seems to stabilize the representation of relative long-term value of the risky option, PFC D2 receptors may facilitate and update modifications in value representations (Onge et al, 2011). Indeed, the role of D1 signaling has been associated to the ability to overcome costs that may be associated with larger rewards (keep "eye on the prize") and, thus, maximize long-term gains. D2 receptors play a crucial role in the ability of animals to inhibit a pre-potent learned response, whereas the dopamine receptors type 3 (D3) are more likely involved in the modulation of the learning process during changing reward contingencies (Boulougouris et al, 2009). Importantly, stimulation of D1 or D2 receptors increased risk choice, whereas activation of D3 reduced risk choice (Onge et al, 2009). Interestingly, however, in another work using a rat gambling task similar to human Iowa Gambling Task, no changes were induced by acute administration of quinpirole or SKF 81297 (dopamine agonists) (Zeeb et al, 2009). These contrasting observations

highlight the differences between decision-making processes based solely on differences in reward probability and those incorporating more complex punishment signals.

In addition to dopamine other neurotransmitters seems to be involved in the decision-making processes. One example is serotonin that although during decades had been conceptualized as working in an apparent antagonism with dopamine, recent data pointed out that these neurotransmitters often work synergistically in decision-making systems (Aronson et al., 1995). Nakamura and colleagues (2008) proposed that tonic activity of serotoninergic neurons of dorsal raphe nucleus code magnitude of rewards while, as stated before, dopaminergic activity encodes differences between predicted and received outcomes. There is also a general agreement of the involvement of serotonin in time-dependent decisions and participation on coding reward value across different time delays (Winstaley et al., 2006). Aside from a role in the integration of time value, serotonin is also proposed to mediate critical aspects of risky decisions, namely in loss aversion (Long et al. 2009; Murphy et al., 2009). Finally, several studies demonstrate the relevance of serotoninergic system in affective modulation of motivated behaviors (Hollander and Rosen, 2000; Crockett et al., 2008), facilitation of reward processing by dopamine (Nakamura et al., 2008) and influencing cooperative responding and mutual cooperation (Wood et al., 2006).

In addition, also norepinephrine has been generally neglected on decision-making processes. However, some observations ruled out relevant functions for a possible role for adrenergic system on decision-making. It has been proposed that phasic noradrenergic activity within *locus coeruleus* might mediate outcome coding, exploratory choices and behavioral flexibility, crucial abilities to update learning processes in changing conditions (Aston-Jones and Cohen, 2005; Dayan and Yu, 2006; Yu and Dayan, 2005). Norepinephrine is also proposed to mediate learning and enhance memory under stress (Kerfoot et al., 2008) as well as the somatic feedback that can influence high brain cognitive and executive processes, proposed by Damasio (1996) in the Somatic Markers Hypothesis.

1.5. Maladaptive Choice Behavior as a Model for Neuropsychiatric Disorders

Impairments in decision making processes can have deleterious consequences for the personal and social well-being. Of notice, they can be recognized in patients affected by prevalent

neuropsychiatric disorders, such as obsessive-compulsive disorders, post-traumatic stress disorder, schizophrenia, substance abuse, depression, anxiety disorders, pathological gambling or Parkinson's disease.

Stress exposure is known to influence emotional and cognitive processes, which are crucial determinants of decision making processes. Exposure to stress elicits neuroendocrine and autonomic adaptive responses that promote coping strategies when dealing with acute conditions. However, when the stressors are extreme or prolonged these responses may have deleterious consequences, affecting the behavior and triggering neuropsychiatric disorders, such as anxiety disorder, post-traumatic stress disorder, depression or dementias (McEwen, 2004). The stress-induced behavioral impairments arise as a consequence of alterations in the brain structure, including the PFC, the hippocampus, the amygdala, the nucleus accumbens and the OFC (Sousa et al., 1998; Kim and Diamond, 2002; Dias-Ferreira et al., 2009; Bessa et al., 2009). Preclinical studies from our group started to unravel the mechanisms through which stress can alter instrumental behavior (Dias-Ferreira et al., 2009), biasing choices from a goal directed to a habit-based pattern. Additionally, recent studies have focused on effects of acute stress on decision making (Table 1), presenting contradictory data (Porcelli et al., 2010; Koot et al., 2013; Pabst et al., 2013; Reynolds et al., 2013) about risk-based decision.

Table 1. Recent studies on stress and decision-making.

Original References	Animal	Age	Task(s)	Reward (Punishment)	Type of Stressor	Duration of Stress	Behavioral effect of stress
Dias Ferreira et al., 2009	Rat	Adult	Outcome devaluation and Contingency degradation	Sucrose pellets and Sucrose solution	Chronic unpredictable stress	Chronic (28 days)	Habit-based behavior
Porcelli et al., 2010	Human	Adult	Card guessing task	Money	Cold pressure task	Acute (2 min.)	No effects
Koot et al., 2013	Rat	Adult	Rat gambling task	Sucrose pellets (Quinine pellets)	Corticosterone injection	Acute (3 days)	More risky, less advantageous choices
Pabst et al., 2013	Human	Adult	Game of Dice Task	Money	Trier Social Stress Test (TSST)	Acute (18 min.)	Less risky (5 and 18 min); More risky (28 min after)
Reynolds et al., 2013	Human	Adolescent	Balloon Analogue Risk Task	Money	Trier Social Stress Test (TSST)	Acute (18 min.)	More risky

Despite these findings, relatively little is known about the effect of chronic stress on the decision-making processes that involve risk. Furthermore, knowledge about the behavioral changes induced by stress and the neuronal mechanisms underlying those patterns could be of interest since individuals often have to take relevant decisions under high levels of stress.

1.6. Aims

The central question addressed in this thesis is whether decision-making processes are affected as a result of exposure to chronic stress, either analyzing animals' models of chronic unpredictable stress or using psychiatric disorders related with stress. Specifically, the experimental work undertaken aimed at:

1. Analyzing the influence of exposure to chronic unpredictable stress (CUS) on pavlovian to instrumental transferring (Chapter 2.1);

2. Characterizing the behavioral, neurochemical and structural effects of stress exposure in decision making processes using an animal model of risk-based decision (Chapter 2.2);

3. Studying whether treatment with dopaminergic agents contributes to reversion of behavioral changes induced by chronic stress on risk-based decisions (Chapter 2.2);

4. Analyzing stress response in patients suffering by Obsessive Compulsive Disorder (OCD) (Chapter 2.3);

5. Characterizing neural mechanisms of decision-making in OCD patients using a fMRI paradigm of risk-based decision-making (Chapter 2.4).

1.7. References

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Chapter 2

Experimental Work

Chapter 2.1.

Morgado P, Silva M, Sousa N, Cerqueira JJ. (2012)

Stress Transiently Affects Pavlovian-to-Instrumental Transfer.

Frontiers in Neuroscience, 6. 93: 1-6.

frontiers in
NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 25 June 2012
doi: 10.3389/fnins.2012.00093

Stress transiently affects Pavlovian-to-instrumental transfer

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Stress has a strong impact in the brain, impairing decision-making processes as a result of changes in circuits involving the prefrontal and orbitofrontal cortices and the striatum. Given that these same circuits are key for action control and outcome encoding, we hypothesized that adaptive responses to which these are essential functions, could also be targeted by stress. To test this hypothesis we herein assessed the impact of chronic stress in a Pavlovian-to-instrumental transfer (PIT) paradigm, a model of an adaptive response in which a previously conditioned cue biases an instrumental goal-directed action. Data reveals that rats submitted to chronic unpredictable stress did not display deficits in pavlovian conditioning nor on the learning of the instrumental task, but were impaired in PIT; importantly, after a stress-free period the PIT deficits were no longer observed. These results are relevant to understand how stress biases multiple incentive processes that contribute to instrumental performance.

Keywords: stress, conditioning, pavlovian-to-instrumental transfer, choices

INTRODUCTION

Exposure to a stressful stimulus activates a physiological response intended to restore the organism's homeostasis. However, when stressors are maintained for long periods of time, this response becomes maladaptive and results in several disruptions at the level of the regulatory systems, of which the brain is a key element. Thus, it is not surprising that chronic stress exposure is associated with significant behavioral impairments such as deficits in spatial reference and working memory (Mizoguchi et al., 2000; Cerqueira et al., 2007), behavioral flexibility (Cerqueira et al., 2007), anxiety (Pêgo et al., 2006), and mood (Bessa et al., 2009). Such functional deficits are paralleled by structural changes in several brain regions (Sousa and Almeida, 2002), that render chronic stress as an important risk-factor for the development of several neuropsychiatric disorders. Importantly, several studies have also shown that the behavioral and structural effects of stress are transient, and important plastic phenomena take place after the removal of the stressful stimuli (Sousa et al., 2000; Bloss et al., 2010).

Recent studies from our laboratory show that chronic stress bias decision-making processes, by favoring the shift from goal-directed actions to habit based behaviors (Dias-Ferreira et al., 2009). These alterations in instrumental behavior are correlated with changes in neuronal circuits involving different areas of the prefrontal cortex (PFC) [including the medial PFC (mPFC) and orbitofrontal cortex (OFC)] and dorsal striatum (Dias-Ferreira et al., 2009). Given that these regions are implicated in action control (Balleine and O'Doherty, 2010) and outcome encoding necessary for adaptive responses (Chudasama and Robbins, 2003; Hornak et al., 2004), we hypothesized that stress-induced changes in neuronal circuits involving the PFC and the striatum could lead to outcome encoding deficits and to changes in the multiple incentive processes that contribute to instrumental performance.

One of these processes is Pavlovian-to-instrumental transfer (PIT; Estes, 1948; Colwill and Rescorla, 1986). PIT encompasses three distinct components:(1) Pavlovian learning, in which stimuli are associated with rewards; (2) instrumental conditioning, in which associations between actions and out comes are learned; and (3) a test phase, in which the impact of previous cues on instrumental response is assessed. The associative value of cue and its motivational significance are determinants found crucial to proper transfer, a phenomenon which resembles cue-mediated increased drive seeking for drugs seen in drug abusers (Dickinson et al., 2000; Corbit and Janak, 2007). Because of that, PIT has been used as a useful model of maladaptive learning observed in several conditions, namely addictive disorders.

The neural basis of PIT is not completely established but several studies implicate regions associated with emotional processing [amygdala and nucleus accumbens (NAc)], executive commands (dorsal striatum), and their integration (mPFC and OFC); importantly, it is known that key regions involved in PIT operate in parallel. In fact, lesion studies in the amygdala and NAc have demonstrated that these brain regions are necessary for the behavioral expression of PIT (Corbit et al., 2001; Hall et al., 2001; de Borchgrave et al., 2002; Holland and Gallagher, 2004). Moreover, it is relevant to note that different regions of the amygdala and NAc display different roles in this process. While the amygdala basolateral nucleus (BLA) mediates the association between specific sensory and emotional features of stimulus and the responses that are elicited by each one (consummatory conditioning), the central nucleus (CN) mediates the association between cues and affective properties of stimuli (preparatory conditioning; Killcross et al., 1997; Balleine and Killcross, 2006). In what concerns the NAc, the core mediates the general excitatory effects of reward-related cues, whereas the shell mediates the effect of outcome-specific reward

predictions on instrumental performance (Corbit and Balleine, 2011). In addition, these regions are known to regulate the activity of cortical sites integrating affective stimuli with executive commands, such as the mPFC and the OFC (Christakou et al., 2004; Kelley, 2004; Pasupathy and Miller, 2005; Saddoris et al., 2005; Stalnaker et al., 2007).

Surprisingly, given the fact that stress influences many of the above mentioned areas involved in PIT, the impact of stress in PIT is largely unknown. Indeed, while acute stress was shown to enhance Pavlovian learning (Shors et al., 2000) and chronic stress failed to influence instrumental learning (Dias-Ferreira et al., 2009), no study addressed the effect of either acute or chronic stress on the interaction between these two key processes and thus on the impact of conditioned clues in goal-directed behavior. Thus, in the present study, we tested the impact of chronic stress in the modulation of instrumental behavior according to cues previously associated with rewards by studying the behavior of control and stressed rats in a PIT paradigm using a two-lever operant chamber. Moreover, to assess whether the impairments are reversible after chronic exposure to stress, PIT was also assessed after a period free of exposure to stressful stimuli.

MATERIALS AND METHODS

ANIMALS

All experiments were conducted in accordance with local regulations (European Union Directive 86/609/EEC) and National Institutes of Health guidelines on animal care and experimentation and approved by Direção Geral Veterinária (DGV; the Portuguese National Institute of Veterinary).

Thirty-two adult male Wistar rats (Charles River Laboratories, Barcelona, Spain; 250–300 g at the start of the experiment), aged 3 months and weighing 400–500 g, were housed in groups of two under standard laboratory conditions with an artificial light-dark cycle of 12:12 h (lights on from 8:00 a.m. to 8.00 p.m.) in a temperature- and humidity-controlled room. Animals were given 2 weeks to acclimate to the housing conditions with *ad libitum* access to food and water. A food deprivation regimen was initiated 24 h before the initiation of training and testing to maintain the subjects at approximately 90% of their free-feeding body weight. Rats had free access to water while in the home cage.

CHRONIC UNPREDICTABLE STRESS PARADIGM

Animals assigned to the chronic unpredictable stress (CUS) group were exposed during 60 min once a day to one of five different stressors: cold water (18°C), vibration, restraint, overcrowding, and exposure to a hot air stream. Stressors were randomly distributed throughout a 28-day period. This type of chronic stress paradigm, mixing different stressors (including physical and psychological components) presented in an unpredictable schedule, was shown previously to result in persistently elevated plasma levels of corticosterone (for details, see Sousa et al., 1998) and is thought to better mimic the variability of stressors encountered in daily life (Sousa et al., 1998). Controls were handled daily during the same period.

To assess the impact of chronic stress exposure in Pavlovian-instrumental transfer but also ascertain its reversibility, a first group of animals (eight stressed and eight controls) were behaviorally characterized immediately after stress while a similar group (eight stressed and eight controls) was left to recover for 6 weeks before being tested. This recovery period was set-up in light of previous studies showing that, at least, 4 weeks are necessary to complete reversion of behavioral and structural changes induced by CUS treatment (Sousa et al., 2000). Importantly, animals were randomly allocated to each of the four groups before the beginning of stress exposure.

PAVLOVIAN-INSTRUMENTAL TRANSFER

Behavior was assessed using the Pavlovian-instrumental transfer protocol as described by Ostlund and Balleine (2007). This task took place in operant chambers (30.5 cm L × 24.1 cm W × 21.0 cm H, MedAssociates, CA, USA) housed within sound attenuating cubicles. Each chamber was equipped with two retractable levers on either side of the food magazine and a house light (3W, 24V) mounted on the opposite side of the chamber. Reinforcers were delivered into the magazine through a pellet dispenser that delivered 45 mg regular "chow" pellets or a liquid dipper that delivered 0.1 ml of 20% sucrose solution. A computer equipped with MED-PC IV software controlled the equipment and recorded lever presses and head entries. As described previously, animals were placed in a food deprivation schedule, having access to food during 1 h per day after the training or testing session, allowing them to maintain a body weight above 90% of their baseline weight. Water was removed for 2 h before each daily session.

Training began with eight daily sessions of Pavlovian conditioning in which each of two auditory conditioned stimuli (tone and white noise) were paired with a different outcome (pellets and sucrose). Each CS was presented four times per session using a pseudo-randomized order and a variable ITI (mean 5 min). In the ninth day, animals were submitted to an outcome devaluation to ensure they were able to associate each outcome to the conditioned stimulus; this was assessed by comparing the number of head entries into the food dispenser during stimuli presentation and during ITI.

Animals were then trained to obtain two different outcomes (pellets and sucrose) by pressing left and right levers. Training was performed in two separate daily sessions and the order of training was alternated during days (average interval between the two daily sessions was 3 h). Each session finished after 15 outcome deliveries or 30 min. In the first 2 days, lever pressing was continuously reinforced (CRF) which means that each action resulted in one outcome delivered ($p = 1.0$). The probability of getting a reward decreased according the following sequence: days 3–4, $p = 0.2$; days 5–6, $p = 0.1$; and days 7–9, $p = 0.05$.

Two sessions of outcome devaluation (by free access to the reward until satiety) were then performed, 48 h apart. In order to do this, one of the two outcomes (pellets or sucrose) was given *ad libitum* during 1 h before each session. Then the rats were placed during 5 min into the testing operant chamber where both levers were inserted but no outcome was delivered.

Forty-eight hours later, subjects were placed in the operant chamber to test Pavlovian-instrumental transfer with both levers inserted. After an initial period of response extinction that lasts for 8 min, four blocks of each auditory conditioned stimulus were

presented randomly over the next 40 min and lever presses were registered. During each stimulus presentation, lever presses where considered correct if encoded the same reward as the audible sound. When different, actions were considered incorrect.

STATISTICAL ANALYSIS

Results are expressed as group means ± SE. Pavlovian, instrumental behavior and results of transfer test were compared between and within groups using two-way ANOVA. Differences were considered to be significant if $p < 0.05$.

RESULTS

Pavlovian training resulted in conditioning of animals both in control and stress groups. Comparison of head entries on CS presentation and on ITI during Pavlovian training (**Figure 1**) shows that all animals associate the stimuli to the outcome[head entries: $F_{(1,28)} = 88.762$, $p < 0.001$] without differences between experimental groups [stress exposure: $F_{(1,28)} = 0.163$, $p = 0.689$], thus implying that CUS does not affect Pavlovian conditioning.

In what regards to instrumental training, the number of lever presses per minute increased during the task indicating that animals in both groups can learn it equally well (**Figure 2**). This is confirmed by the results of the outcome devaluation test performed at the end of instrumental conditioning, in which animals of both groups [stress exposure: $F_{(1,28)} = 1.019$, $p = 0.321$]could correctly associate each reward to a specific lever[lever: $F_{(1,28)} = 25.787$, $p < 0.001$; **Figure 2**. These results are in accordance with our previous data (Dias-Ferreira et al., 2009) showing that CUS does not impair outcome devaluation when performed early during the period of training.

Subsequently, we assessed the Pavlovian-instrumental transfer. **Figure 3** displays the number of lever presses per minute when the conditioned sound predicted the same outcome as the response (same) and the number of lever presses per minute when the conditioned sound predicted a different outcome (diff). Our results (**Figure 3A**) show that stress significantly impairs the transfer [stress exposure: $F_{(1,28)} = 5.397$, $p = 0.028$], preventing exposed animals, contrary to controls, [interaction: $F_{(1,28)} = 7.558$, $p = 0.010$] from associating levers to the corresponding sound cues [lever: $F_{(1,28)} = 7.630$, $p = 0.010$].

Importantly, we also assessed whether these stress-induced effects were sustainable in time after the end of the exposure to stress and found that these effects of stress were reversible. In fact, a similar assessment of stressed-recovered animals and controls (**Figure 3B**) failed to show any difference between groups [stress-recovery exposure: $F_{(1,28)} = 0.976$, $p = 0.332$], with all animals from both groups [interaction: $F_{(1,28)} = 0.178$, $p = 0.676$] being able to associate conditioned sound and appropriate responses[matching vs. non-matching lever: $F_{(1,28)} = 18.217$, $p < 0.001$].

DISCUSSION

The present results show for the first time that chronic stress disrupts the modulation of instrumental responses by conditioned cues and that these stress-induced impairments are transient, being absent after a 6-weeks recovery period. This is of relevance for decision-making, as it is well established that environmental cues can have a strong modulatory effect upon instrumental responses (Estes, 1948), which are the basis of most decision-making processes.

Chronic stress has a strong modulatory influence (either negative or positive) on learning processes, including spatial memory (Sousa et al., 2000), working memory (Mizoguchi et al., 2000; Cerqueira et al., 2007), and behavioral flexibility (Cerqueira et al., 2007), but also in decision-making processes by biasing instrumental actions to habits (Dias-Ferreira et al., 2009). In the present study we show that chronic stress does not affect Pavlovian conditioning nor instrumental learning. Although the effects of chronic stress upon Pavlovian conditioning have never been reported, the latter finding is in accordance with a previous report showing that chronic stress promotes the transfer from goal-directed actions to habit based behaviors without affecting instrumental learning *per se* (Dias-Ferreira et al., 2009). Indeed, in that study, when tested on a devaluation paradigm after 8 days of training (similar to the

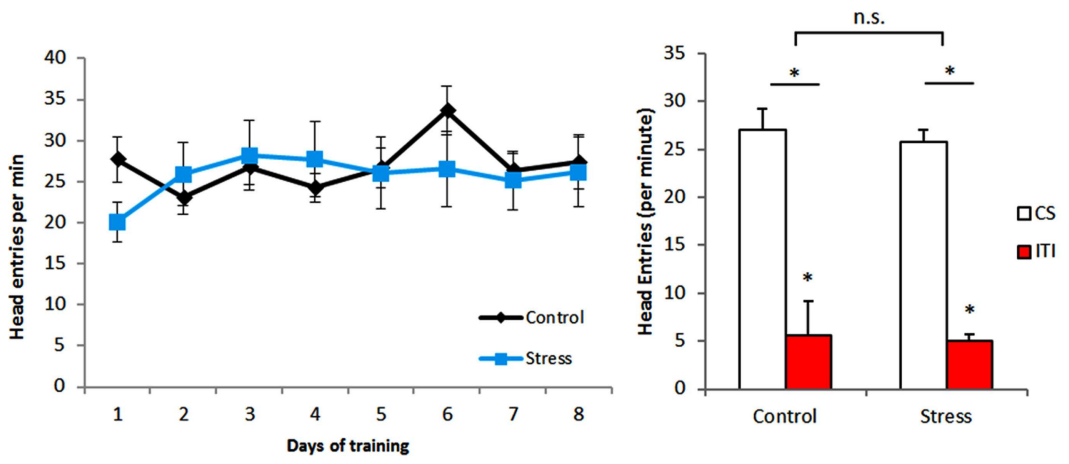

FIGURE 1 | Pavlovian conditioning. There were no differences between groups, with all animals increasing the number of head entries during conditioned stimulus exposure. ITI – intertrial interval between presentations of conditioned stimuli CS – conditioned stimulus. *$p < 0.05$.

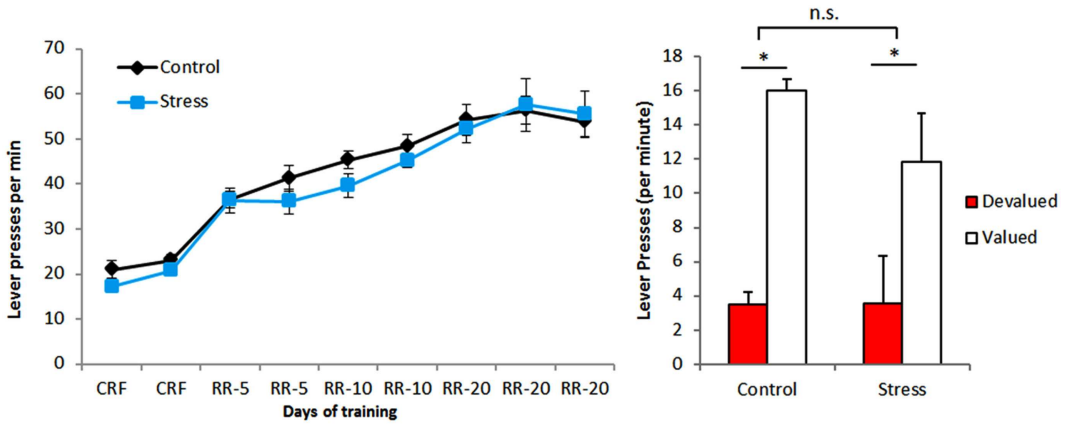

FIGURE 2 | Instrumental conditioning. Both experimental groups acquired the lever pressing task and were able to correctly distinguish devalued from valued levers. $*p < 0.05$.

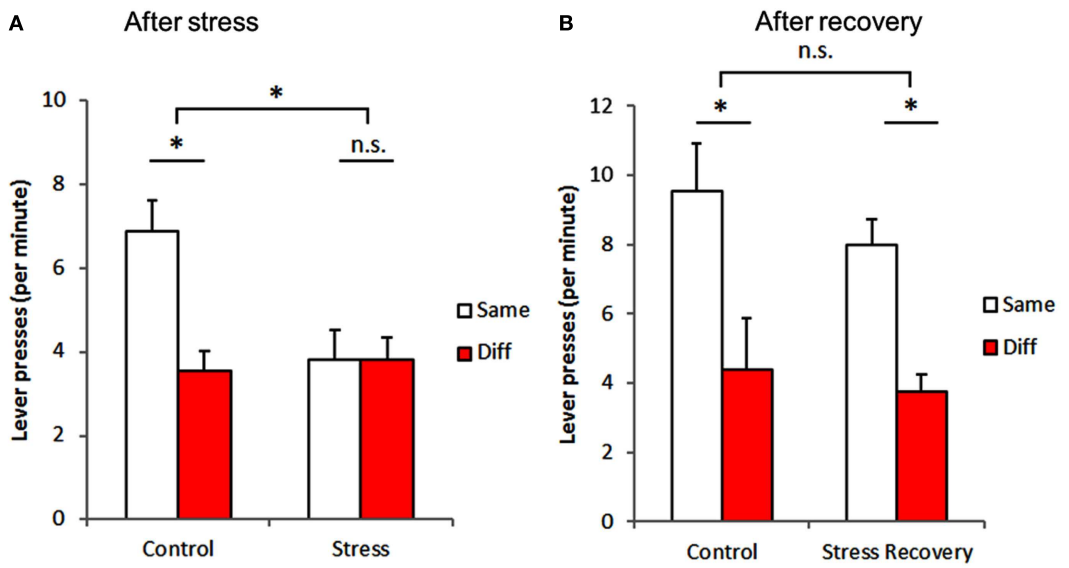

FIGURE 3 | Stress-induced impairment of Pavlovian-to-instrumental transfer is reversible. Stress exposure resulted in an impairment of the association between Pavlovian and instrumental behavior **(A)**, which was no longer observed after a 6-week period without exposure to stressful stimuli **(B)**. Same – conditioned stimulus (sound) predicted the same outcome as the lever pressed; Diff – conditioned stimulus (sound) predicted a different outcome as the lever pressed. $*p < 0.05$.

present protocol), stressed animals were still able to effectively suppress the devaluated response, which was not the case when the test was performed later during training (Dias-Ferreira et al., 2009).

Since neither Pavlovian conditioning nor instrumental learning are affected by chronic stress, the most likely explanation for the herein observed stress-induced impairment of PIT seems to be a deficit in the transfer between the two networks. The precise neuronal networks implicated in PIT are still being described. As stated before, several regions that are known to be susceptible to chronic stress are crucial to PIT. In fact, several studies demonstrate that the mPFC and OFC encode distinct components of both Pavlovian and instrumental processes (Gallagher et al., 1999; Chudasama and Robbins, 2003; Ostlund and Balleine, 2007; Homayoun and Moghaddam, 2008) and a recent study reveals that the OFC and mPFC orchestrate the integration of Pavlovian and instrumental processes during PIT (Homayoun and Moghaddam, 2009). This integration involves distinct operations as mPFC and OFC display predominantly inhibitory and excitatory phasic responses to the same events, respectively. Taken into account our previous observations that stress triggers atrophy in the mPFC and hypertrophy in the OFC (Dias-Ferreira et al., 2009), we suggest the existence of an imbalance in these inhibitory/excitatory responses and, as a consequence, a failure in the reinforcement of goal-directed actions by conditioned stimuli.

While the excitatory response of lateral OFC neurons may signify a positive motivational signal associated with the expected reward (Tremblay and Schultz, 1999; Schoenbaum et al., 2003), the inhibitory response of mPFC neurons evokes their pattern of activity in goal-directed actions (Homayoun and Moghaddam, 2006; Moghaddam and Homayoun, 2008). The fact that there is a re-emergence of inhibitory pattern in prelimbic mPFC neurons re-activates its representation of instrumental action under the influence of the Pavlovian incentives (Homayoun and Moghaddam, 2009). This integration of Pavlovian and instrumental processes, where cue-evoked incentives recruit instrumental representations, may provide a mechanism for the prelimbic mPFC, to execute motivational control over goal-directed behavior; importantly, this re-activation is likely to be compromised after chronic stress as the present results demonstrate. Of course, other regions targeted by stress, such as the striatum, could also be implicated in the stress-induced PIT impairment; in fact, there are studies demonstrating that the dorsolateral striatum is critical for the formation of specific stimulus-outcome associations, whereas the dorsomedial striatum is involved in the formation of specific response-outcome associations. Disruption of either form of learning impairs PIT (Corbit and Janak, 2010) and stress is known to influence the structure and function of both divisions of the dorsal striatum (Dias-Ferreira et al., 2009). In the same vein, ventral striatal areas could also play an important role in the observed impairments as integrity of NAc shell was found to be critical to the transfer effect (Corbit et al., 2001). In fact, previous studies of our lab showed that stress reduces the total volume of this region, impairing its function (Leão et al., 2007). These reported alterations could underlie impairments we have found.

Additionally, current evidence suggests that BLA is involved in the formation of stimulus-reward associations by assigning an affective value to associated rewards (Everitt et al., 1991) and in the production and direction of instrumental actions (Everitt and Robbins, 1992). Although BLA lesions completely abolish both outcome-selective PIT and outcome devaluation, this area integrates different circuits that connect differently with other relevant brain structures. Anterior BLA connects with OFC and shell NAc and posterior BLA connects with prelimbic cortex, medial accumbens core, and key components of instrumental conditioning circuitry (Balleine, 2005). Structural stress-induced alterations described by Vyas et al. (2002) could configure an interesting possibility to explain our results.

Stress has a strong impact in hippocampal structure and function, impairing the learning and storage of newly acquired information (Sousa et al., 2000). In this regard, the herein observed PIT deficits could be due to a disruption of these hippocampal functions, interfering with the consolidation of stimulus-outcome associations. Alternatively, the stress-induced hippocampal dysfunction could also interfere with the hippocampal role in appetitive Pavlovian conditioning (Ito et al., 2005). However, neither of these hypotheses is supported by the fact that chronic stress did not impair Pavlovian conditioning.

Importantly, the stress-induced impairment of PIT was no longer evident after a stress-free period. This reversibility of stress effects is in accordance with previous studies showing the recovery of other stress-induced deficits, including spatial memory (Sousa et al., 2000), and behavioral flexibility (Bloss et al., 2010), after similar stress-free periods. Of note, recovery of these functions is paralleled by synaptic regrowth and reorganization on the hippocampus and the mPFC, which are also involved in PIT. Altogether, these results highlight the extreme plastic capabilities of areas involved in PIT and explain why most stress-induced deficits, including those described in the present paper, are, at least in part, reversible. A better knowledge of the mechanisms underlying these events, to be pursued in future studies, is crucial to optimize therapeutic interventions in altered cue-controlled behaviors, particularly in those situations in which spontaneous recovery is not likely.

ACKNOWLEDGMENTS

The authors acknowledge the discussions with Osborne Almeida. Pedro Morgado is supported by a fellowship "SFRH/SINTD/60129/2009"funded by FCT – Foundation for Science and Technology. Supported by FEDER funds through Operational program for competitivity factors – COMPETE and by national funds through FCT – Foundation for Science and Technology to project "PTDC/SAU-NSC/111814/2009."

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 March 2012; accepted: 07 June 2012; published online: 25 June 2012.

Citation: Morgado P, Silva M, Sousa N and Cerqueira JJ (2012) Stress transiently affects Pavlovian-to-instrumental transfer. Front. Neurosci. ***6****:93. doi: 10.3389/fnins.2012.00093*

This article was submitted to Frontiers in Decision Neuroscience, a specialty of Frontiers in Neuroscience.

Chapter 2.2.

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Stress induced risk-aversion is reverted by D2/D3 agonist

Manuscript under preparation.

Stress induced risk-aversion is reverted by D2/D3 agonist

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Number of pages: 23

Number of figures: 4

Keywords: decision-making, stress, orbitofrontal, insula, dopamine, quinpirole

Abstract

Exposure to stress can lead to cognitive and behavioral impairments that can influence the decision-making processes. Risk-based decisions require complex processes that are known to be mediated by the mesocorticolimbic dopamine (DA) system through brain areas sensible to deleterious effects of chronic stress. Using a new behavioral decision-making task, we shown that chronic stress bias risk-based decision-making to safer options which could be related with hyperactivation of lateral part orbitofrontal and insular cortices. Additionally, chronic stress induced morphological changes in orbitofrontal pyramidal neurons, specifically recruited by this task, and a hypodopaminergic status with low DA levels and high mRNA levels of dopamine receptor type 2 (Drd2). Treatment with D2/D3 agonist quinpirole reverted behavioral impairments induced by stress on decision-making. These data suggests that risk-aversion induced by stress is mediated by dopaminergic orbitofrontal dysfunction, a link that could support new perspectives in the field of neuroeconomics and challenge current therapeutic approaches to neuropsychiatric disorders with known several decision-making impairments such as obsessive compulsive and related disorders.

1. Introduction

Decision-making processes are complex and influenced by multiple factors, but can be described as a basic algorithm consisting of representation, valuation and action selection steps, in which computation of the value associated with each potential action is the determinant element (Rangel et al., 2008). Research in both animal models and humans has revealed that the attribution of value, factoring expectation (the balance between value and effort), predictability (the waiting time until an outcome is attained) and uncertainty (the probability of a given outcome), is carried by a network comprising the medial prefrontal cortex (mPFC) and the orbitofrontal cortex (OFC), as well as subcortical limbic regions, including the dorsal striatum and nucleus accumbens (Doya, 2008). In addition, manipulations of the dopamine (DA) system, the main neurotransmitter modulating mPFC/OFC activity, have also been shown to impair decision-making processes (St Onge et al., 2011; Simon et al., 2009; St Onge et al., 2010; Zeeb et al., 2009), including those involving risk (St Onge et al., 2009).

Chronic stress exposure triggers plastic changes in the brain, particularly targeting the areas involved in valuation and decision-making (Cerqueira et al., 2007, Dias-Ferreira et al., 2009). Accordingly, we have shown that prolonged stress alters decisions by promoting the shift from goal-directed to habit-based choices (Dias-Ferreira et al., 2009) and impairing pavlovian-to-instrumental transfer (Morgado et al., 2012). Interestingly, while initially chronic stress induced hypodopaminergic status has been correlated with prefrontal cortical dysfunction (Mizoguchi et al., 2000), there is presently a growing body of evidence suggesting that stress-induced dopaminergic dysfunction also interferes with the cognitive processes involved in valuation (Rodrigues et al., 2011). However, to the best of our knowledge, no study has addressed the impact of chronic stress on decisions involving risk.

In the present work we investigated the impact of chronic stress exposure on risk-taking behavior and explored its neuronal substrate, focusing on changes in the corticostriatal circuits and their dopaminergic innervation. In particular, we were interested in exploring the valuation of uncertainty, independently of expectation and predictability. To achieve these objectives, we first established a new risk-based decision-making paradigm in which rats choose between certain (safe) and uncertain (risky) options, with similar overall expectations and predictability, and mapped the brain regions it activates; subsequently, we assessed stress-induced changes in task performance and correlated them with alterations in structure and dopaminergic content of

regions differentially activated between stressed and control animals; finally, we tested whether treatment with a DA agonist was able to pharmacologically revert the behavioral changes induced by stress.

2. Materials and Methods

Animals

Sixty adult male Wistar rats (Charles River Laboratories, Barcelona, Spain), aged 2 months and weighting 250-300g at the start of the experiment, were housed in groups of two under standard laboratory conditions with an artificial light–dark cycle of 12:12 h (lights on from 8:00 A.M. to 8.00 P.M.) in a temperature- and humidity-controlled room. Animals were given 2 weeks to acclimate to the housing conditions with ad libitum access to food and water. A food deprivation regimen was initiated twenty-four hours before the initiation of behavioral training and testing to maintain the subjects at approximately 90% of their free-feeding body weight. Rats had free access to water while in the home cage.

All experiments were conducted in accordance with local regulations (European Union Directive 86⁄609⁄EEC) and National Institutes of Health guidelines on animal care and experimentation and approved by Direção Geral Veterinária (DGV; the Portuguese National Institute of Veterinary).

Chronic unpredictable stress

Animals assigned to the stress group were exposed during sixty minutes once a day to one of five different stressors: cold water (18°C), vibration, restraint, overcrowding and exposure to a hot air stream. Stressors were randomly distributed throughout a 28 day period. This type of chronic stress paradigm, mixing different stressors (including physical and psychological components) presented in an unpredictable schedule, was shown previously to result in persistently elevated plasma levels of corticosterone (for details, see Sousa et al., 1998) and is thought to better mimic the variability of stressors encountered in daily life (Sousa et al., 1998; Joels et al., 2004). Controls were carefully handled daily during the same period.

Biometric parameters

To assess stress treatment efficacy, corticosterone levels were measured in serum. For that blood was collected via tail venipuncture at least 8 h after the last stress exposure (4 h before “lights off”) and before initiation of food deprivation. The collected blood was centrifuged at

13.000 rpm for 10 min and supernatant removed and stored at -80°C until use. Serum total corticosteroids levels were measured by radioimmunoassay using a commercial kit (R&D Systems, Minneapolis, MN, USA), according to manufacturer's instructions.

Risk-based decision-making paradigm

Behavioral training and testing took place in 5-hole operant chambers (30.5 cm L × 24.1 cm W × 21.0 cm H) housed within sound attenuating cubicles. Each chamber has five apertures mounted into a curved wall, each hole equipped with a light and crossed by an infra-red detector that monitored animal nose pokes. In the opposite side, one pellet dispenser is used to deliver rewards into a hole crossed by an infra-red detector to check pellet dispenser entries.

The decision-making paradigm is presented in Figure 1A. Each daily session was initiated by switching the home light on, five seconds after the animal was placed in the chamber, and lasted for 30 minutes or 100 trials, whichever occurred first. In each trial, rats could choose between a "safe" hole (resulting in the delivery of 1 pellet with 100% probability) and 4 "risk" holes (resulting in the delivery of 4 pellets with 25% probability); light was used to cue risk options, which were randomly allocated to 4 of the 5 apertures. Importantly, this design of risky and safe choices evens the overall outcome of either option, allowing an analysis of risk-taking behaviors independently of reward value or delay. After each choice, animals had to check the amount of reward received at the pellet dispenser (they were taught to do it by applying a 10s "lights off, holes inactive" penalty if they failed to do so), home cage light was switched off and a new trial started 5 seconds later. Number of trials completed, total time spent, animals' choices and omissions as well as pellets received in each trial were automatically registered by the software and analyzed.

c-Fos immunohistochemistry

Animals were sacrificed 90 minutes after the end of the behavioral task with a lethal injection with pentobarbital and then were transcardially perfused with PBS followed by 4% paraformaldehyde. Control animals were exposed to the same conditions with the sole exception of the behavioral task. Brains were removed and post-fixed in PFA for 4h and then transferred to an 8% sucrose solution and kept at 4°C. 50 µm coronal sections of the forebrain were serially cut

on a vibrotome (Microm HM-650V, Thermo Fisher Scientific, Waltham, MA, USA) at and collected in phosphate buffer (PBS; 0.1M; pH7.2). For c-fos immunohistochemistry, sections were firstly incubated in H2O2 (3.3% in PBS) solution for 30 minutes and then sequentially washed in PBS and PBS-T (0.3% triton X-100; Sigma-Aldrich). Sections were first incubated in 2.5% (in PBS-T) of fetal bovine serum for 2h and then in anti-Fos primary antibody (1:2000 in the same solution; PC38 Anti-c-Fos (Ab-5), Calbiochem, Darmstadt, Germany) overnight. After several washes in PBS-T, sections were incubated with secondary antibody (1:200 in PBS-T; polyclonal swine anti-rabbit E0353, DAKO) for 1h, again washed in PBS-T and incubated in avidin-biotin complex (ABC, 1:200, Vector Laboratories) for 1h. Sections were then sequentially washed with PBS-T, PBS and Tris-HCl (0.05M, pH 7.6) and incubated in 0.0125% diaminobezidine tetrahydrochloride (DAB; Sigma, St. Louis, USA) and 0.02% H2O2 in Tris-HCl for 3-5 minutes to reveal the labeling. All procedures were performed at room temperature. Sections were placed on SuperFrost Plus slides (Braunschweig, Germany), dehydrated, counterstained with hematoxylin.

The number of c-fos positive cells was counted within the boundaries of the medial prefrontal cortex [prelimbic cortex (PrL), infralimbic cortex (IL) and cingulate cortex (Cg1)], OFC [medial (MO), ventral (VO) and lateral (LO) parts], somatosensory cortex (SSC), motor cortex (MC), insula, dorsal striatum [dorsolateral striatum (DLS) and dorsomedial striatum (DMS)] and nucleus accumbens [shell (NAcS) and core (NAcC)] as defined by the Paxinos and Watson atlas (1998). c-fos positive cells densities (number of positive cells / cross sectional area of the region of interest) were calculated for comparisons between groups. Cross sectional area of each region was calculated according to the Cavalieri principle (Gundersen, 1988). For this, we randomly superimposed onto each area a test point grid in which the interpoint distance, at tissue level, was: 100 µm for IL and MO; 150 µm for PL, VO and LO; 350 µm for MC, SSC, NAcS and NAcC; and 500 µm for DLS and DMS, and counted the points that fell into the boundaries of the region of interest. These procedures were done using using StereoInvestigator software (MicroBrightField Bioscience, Magdeburg, Germany) and a camera attached to a motorized microscope.

Gene expression measurements by quantitative Real time PCR (qRT-PCR)

Total RNA was isolated from the OFC using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The isolated total RNA was reverse transcribed using the iScript cDNA Synthesis Kit for RT-PCR (Bio-

Rad, Laboratories, Hercules, CA, USA). Primers used to measure the expression levels of selected mRNA transcripts by qRT-PCR were designed using the Primer3 software (Rozen and Skaletsky, 2000), on the basis of the respective GenBank sequences. qRT-PCR analysis was used to measure the mRNA levels of the following genes: dopamine receptor D1A (*Drd1a*), dopamine receptor D2 (*Drd2*) and dopamine receptor 3 (*Drd3*). The reference gene for hypoxanthine guanine phosphoribosyl transferase (*Hprt*) (accession number from GenBank: NM_012583) was used as an internal standard for the normalization of the expression of selected transcripts, since we have first confirmed that its expression is not influenced by the experimental conditions. All accession numbers and primer sequences are available on request. qRT-PCR was performed on a CFX 96TM real time system instrument (Bio-Rad), with the QuantiTect SYBR Green RT-PCR reagent kit (Qiagen, Hamburg, Germany) according to the manufacturer's instructions, using equal amounts of RNA from each one of the samples. Product fluorescence was detected at the end of the elongation cycle. All melting curves exhibited a single sharp peak at the expected temperature.

Dopamine quantification by high-performance liquid chromatography (HPLC)

Following decapitation, the brains were rapidly removed and discrete brain regions, specifically the OFC and insula were dissected. The dissected tissues were weighted, homogenized and deproteinized in 500 μl of 0.2 N perchloric acid solution (Merck KgaA, Darmstadt, Germany) containing 7.9 mM Na2S2O5 and 1.3 mM Na2EDTA (both by Riedel-de Haën AG, Seelze, Germany). The homogenate was centrifuged at 14,000 rpm for 30 min in 4°C and the supernatant was stored at −80°C, until analysis. The analytical measurements were performed using a Pharmacia-LKB 2248 high-performance liquid chromatography (HPLC) pump coupled with a BAS LC4B electrochemical detector (Bioanalytical Systems Inc., West Lafayette, IN, USA), as described by Dalla et al (2004). All samples were analyzed within one month after homogenization. The mobile phase consisted of a 50 mM phosphate buffer regulated at pH 3.0, containing 5-octylsulfate sodium salt at a concentration of 300 mg/L as the ion pair reagent and Na2EDTA at a concentration of 20 mg/L (both by Riedel-de Haën AG, Seelze, Germany). Further on, acetonitrile (Merck &Co., Darmstadt, Germany) was added at a 7–10% concentration. The reference standards were prepared in 0.2 N perchloric acid (Merck KgaA, Darmstadt, Germany) solution containing 7.9 mM Na2S2O5 and 1.3 mM Na2EDTA (both by Riedel-de Haën AG,

Seelze, Germany). The sensitivity of the assay was tested for each series of samples using external standards. The working electrode was glassy carbon and the reference one was Ag/AgCl; the columns were Thermo Hypersil-Keystone, 150× 2.1 mm 5 µHypersil, Elite C18 (Thermo Electron, Cheshire, UK). Sampleswere quantified by comparison of the area under the curve (AUC) against reference standards using a PC compatible HPLC software package (Chromatography Station for Windows ver.17 Data Apex Ltd). The limit of detection was 1 pg/27 µl (volume of HPLC injection loop) and the signal to noise ratio was more than 3:1.

Neuronal 3D-dendritic structure analysis

Pyramidal neurons of lateral part of OFC (lOFC) cortex and of insula (Zilles and Wree 1995) were analyzed. Within the lOFC and insula, layers II-III are readily identifiable in Golgi-stained material on the basis of its characteristic cytoarchitecture. It is positioned immediately ventral to the relatively cell-poor layer I (which also contains the distal dendritic tufts of layer II/III pyramidal cells) and immediately superficial to layer V; this boundary is pronounced because of the greater cell-packing density and smaller somata of pyramidal cells in layers II-III relative to layer V in this region of the brain (Van Eden and Uylings 1985; Cajal 1995; Zilles and Wree 1995). Golgi-impregnated pyramidal neurons of the lOFC and insula were readily identified by their characteristic triangular soma, apical dendrites extending toward the pial surface, and numerous dendritic spines. The criteria used to select neurons for reconstruction were those described by Uylings et al. (1986): 1) location of the cell soma in layer II-III of the lOFC, approximately in the middle third of the section; 2) full impregnation of the neurons; 3) apical dendrite without truncated branches (except on the most superficial layer); 4) presence of at least 3 primary basal dendritic shafts, each of which branched at least once; and 5) no morphological changes attributable to incomplete dendritic impregnation of Golgi-Cox staining. In order to minimize selection bias, slices containing the region of interest were randomly searched and the first 10 neurons fulfilling the above criteria (maximum of 3 neurons per slice) were selected. For each selected neuron, all branches of the dendritic tree were reconstructed at 6003 magnification using a motorized microscope (Axioplan 2, Carl Zeiss, Germany), with oil objectives, and attached to a camera (DXC-390, Sony Corporation, Tokyo, Japan) and Neurolucida software (MicroBrightField Bioscience). A 3D analysis of the reconstructed neurons was performed using NeuroExplorer software (MicroBrightField Bioscience).

Imuno-golgi staining

Stressed and control rats were sacrificed 90 minutes after the end of the behavioral task and were transcardially perfused with 0.9% saline under deep pentobarbital anesthesia and processed according to the protocol described by Pinto et al. (2012).

Brains were removed, dropped into Golgi-Cox solution and kept in the dark for 15 days. Next, they were transferred to a 30% sucrose solution and kept in the refrigerator for 2 to 5 days in the dark until they sink. Sections (200 µm) were obtained in a vibratome (Microm HM-650V) and transferred to 24-well multiwell plate filled with distilled water for 15 min and then dipped in ammonium hydroxide (Sigma) for 5 min in the dark. Sections were washed with distilled water twice, 10 min each, and dipped in Kodak Fix solution (Rapid fixer; Sigma) for 20 min. After washes in distilled water, 10 min each, sections were dipped in PBS, and kept cool in the refrigerator.

After Golgi-Cox staining, sections were transferred to 6-well multi-well plates with citrate buffer (10 mM; pH = 6). For antigen retrieval sections were heated for 5 min in the microwave to near 100° to expose the cFOS epitope in the tissue. Sections were then rinsed in PBS-T (0,3 % of Triton®-X 100) 3 times, for 10 min and blocked during 1h with 2,5 % FBS in PBS-T and incubated with primary cFOS antibody (1:1000 in PBS-T and 2 % of fetal bovine serum; Calbiochem) overnight at 4 °C. The next day, sections were rinsed with PBS-T and incubated with secondary antibody anti-rabbit Alexa Fluor 594 (1:500 in PBS-T; Invitrogen, Carlsbad, CA, USA) for 2 h at RT. Finally, sections were incubated in DAPI (1 µg/ml) for 10 min at RT and then rinsed in PBS. Sections were mounted in superfrost slides using Vectashield mounting medium (Vector Labs, Burlingame, CA, USA)

Treatment with the D2/D3 agonist

Quinpirole hydrochloride (0.15 mg/kg; Sigma), dissolved in 0.9% sterile saline to a volume of 1 ml/Kg, was administered intraperitoneally. Injections were given 15 minutes before behavioral testing and dose was selected in accordance with previous reports showing behavioral effects of the drug (Kurylo and Tanguay S, 2003; Boulougouris et al., 2009).

Animals were trained for 20 days in the risk-based decision-making paradigm and then half of animals were submitted to a chronic unpredictable stress protocol during 4 weeks while others were carefully handled. At this point, 5 animals of each group were sacrificed to perform c-fos expression, HPLC, rT-PCR and structural analysis. The remaining 40 animals were then tested in 3 consecutive 8-day decision-making paradigms: in the first, safe and risk choices were rewarded with 1 and 4 pellets, respectively, resulting in no net gain; in the second, only the risk choice reward was doubled (8 instead of 4), resulting in an average long-term profit for those who risk; in the third, only the reward in safe choices was doubled (2 instead of 1), resulting in a long-term profit for those who tend to choose safe. Thirty minutes before each daily session, half of the animals of each group (controls and stressed) received i.p. injections of the DA D2/D3 agonist quinpirole (0.15 mg/kg) while the remainder received vehicle.

Statistical Analysis

Data was analyzed using SPSS (version 19.0; IBM). Results are expressed as group means ± SE. Control and stress groups were compared using paired Student's t test. To test for the effects of stress and quinpirole two-way ANOVA was used; groups comparisons were determined using Tukey's honestly significant difference post hoc analysis. For all analysis, differences were considered to be significant if $p < 0.05$.

3. Results

Acquisition of the Risk-Based Decision-Making Task

As expected, during training, animals increased the number of completed trials in each session, inversely decreasing total time spent to do so (Figure 1, panel B); total completed trials were achieved by all animals on the 8th day of training. Animals performing the task did not display any preference between safe and risk options, as they choose approximately 20% of times the safe hole and 80% of times the four risk holes. This pattern was established relatively early and maintained during the entire protocol (Figure 1, panel C).

When rewards, for either the risk or the safe options, were increased (risk favorable or safe favorable conditions, respectively), animals switched their pattern of choices accordingly, decreasing (-15.3% ± 6.68) or increasing (16.0% ± 8.70) the percentage of safe choices relative to the baseline (Figure 1, panel D).

The analysis of c-fos expression, a marker of cell activation, by performance of the task, revealed significant activation of several brain areas, including the medial prefrontal cortex (Prelimbic cortex: $t = -3.61$, $P < 0.05$; Infralimbic cortex: $t = -1.58$, $P < 0.05$; Cingulate cortex: $t = -2.22$, $P < 0.05$), OFC (Medial OFC: $t = -3.14$, $P < 0.05$; Ventral OFC: $t = -3.97$, $P < 0.05$; Lateral OFC: $t = -7.28$, $P < 0.05$), insular cortex ($t = -4.45$, $P < 0.05$), dorsal striatum (DLS: $t = -5.45$, $P < 0.05$; DMS: $t = -2.86$, $P < 0.05$) and nucleus accumbens (NAcc Shell: $t = -2.41$, $P < 0.05$; NAcc Core: $t = -3.76$, $P < 0.05$), when compared with animals that were not exposed to the task but were placed in the chamber. Interestingly, no differences were found in principal somatosensory ($t = -0.88$, $P = 0.40$) and motor cortices ($t = -1.78$, $P = 0.09$) (Figure 1, panel E).

Stress bias to safe options and is associated with changes in the orbitofrontal and insular cortices

Chronic unpredictable stress significantly altered the pattern of choices, leading to an increased preference for safe options in all three different paradigms (basal: $t = -6.206$, $P < 0.05$; risk favorable: $t = -3.43$, $P < 0.05$ and safe-favorable: $t = -4.03$, $P < 0.05$) (Figure 2, panel A).

This altered pattern of choice was accompanied by differential c-fos activation during the task. Chronic stressed animals displayed significantly increased activation on the lateral part of the OFC ($t = -2.32$, $P < 0.05$) and on the insula ($t = -2.50$, $P < 0.05$) when compared to controls;

interestingly, no other significant differences were found in any of the brain regions analyzed (Figure 2, panel B).

Given the over-activation observed on orbitofrontal and insular cortices, we measured DA levels in these regions by HPLC. Data shows a significant decrease on DA concentration after chronic stress in the OFC ($t = 3.32$, $P < 0.05$). In contrast, no significant differences were found in insular cortex ($t = -1.30$, $P = 0.24$) (Figure 3, panel A).

Subsequently, we quantified the different DA receptors in the OFC. Expression levels of the mRNAs encoding the *Drd1* ($t = -0.04$, $P = 0.97$) and Drd3 (-0.49, $P = 0.64$) receptors did not differ between controls and stressed animals. However, there was a significant up-regulation of *Drd2* mRNA in this brain region ($t = -3.42$, $P < 0.05$) (Figure 3, panel B).

Finally, we performed a three-dimensional morphometric analysis of pyramidal neurons from the lateral part of the OFC and insular cortex. When compared with controls, chronically stressed animals displayed a significant increase in the length of apical dendrites ($t = -2.96$, $P < 0.05$), but no differences were found in basal dendrites ($t = -1.59$, $P = 0.15$) (Figure 3, panel D) of lOFC neither in apical and basal dendrites of insula (Figure 3, panel C). To test whether the neurons activated by the behavioral task also displayed the same morphological changes, we performed the immuno-golgi staining that revealed that similar differences in the apical dendrites of lOFC were present when considering only those cells activated by performance of the task (c-fos positive cells: $t = -2.60$, $P < 0.05$) (Figure 3, panel C).

D2/D3 agonist quinpirole reverts stress effects on behavior

Given our prior observations, but also other studies (Onge and Floresco, 2008; Zeeb et al., 2009; Onge et al., 2010), we decided to test the effect of quinpirole, a D2/D3 agonist, on risk-based decision-making behavior. As mentioned before, chronically stressed animals displayed an increased preference for safe choices in all three different paradigms. Interestingly, administration of quinpirole reverted this bias; indeed, the D2/D3 agonist reverted the risk-aversion induced by stress in all three different conditions [Basal: $F(3,39) = 7.26$, $P<0.01$; stress+quinpirole vs stress, $P<0.01$; Risk favorable: $F(3,39) = 4.30$, $P<0.01$; stress+quinpirole vs stress $P<0.05$; Safe favorable: $F(3,39) = 4.45$, $P<0.01$; stress+quinpirole vs stress, $P<0.05$], making their pattern of choices undistinguishable from that of untreated controls (Basal: untreated controls vs stress+quinpirole, $P=0.80$; Risk favorable: untreated controls vs

stress+quinpirole, P=0.95; Safe favorable: untreated controls vs stress+quinpirole, P=0.63) (Figure 4). Of notice, continued treatment with quinpirole had no effect on the choices of non-stressed animals in any of the paradigms (Basal: non-stressed+quinpirole vs untreated controls, P=0.96; Risk favorable: non-stressed+quinpirole vs untreated controls, P=0.99; Safe favorable: non-stressed+quinpirole vs untreated controls, P=0.97) (Figure 4).

4. Discussion

The present study shows, for the first time, that risk-aversion induced by chronic stress exposure correlates with overactivation, increased dendritic arborization and decreased dopaminergic activity in the OFC; more importantly, we also show that this behavioral change can be corrected by administration of the D2/D3 agonist quinpirole, leading to a complete restoration of risk-taking preferences.

In order to analyze the impact of chronic stress on risk-taking behaviors and dissect its correlates, we developed a new decision-making task in which animals are given the choice between 1 certain option and 4 uncertain but otherwise similar options, the latter resulting in a ¼ probability of receiving a 4 times bigger reward, all rewards being delivered simultaneously. By leveling the expectations (balance between value and effort) and predictability (time until reward delivery) of the task, and contrary to previously published risky-behavior tasks (Cardinal and Howes 2005, den Bos et al., 2006, Simon et al., 2009, Boulougouris et al., 2009, Zeeb et al., 2009), the design of the herein described paradigm makes the choice between a certain (safe) and an uncertain (risky) reward to be dependent only of "risk-taking behavior", and thus a better readout of the latter. In the initial characterization of the task, we found out that, in basal conditions, animals had a performance at chance level (1 out of 5) and thus did not show a preference for either option. Importantly, this allowed us, in subsequent experiments, to assess the impact of chronic stress and/or pharmacological manipulations on risk-taking behavior, by quantifying the preference shift from this baseline condition. In addition, we also showed that this baseline behavior could be manipulated by modifying the expectations associated with each option, thus highlighting the importance of independently manipulating each variable.

The next step, involved the topographical analysis of the corticostriatal regions engaged in this task. As expected, this analysis revealed activation in almost all key areas known to be involved in decision making processes, including the orbitofrontal and medial prefrontal cortices, the insular cortex, the dorsal striatum and the nucleus accumbens; of notice, areas not usually associated with such processes, such as the somatosensory and the motor cortices, were also not activated by our task, increasing the specificity of the activated areas. All together, these features suggest this new behavioral task to be highly valuable in exploring animal preferences based on certainty of different options available and in the study of mechanisms underlying the modulations of such behaviors.

As we have previously shown, chronic stress has a strong impact on behaviors and can profoundly alter decision-making, impairing behavioral flexibility (Cerqueira et al., 2007) and biasing decisions to habits (Dias-Ferreira et al., 2009). Surprisingly, however, no study to our knowledge has explored the impact of chronic stress on the willingness to take risks, as we have done in the present work. A recent paper by Pabst et al. (2013) has shown that, in humans, acute stress exposure before the task increases the preference for risky options, which can be correlated with an increase in salivary cortisol reflecting the activation of the hypothalamus-pituitary-adrenal (HPA) axis. Interestingly, these data are in line with results by Koot and collaborators (2013) revealing that acute corticosterone administration, which partially mimics HPA axis activation, promotes the choice of unfavorable conditions. However, both studies seem to be in contradiction with the present findings that chronic stress increases the preference for safe options. These contrasting and opposing effects of acute versus chronic stress have been described in other behavioral domains (more importantly in cognition, where acute stress enhances while chronic stress impairs memory, see Lupien 2009 for a review) and might represent a key feature of its action. Indeed, while acute stress can be considered adaptive (Diamond et al., 1992), chronic or prolonged stress becomes maladaptive, in line with its negative impact in memory (Cerqueira et al., 2007), executive function (Sousa and Almeida, 2012), goal-directed behaviors (Dias-Ferreira et al., 2009) and, herein, risk preference.

Despite these considerations, our observations suggest that animals submitted to chronic stress change their valuating systems, overrating losses and, subsequently, avoiding 'risk' options that imply the possibility of not receiving any reward. Importantly, in the present paper we also show, using the expression of the c-fos protein, that this behavioral effect seems to be mediated by an over-activation of lateral part of OFC and insular cortex. Intriguingly, but significantly, these are exactly the same two regions that mediate the effect of acute corticosterone administration on a rodent Iowa gambling task described above (Kloot et al., 2013), which strongly suggests these areas to be key to the impact of stress and glucocorticoids on such tasks involving risk. The OFC is critically involved on assigning and updating reward values, encoding a wide range of other variables indispensable for decision-making, including expected outcomes (Schoenbaum et al., 1998), effort associated to each option (Roesch and Olson, 2005; Kennerley et al., 2009), confidence in the decision (Kepecs et al., 2008) and the probability of win (Kennerley et al., 2008). Interestingly, rodent lesion studies have highlighted that the OFC encodes specific information about the outcome rather than its general affective value (Burke et al., 2008).

Additionally, we had previously shown that chronic stress biased behavior from goal-directed to habit based choices, which was mediated by a shift from an atrophied medial prefrontal loop to a hypertrophied orbitofrontal network (Dias-Ferreira et al., 2009). In accordance with this finding, we have also identified a deleterious impact of chronic stress in pavlovian to instrumental transfer, an ability highly dependent on the integrity of the OFC (Morgado et al., 2012). Furthermore, the insular cortex was also over-activated during the task in stressed animals. This brain region is involved in representations of bodily internal states and needs (Naqvi and Bechara 2009) and in risk-aversion signaling (Clarke et al., 2008; Preuschoff et al., 2008). The first is crucial in chronic stress, where body states and bodily perception are significantly altered, and the second is of relevance in the context of this specific task. Insula lesion studies have shown an increased in risk non-advantageous choices (Clarke et al., 2008), which made expectable that insular over-activation could lead to a risk-aversion pattern of choice.

Significant DA depletion was found in OFC, but not in the insular cortex, associated with over expression of Drd2 mRNA, suggesting that DA depletion and subsequent overregulation of Drd2 are the underlying mechanisms of OFC over-activation. This is in line with previous studies revealing a role for a stress-induced hypodopaminergic status in the PFC in the genesis of working memory (Mizoguchi K et al., 2000) and decision making deficits (Tseng and O'Donnell 2004, Gruber et al., 2010); of notice, the latter were ascribed to a lack of inhibitory actions of D2 receptors on NMDA-induced responses (Tseng and O'Donnell, 2004). Irrespective of the underlying mechanism, our observation that the stress-induced bias on risk-based decision-making can be pharmacologically reverted by a D2/D3 agonist quinpirole clearly proves their role in this process. These observations are in accordance with the hypothesis that stress induced hypodopaminergic state could mediate its behavioral effects on risk-based decision-making through hyperactivation of OFC. Indeed, two recent papers pointed out the role of dopaminergic system in stress resilience (Zurawek et al., 2013) and social aversion induced by chronic stress (Barik et al., 2013).

Previous studies have reported effects of dopaminergic agents on decision-making behaviors, associating dopaminergic agonists with increased rates of risk choices (Onge and Floresco, 2008; Riba et al., 2008; Onge et al., 2010). As chronic stressed animals were risk-aversive, it could be argued that quinpirole effects observed in our study could be explained by an unspecific increasing of risk-prone behavior induced by dopaminergic activation. However, if it was a non-specific effect of dopaminergic activation it would be expectable that non-stressed animals

treated with quinpirole also increased their frequency of risk choices, which was not verified herein. Additionally, contradictory data on literature reported impaired performance on gambling tasks induced by dopaminergic agonists (Zeeb et al., 2009) and related lower dopaminergic levels with higher risk choices in IGT (Sevy et al., 2006) which supports the idea that effects of dopaminergic drugs on decision-making cannot be explained in an oversimplistic way and could be dependent on basal levels of DA, available dopaminergic receptors, specific features of decision-making tasks and duration of treatment.

Our results suggest, for the first time, that risk-aversion induced by chronic stress is due to reduced DA levels in OFC and that this impairments on decision-making can be reverted with dopaminergic agents. These findings can have a strong impact not only for unveiling specific mechanisms underlying stress-induced decision-making impairments but also for proposing a pharmacological intervention that can restore risk-based decision-making. Since decision-making impairments are core symptoms in several neuropsychiatric disorders such as gambling, obsessive and impulsive disorders, our data could support the possibility of explore alternative pathological mechanisms and develop new and more effective treatments and interventions.

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Figures

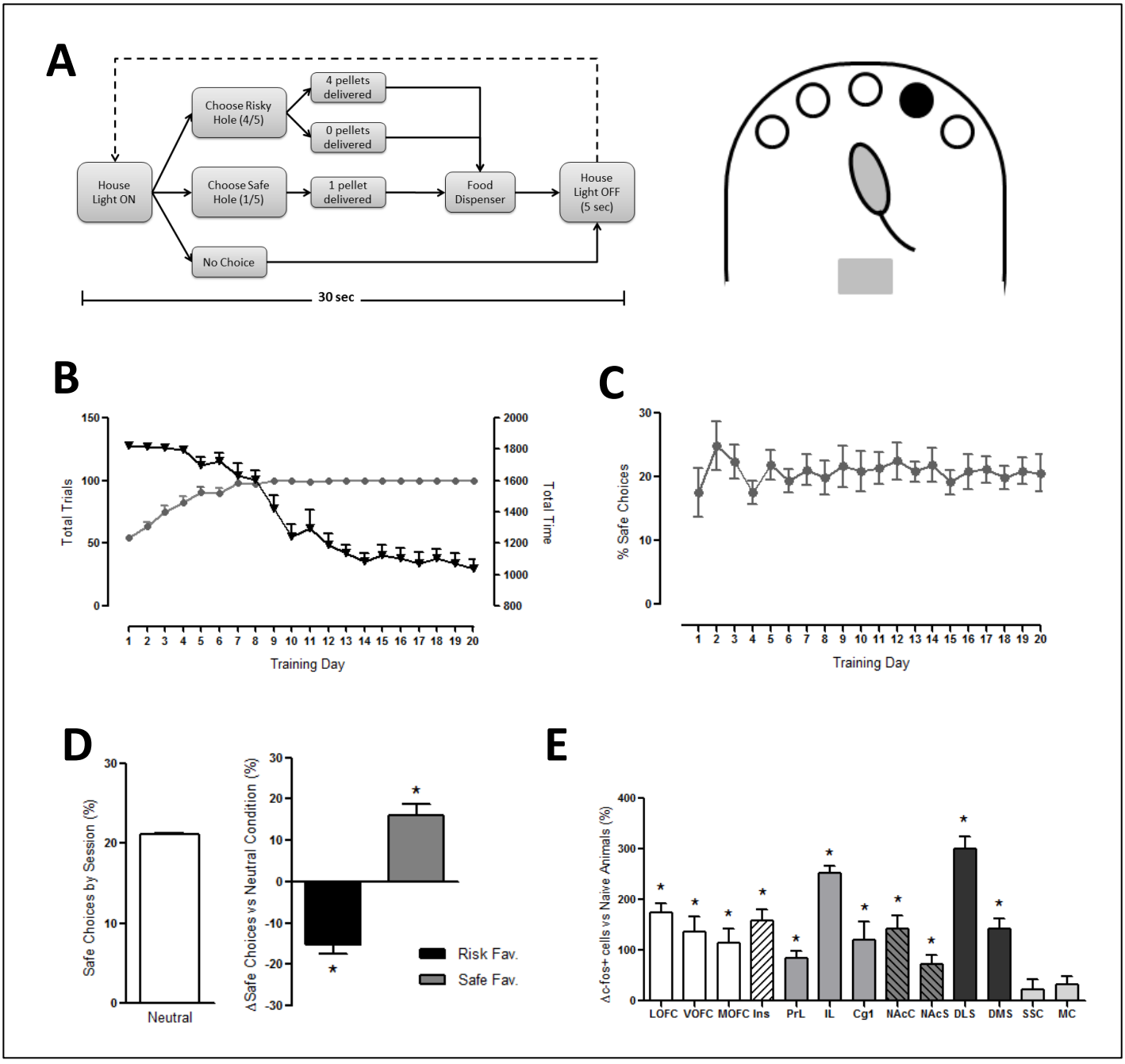

Figure 1. Risk-taking task. (A) Flow-chart of one trial in the neutral condition, in which the overall gain is the same for risky or safe choices; each daily session consisted of 100 trials or 30 minutes of testing. **(B)** Animals significantly decreased the total session time and increased the number of trials per session until the maximum of 100 in the first two weeks. **(C)** Under the neutral condition, animals stabilize their performance at around 20% of safe choices, without a net preference for risk or safe. **(D)** Increases (doubling) in the amount of reward of the risky

choices (risk favourable condition) or the safe choices (safe favourable condition) lead to a reduction (risk favourable) or increase (safe favourable) in the % of safe choices, compared with the neutral condition, which is of similar magnitude. *p<0,05 vs neutral condition. **(E)** Increase (in %) in the density of c-fos positive cells in several brain areas in animals that performed the task, as compared with home cage controls. Areas activated by the task include the orbitofrontal cortex (OFC), the insula, the prefrontal cortex (PFC), the Nucleus accumberns (Nacc) and the dorsal striatum (DS). L/V/MOFC (lateral/ventral/medial OFC), PrL (Prelimbic cortex), IL (infralimbic cortex), Cg1 (cingulate cortex), NaccC/S (Nacc Core/Shell), DLS/DMS (Dorsomedial/lateral striatum). *p<0,05 vs home cage controls.

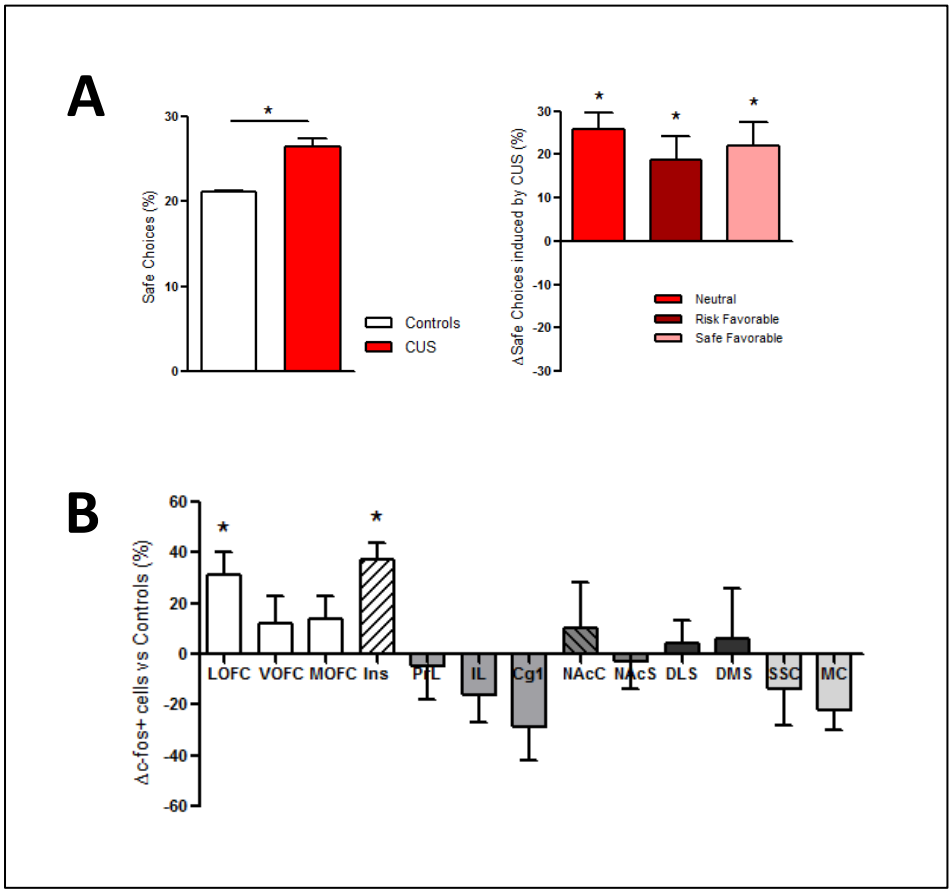

Figure 2. Effects of chronic stress on risk-taking behavior. (A) Chronic stress significantly decreased risk choices among the three different protocols tested. *p<0,05 vs non-stressed controls. **(B)** Increased (in %) in the density of c-fos positive cells in orbitofrontal cortex (OFC) and insular cortex in chronically stressed animals performing the task when compared with non-stressed controls. No significant differences were found in other brain areas examined. *p<0,05 vs non-stressed controls.

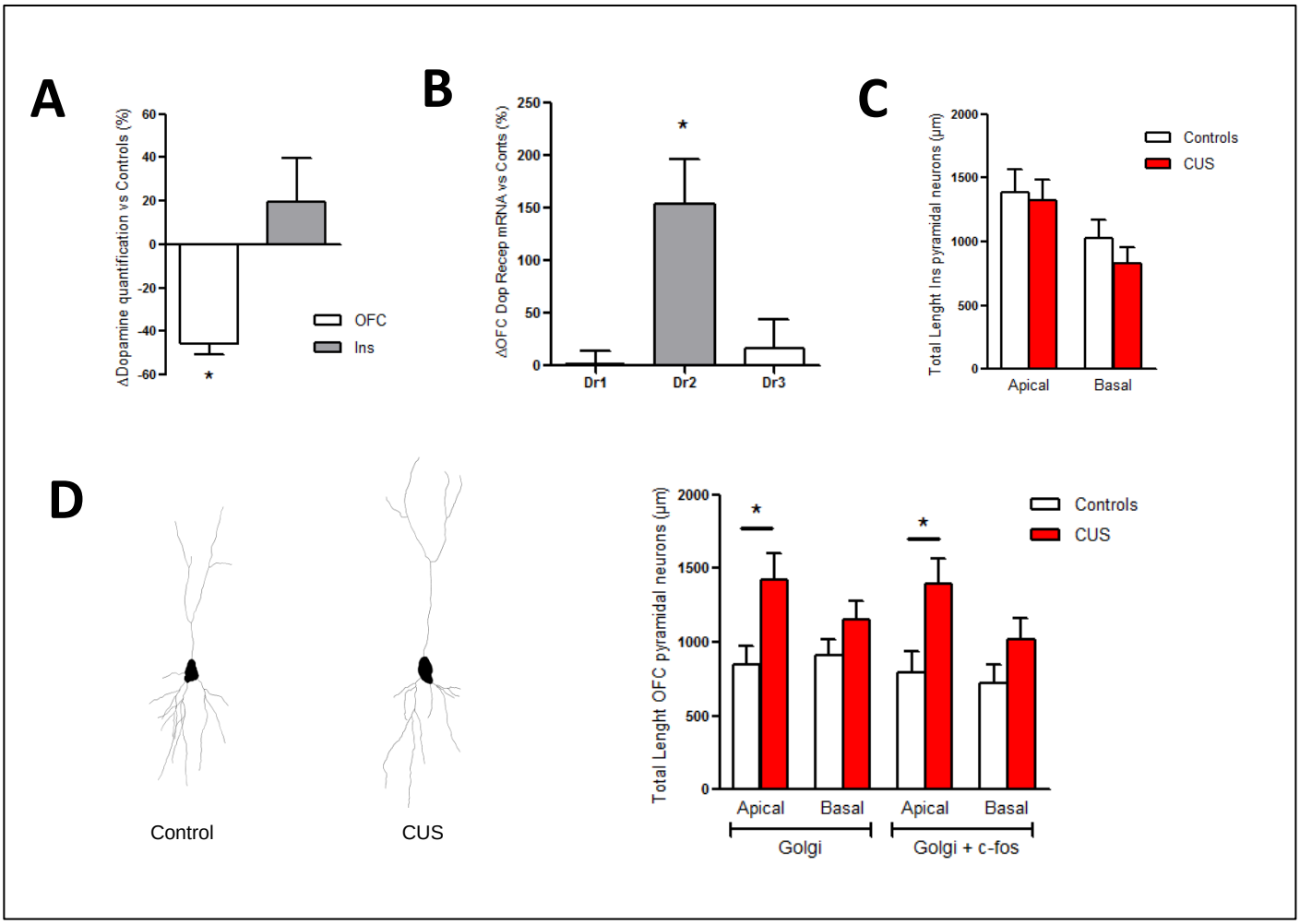

Figure 3. Neurochemical and neural effects of chronic stress. (A) The effects of chronic stress on HPLC DA levels in orbitofrontal cortex (OFC) and insula as compared with non-stressed controls. *p<0,05 vs non-stressed controls. **(B)** The effects of chronic unpredictable stress on dopamine D1, D2 and D3 receptors mRNA levels (in %) in orbitofrontal cortex (OFC) when compared with non-stress controls. *p<0,05 vs non-stressed controls. **(C)** Morphometric analysis of apical and basal dendrites in the insular pyramidal neurons in animals submitted to chronic stress when compared with non-stressed controls. **(D)** Morphometric analysis of apical and basal dendrites in the OFC pyramidal neurons in animals submitted to chronic stress when compared with non-stressed controls. Neurons specifically recruited by the task were assessed with imunogolgi staining (golgi+c-fos). *p<0,05 vs non-stressed controls.

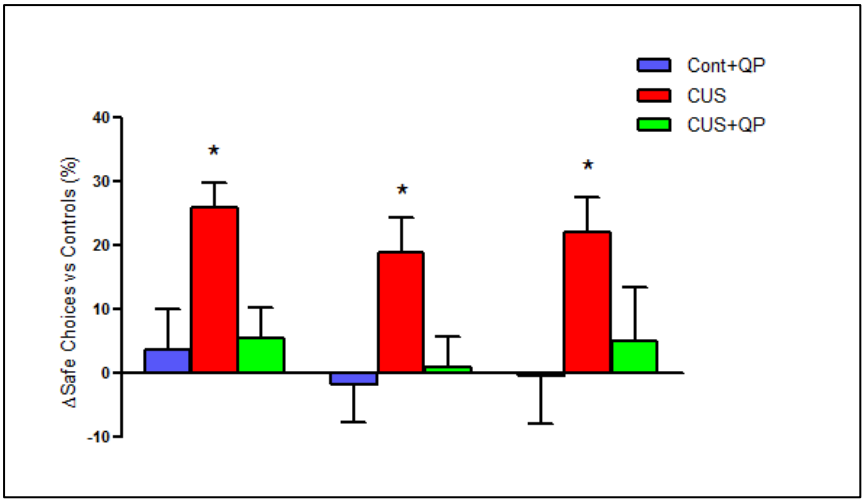

Figure 4. Quinpirole effects on decision-making. Chronic stress significantly increases frequency of safe choices (in %) as compared with non-stressed controls, an effect that is reverted by D2/D3 agonist quinpirole. *p<0,05 vs non-stressed controls.

Chapter 2.3.

Morgado P, Freitas D, Bessa JM, Sousa N, Cerqueira JJ. (2013)

Perceived Stress in Obsessive Compulsive Disorder is Related with Obsessive but Not Compulsive Symptoms.

Frontiers in Psychiatry 4, 4: 1-6.

frontiers in PSYCHIATRY

ORIGINAL RESEARCH ARTICLE
published: 02 April 2013
doi: 10.3389/fpsyt.2013.00021

Perceived stress in obsessive–compulsive disorder is related with obsessive but not compulsive symptoms

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Obsessive–compulsive disorder (OCD) is achronic psychiatric disorder characterized by recurrent intrusive thoughts and/or repetitive compulsory behaviors. This psychiatric disorder is known to be stress responsive, as symptoms increase during periods of stress but also because stressful events may precede the onset of OCD. However, only a few and inconsistent reports have been published about the stress perception and the stress-response in these patients. Herein, we have characterized the correlations of OCD symptoms with basal serum cortisol levels and scores in a stress perceived questionnaire (PSS-10). The present data reveals that cortisol levels and the stress scores in the PSS-10 were significantly higher in OCD patients that in controls. Moreover, stress levels self-reported by patients using the PSS-10 correlated positively with OCD severity in the Yale–Brown Obsessive–Compulsive Scale (Y–BOCS). Interestingly, PSS-10 scores correlated with the obsessive component, but not with the compulsive component, of Y–BOCS. These results confirm that stress is relevant in the context of OCD, particularly for the obsessive symptomatology.

Keywords: obsessive–compulsive disorder, stress, cortisol, Y–BOCS, PSS-10

INTRODUCTION

Obsessive–compulsive disorder (OCD) is a psychiatric disorder that affects 2–3% of population worldwide (Ruscio et al., 2010) and carries high levels of morbidity (Murray and Lopez, 1996; Hollander et al., 2010). It is characterized by obsessions (persistent, intrusive, and inappropriate thoughts, as well as impulses or images that cause anxiety) and compulsions (repetitive behaviors or thoughts performed in order to decrease the anxiety caused by the obsessions). Although genetic factors play an important role in the etiology of disease, several reports implicate environmental influences such as relevant life events and traumatic events in the onset of the disease (Zohar et al., 2007; Forray et al., 2010). Analyzing a group of 74 female OCD patients, Lochner et al. (2002) found them to have higher rates of childhood trauma than healthy controls. Interestingly, subsequent studies demonstrated that frequency, clinical pattern, and severity of OCD symptoms correlated not only with a history of one or more traumatic life events (Gershuny et al., 2003; Cromer et al., 2007; Real et al., 2011) but also with their intensity (Jordan et al., 1991).

Importantly, the stress-response and the activity of the hypothalamic-pituitary-adrenal (HPA) axis have been shown to be relevant in the context of several psychiatric disorders (Holsboer, 1983). This also holds true for OCD as it is known that stressful events may precede the onset of OCD (Toro et al., 1992) and that, in addition, OCD symptoms increase at times of stress (Findley et al., 2003). Nevertheless, it is also true that core symptoms of the disease, namely obsessions, cause significant distress, and as a consequence, may trigger physiological stress-related systems such as the HPA axis. However, the characterization of the activity of the HPA axis in OCD is still a matter of dispute, with some studies reporting normal levels of cortisol (Kuloğlu et al., 2007) and a normal dexamethasone suppression response (Monteiro et al., 1986; Jenike et al., 1987), while others observe high levels of cortisol (Gehris et al., 1990; Kluge et al., 2007) and non-suppression of cortisol during suppression tests (Cottraux et al., 1984; Catapano et al., 1990). Yet, the discrepancies extend beyond the measurement of cortisol levels; in fact, while one study showed increased corticotrophin releasing hormone (CRH) levels in the cerebrospinal fluid (Altemus et al., 1992) of OCD patients, which might be indicative of hyperactivation of stress-response systems, another found a decreased pituitary volume in OCD patients (Jung et al., 2009), which is suggestive of hypofunction of the adenohypophysis.

In light of such controversy, we thought of interest to further characterize the link between OCD and stress. In order to achieve this aim, we measured basal serum cortisol levels and assessed the perception of stress using a validated perceived stress scale 10 (PSS-10) in a group of OCD patients (without depression) and in a cohort of age- and sex-matched controls. In patients, such measurements were subsequently correlated with OCD symptoms, namely by discriminating the obsessive and compulsive components using the Yale–Brown Obsessive–Compulsive Scale (Y–BOCS).

MATERIALS AND METHODS

PARTICIPANTS

The cohort under analysis comprised 18 patients with OCD and 18 healthy controls. Patients were admitted to the Psychiatric

Department of Hospital de Braga as outpatients with a diagnosis of OCD. All patients were aged >18 years and able to communicate in Portuguese. Diagnosis was established by experienced psychiatrists with a semi-structured interview based on Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV)-TR and corroborated by a severity score of 7 or greater on the Y–BOCS. Exclusion criteria included: any other mental disorder revealed by the Mini-International Neuropsychiatric Interview (MINI Plus; except for OCD), any acute and/or chronic medical illness as assessed by a physical examination and routine laboratory examination, females who are pregnant or lactating and substance dependence within the previous 12 months. From 21 patients initially enrolled, 3 dropped out and thus only 18 were analyzed (2 patients were not included because they did not attend for blood collection and in the remainder case, blood sample was not collected properly). The three matching controls were also excluded from the analysis.

Healthy controls were carefully recruited to match OCD patients for age, sex, educational level, ethnical origin, and dominance. Exclusion criteria included previous history of neuropsychiatric disorder, any present mental disorder revealed by MINI Plus and use of any medication (excluding oral contraceptives).

All subjects provided written informed consent following a description of the procedures. The study protocol was approved by the Ethics Committee of the Hospital of Braga, Portugal. The study was performed in accordance with the Declaration of Helsinki.

INSTRUMENTS

Sociodemographic form

All subjects were evaluated by a semi-structured questionnaire form in order to characterize gender, age, marital status, educational level, professional status, ethnical origin, and previous medical history of the cohorts. OCD patients were also evaluated in terms of duration of illness, type of obsessions, and compulsions and medication taken. **Table 1** summarizes the characteristics of patients and healthy controls.

Mini-international neuropsychiatric interview

Patients were assessed with MINI Plus, a short structured diagnostic interview (Sheehan et al., 1998), design to screen for neuropsychiatric diagnosis according to the DSM-IV.

Yale–Brown obsessive–compulsive scale

Yale–Brown Obsessive–Compulsive Scale was used to assess the severity of OCD and to discriminate the symptoms subcomponents of the disorder. The Y–BOCS is composed of 10-items, half related with obsessions and the other half related with compulsions. Each item is assessed by a clinician and rated on a five-point likert-type scale from 0 to 4 (Goodman et al., 1989).

Perceived stress scale 10

The Portuguese version of the 10-items Perceived Stress Scale, filled-out on the same day of blood collection, was used to assess perception of stress (Cohen et al., 1983). Items were classified on a five-point likert-type scale from 0 (never) to 4 (very often), and refer to the last month. The higher the total score, the greater the intensity of stress perceived by the subject.

Hamilton depression rating scale

This 17-items scale is used to rate the severity of depression (Hamilton, 1960). Scores higher than 25 indicate severe depression while scores below 7 indicate no depression.

Hamilton anxiety rating scale

Fourteen-items scale used to evaluate severity of anxiety (Hamilton, 1959). Each item is scored on a scale of 0 (not present) to 4 (severe). Scores higher than 25 indicate moderate/severe anxiety while scores below 17 indicate mild symptoms.

Blood sampling

Venous blood samples from left forearm vein were collected into 5 mL tubes containing potassium EDTA, between 1:00 and 4:00 p.m. Precise instructions about sleep and alimentation were given to volunteers that should not have any food neither drink except water in the 6-h prior the blood collection. Plasma was separated by centrifugation and stored at −70°C. Serum cortisol was determined by standard radioimmuno assays.

STATISTICAL ANALYSIS

Data was analyzed using SPSS (version 19.0; IBM). Demographics, clinical measures, psychometric scales, and laboratory values were reported using descriptive statistics (frequencies, means, and standard deviation). Group comparisons were carried out by non-parametric Mann–Whitney U test to compare means. For correlation evaluations, the Pearson correlation test was used. Differences were considered to be significant if $p < 0.05$.

RESULTS

No significant differences in age, gender, education, or body mass index were found between the OCD group and controls (**Table 1**). Three OCD patients and four healthy controls were taking oral contraceptives, but no major differences/trends were observed between those on and without them.

The mean OCD severity was 25.61 as measured by Y–BOCS; there was no significant difference between the obsessive and the compulsive sub-scores in OCD patients (**Table 1**). The mean depression score [measured by Hamilton Depression Rating Scale (HDRS)] was 3.83; importantly, no subject displayed values above 7. The mean anxiety score [measured by Hamilton Anxiety Rating Scale (HARS)] was 4.33. The mean age of onset of the disease was 21.61 years and mean duration of illness was 5.72 years. All patients were medicated at the time of study, 77.8% with fluvoxamine alone (200–300 mg/day) and 22.2% with fluvoxamine and clomipramine (200–300 and 75–150 mg/day, respectively). The clinical characteristics of patients are summarized in **Table 1**.

The basal serum concentration of cortisol was significantly higher in OCD patients than in healthy controls ($P = 0.011$) (**Figure 1A**). Stress perception, as assessed by PSS-10, was significantly higher in OCD patients than in control subjects ($P \leq 0.001$) (**Figure 1B**). Importantly, we found a positive correlation between these two measurements of response to stress in our entire population ($r = 0.385$, $P = 0.020$) (**Figure 1C**) that was only replicated in control subjects ($r = 0.717$, $P = 0.001$) (**Figure 1D**) but not in OCD patients ($r = 0.040$, $P = 0.874$) (**Figure 1E**).

Stress self-reported by patients using PSS-10 positively correlated with OCD severity as assessed by Y–BOCS ($r = 0.596$,

Table 1 | Sociodemographic and clinical characteristics of patients with obsessive–compulsive disorder and healthy comparison subjects.

Characteristics	Subjects with OCD ($n = 18$)	Healthy comparison subjects ($n = 18$)	Statistics
Age, years [mean ± SD (range)]	27.33 ± 6.11 (21–38)	26.28 ± 5.21 (20–38)	$P = 0.691$
Male/female	12/6	12/6	
Education, years [mean ± SD (range)]	13.22 ± 1.99 (12–18)	14.06 ± 3.37 (12–18)	$P = 0.346$
Body mass Index [mean ± SD (range)]	23.70 ± 4.18 (17–31)	22.78 ± 2.18 (19–29)	$P = 1.000$
Age of onset [mean ± SD (range)]	21.61 ± 7.05 (9–35)		
Duration of illness [mean ± SD (range)]	5.72 ± 6.70 (0–21)		
Y–BOCS (total score)	25.61 ± 5.90 (12–30)		
Y–BOCS (obsession score)	13.50 ± 3.17 (7–20)		
Y–BOCS (compulsion score)	12.11 ± 3.27 (5–17)		
HDRS (global score)	3.83 ± 2.53 (0–7)		
HARS (global score)	4.33 ± 3.20 (0–16)		
Medication	Only SSRI – 14 (77.8%); SSRI with TCA – 4 (22.2%)		

OCD, obsessive–compulsive disorder; Y–BOCS, Yale–Brown obsessive–compulsive scale; HDRS, Hamilton depression rating scale; SSRI, serotonin selective reuptake inhibitors; TCA, tricyclic antidepressant; HARS, Hamilton anxiety rating scale.

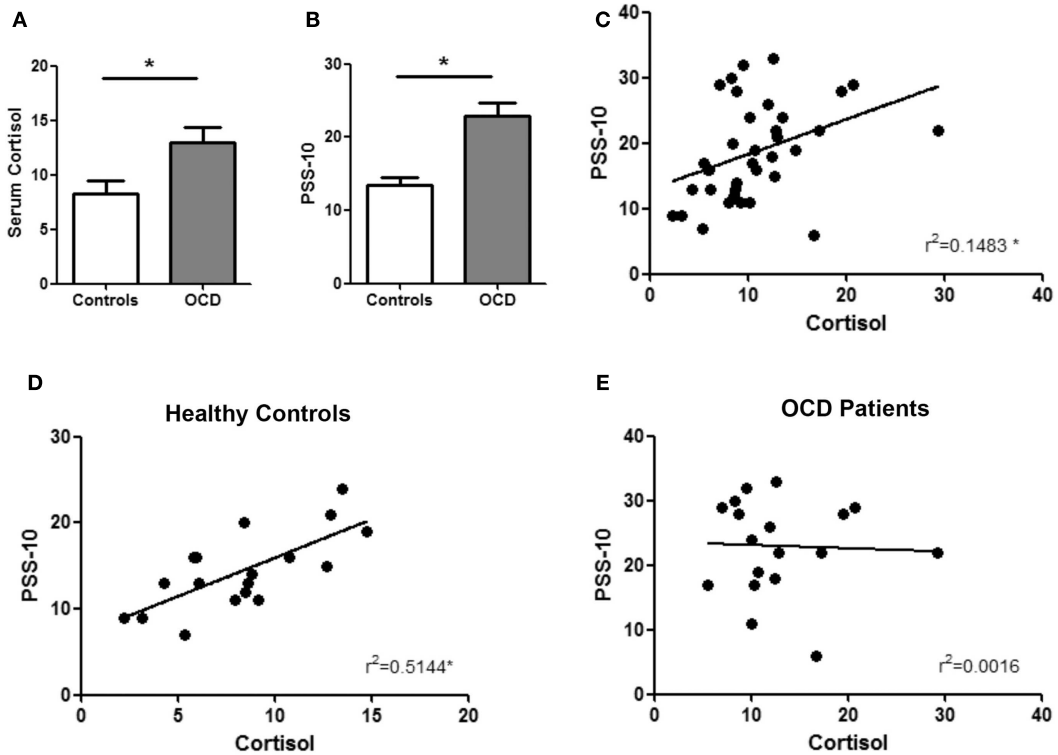

FIGURE 1 | Stress response in OCD patients and controls. OCD patients shown high basal levels of serum cortisol (12.98 ± 5.77 mg/dL) when compared with healthy controls (8.28 ± 3.60 mg/dL) **(A)**. In accordance, OCD patients score higher in the perceived stress scale (PSS-10) questionnaire **(B)**. Importantly, these two measurements were positively correlated **(C)**. Looking at each group separately, cortisol and PSS-10 score correlate in healthy controls **(D)** but not in OCD patients **(E)** $*p < 0.05$.

$P = 0.009$) (**Figure 2A**). Interestingly, no significant correlation was found between cortisol levels and OCD severity as assessed by Y–BOCS ($r = 0.222$, $P = 0.376$) (**Figure 2B**). Importantly, the score on the PSS-10 correlated significantly with obsessive component of Y–BOCS ($r = 0.669$, $P = 0.002$) (**Figure 2C**), but not with the compulsive sub-score ($r = 0.326$, $P = 0.187$) (**Figure 2D**).

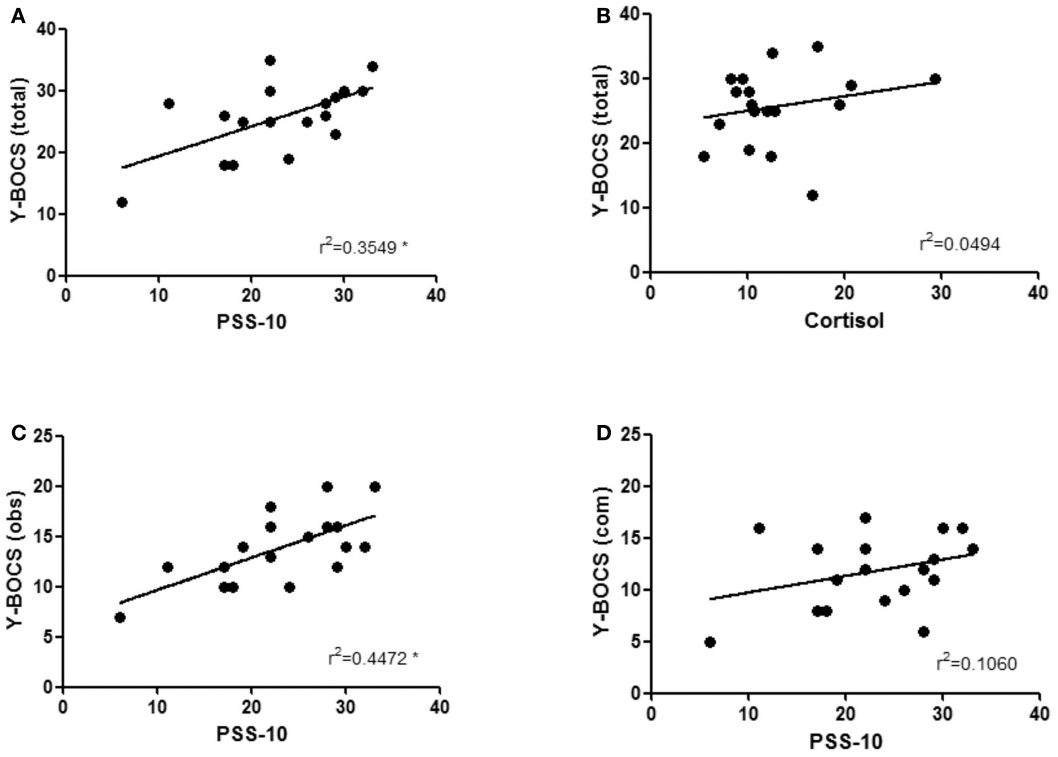

FIGURE 2 | Obsessive–compulsive disorder severity and stress-related measures. OCD severity, as measured by Y–BOCS, positively correlate with PSS-10 score **(A)**, but not with serum cortisol levels **(B)**. Looking at each dimension of the Y–BOCS scale separately, there is a positive correlation between perceived stress and the obsessive score **(C)** but not with the compulsive score **(D)** $*p < 0.05$.

DISCUSSION

In this study, we show that OCD patients report significantly higher levels of perceived stress than healthy controls, and that these are accompanied with higher serum cortisol levels. These findings support the hypothesis that dysregulated stress-response mechanisms are of relevance to this disorder. In this regard, it is important to note that, in our study, self-reported perceived stress levels also correlated positively with global severity of OCD, further strengthening the relevance of our data. Interestingly, these results are in line with a study by Jordan et al. (1991) in which previous traumatic events correlated with the intensity of OCD symptoms. Our data also shows that perceived stress is significantly correlated with the intensity of obsessive symptoms, but not with the intensity of compulsions. Indeed, while obsessions are highly stressful and anxiogenic ideas, compulsive actions are usually perceived as stress relieving. Of note, this finding is in accordance with previous studies that reports that OCD patients suffer significantly more stress by daily events (Coles et al., 2005) and that there is an important relationship between distress tolerance and obsessions (Cougle et al., 2011).

Although self-reported stress was highly correlated with illness severity and obsessive component of Y–BOCS, this study fails to demonstrate correlations between cortisol levels and OCD global severity or each OCD specific component. These can be explained by the recruitment of alternative systems of stress-response but also by the dynamic balance between obsessions and compulsions. High levels of cortisol were reported in previous studies (Gehris et al., 1990; Kluge et al., 2007), even though one study has observed that cortisol elevation was only related with co-morbid depressive symptoms (Kuloğlu et al., 2007). Despite these inconsistent reports, several findings such as non-suppression on dexamethasone test (Cottraux et al., 1984; Catapano et al., 1990), elevation of nocturnal ACTH (Kluge et al., 2007), and reduced pituitary volumes in non-treated OCD patients (Jung et al., 2009) support the altered functioning of HPA axis. Additionally, results from a study that analyzes therapeutic effects and hormonal changes induced by intravenous citalopram treatment suggest that the drug effects are dependent on cortisol response to SSRI (Corregiari et al., 2007) which can be related with cortisol modulation of 5-HT1A post-synaptic activity (Karten et al., 1999; Bijak et al., 2001). Interestingly, we report significant elevations of cortisol levels in a group of OCD patients that are receiving treatment for more than 6 weeks but remain with significant symptoms of disease.

The stress-related findings pointed out by this work are not specific of OCD and can be found in other psychiatric disorders such as depression. However, dysfunction in orbitofronto-striatal circuits has been the most common finding in the pathophysiology of OCD and previous animal and human studies have shown that these circuits are highly sensitive and can be disrupted by chronic stress, inducing a shift in observed decision-making behaviors through habits (Dias-Ferreira et al., 2009; Soares et al., 2012) and impairing ability of associate environmental cues to

goal-directed behaviors (Morgado et al., 2012). Altogether, these observations support a possible role for chronic stress in the etiology of OCD.

By using a significantly homogeneous group of patients that did not display any comorbidity, we eliminate some frequent biases observed in other studies. However, this study has some methodological limitations that should be taken into account: first, we included only medicated patients with OCD, which might bias results; second, this study has a cross-sectional design; and finally, the size of the sample is relatively small.

In summary, this work highlights the dysfunction of stress perception and stress-response systems in the OCD. However, more studies are necessary to clarify whether these findings are implicated in the onset of the symptomatology or are a mere consequence of the symptoms.

ACKNOWLEDGMENTS

The authors acknowledge the discussions with Osborne Almeida. Pedro Morgado is supported by a fellowship "SFRH/SINTD/60129/2009" funded by FCT – Foundation for Science and Technology. Supported by FEDER funds through Operational program for competitive factors – COMPETE and by national funds through FCT – Foundation for Science and Technology to project "PTDC/SAU-NSC/111814/2009."

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 08 February 2013; accepted: 18 March 2013; published online: 02 April 2013.

Citation: Morgado P, Freitas D, Bessa JM, Sousa N and Cerqueira JJ (2013) Perceived stress in obsessive–compulsive disorder is related with obsessive but not compulsive symptoms. Front. Psychiatry ***4****:21. doi: 10.3389/fpsyt.2013.00021*

This article was submitted to Frontiers in Addictive Disorders and Behavioral Dyscontrol, a specialty of Frontiers in Psychiatry.

Chapter 2.4.

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Obsessive compulsive disorder patients display indecisiveness and are more sensitive to negative outcomes in risky decision-making: an fMRI study.

Manuscript under preparation.

Obsessive compulsive disorder patients display indecisiveness and are more sensitive to negative outcomes in risky decision-making: an fMRI study

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Number of pages: 12

Number of figures: 5

Number of tables: 4

Keywords: decision-making, obsessive-compulsive, fMRI, insula, amygdala, striatum

Abstract

Decision-making processes are affected in obsessive-compulsive disorder (OCD). Previous studies have shown decision-making impairments in tasks with implicit rules, but not in those in which explicit and stable rules are provided. Using a gambling task, herein we explored risk-based decision-making in a functional magnetic resonance imaging study, 20 OCD patients and 20 healthy controls, matched for gender, age and educational level. Data revealed that patients with OCD showed higher levels of indecisiveness as assessed by longer times to decide and decreased differential reaction times throughout the experimental paradigm; interestingly, this pattern of altered temporal dynamics in decision-making was not associated with differences in choice preferences between OCD patients and controls. Noticeably, when compared with controls, OCD subjects displayed an inverse pattern of amygdalar activation: on one hand, there was a significant deactivation of the amygdala before high-risk choices and on the other hand, an increased activation of this brain region before low-risk choices. Moreover, in the decision phase of the paradigm there was lower activity on the caudate nucleus in OCD patients. Finally, upon receiving a negative outcome, OCD patients showed an increased activation of (orbito)fronto-striatal regions and the anterior cingulate cortex. These results contribute for the comprehension of decision-making impairments among OCD patients, although more studies are needed to detail the brain circuits involved.

Introduction

Obsessive compulsive disorder (OCD) is psychiatric characterized by intrusive and repetitive thoughts that cause anxiety (obsessions) and repetitive behaviors or mental acts driven to reduce anxiety induced by obsessions (compulsions) (Abramowitz et al., 2009). OCD is also known as the disorder of doubt (Janet, 1903) as a result of severe decision-making impairments. In fact, OCD patients are unable to decide when an action has been satisfactorily executed resulting in repetitive rituals like washing or checking, or they are unable to choose among different alternatives leading to endless ruminations. Interestingly, this inability to take decisions is typically observed in tasks with implicit rules, such as the Iowa Gambling Task (IGT), but not in tasks with explicit and stable rules such as in the Game of Dice Task (GDT) (Starke et al., 2010).

As in other neuropsychiatric conditions, a combination of structural and functional abnormalities is known to underlie the disruption of real life decision-making strategies in OCD patients. While the former involve particularly the orbitofrontal cortex (OFC), basal ganglia and parts of the limbic system (Graybiel and Rauch, 2000), the later range from changes in neurotransmitters systems (namely in serotoninergic and dopaminergic systems (Westenberg, et al., 2007) to metabolic activity both in resting and symptom provocation conditions. Yet, there is a paucity of studies combining multimodal approaches that characterize, in parallel, the structural and functional changes observed in OCD patients in risky decision-making conditions.

Herein, we designed a functional magnetic resonance imaging (fMRI) study to contrast the behavior of OCD patients with that of a cohort of controls on a decision-making paradigm in which explicit rules for rewards and losses, and obvious probabilities were provided (decisions under risk conditions). The activation patterns in brain regions relevant for these behaviors were analyzed as well as their volumes in order to better understand the morphofunctional correlates of decision-making deficits in OCD patients.

Materials and methods

Participants

The study sample consisted of 20 OCD patients and 20 healthy controls. Patients were recruited for this study thorough their ongoing contact as outpatients with Psychiatric Department of Hospital de Braga. All patients were aged >18 and required to satisfy Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM IV) TR diagnostic criteria for OCD. Diagnosis was established by experienced psychiatrists with a semi-structured interview based on DSM-IV TR and corroborated by a severity score of 7 or greater on the Y–BOCS (Goodman et al., 1989). Comorbid symptoms of depression and anxiety were measured by Hamilton Depression (Hamilton, 1960) and Anxiety (Hamilton, 1959) Rating Scales (HDRS and HARS, respectively). Exclusion criteria included: any other mental disorder revealed by the Mini-International Neuropsychiatric Interview (MINI Plus; except for OCD) (Sheehan et al., 1998), any acute and/or chronic medical illness as assessed by a physical examination and routine laboratory examination, females who are pregnant or lactating and substance dependence within the previous 12 months. From 26 patients initially enrolled, 3 dropped out, 3 were not considered due to technical issues related with images acquisition and thus only 20 were analyzed. The three matching controls were also excluded from the analysis.

Healthy controls were carefully recruited to match OCD patients for age, sex, educational level, ethnical origin, and dominance. Exclusion criteria included previous history of neuropsychiatric disorder, any present mental disorder revealed by MINI Plus and use of any medication (excluding oral contraceptives).

The present study was conducted in accordance with principles expressed in the Declaration of Helsinki and the Ethics Committee of Hospital de Braga (Braga, Portugal) approved it. The study goals and tests were previously explained to all participants and all gave informed written consent.

fMRI Paradigm: Gambling Task

During fMRI participants performed a gambling task (Figure 1), adapted from Macoveanu and colleagues (2013), that required subjects to make a choice between two sets of playing cards displayed face down. The choice was made using a response box on the right hand by clicking on

the left button with the index finger to choose the set on the left side of the screen or clicking on the right button with the middle finger to choose the set on the right side of the screen. One of the sets included the “ace of hearts” and subjects were required to choose in which set it was hidden. If the subject chose correctly they won the associated reward. If not, they lost the bet. The objective was to maximize the overall profit.

Each gamble had a stable trial structure consisting of an information, decision and outcome phase (Figure 1A). In the information phase, participants were provided with information about their total amount, which started with 0.50€ (approx. 0.4 USD) and a fixed bet of 5. In the decision phase, two sets of cards were presented face down together with the associated reward and subjects made their choice. The outcome phase revealed the “ace of hearts”, giving the subjects feedback about whether they had won or lost. In each gambling trial, seven cards were divided in two sets (Figure 1B), resulting in six possible risk scenarios with a parametric variation of the odds, ranging from 1/7 (low probability to win) to 6/7 (high probability to win). Choosing the set with the lower number of cards was associated with a higher risk but also with a correspondingly higher reward when the subjects had chosen correctly. Thus, participants would repeatedly choose between a larger set of cards associated with a smaller, likely reward and a smaller set associated with a larger, but less likely reward. For choices with winning probability of more than 50% (i.e., odds of 6/7, 5/7 or 4/7), the reward was matched to the amount of the bet. For choices with a winning probability of less than 50%, the possible reward exceeded the bet by the factor 11 for a winning probability of 1/7, 4 for bets with a winning probability of 2/7, or 1.66 for a winning probability of 3/7. The magnitude of losses was matched to the bet independent of the chosen risk. The gambling task was performed in two sessions each lasting 11 min. In each of the two sessions there were 28 choices between one and six cards, 28 choices between two and five cards, 28 choices between three and four cards and 28 null events of the same length as a real event where a fixation cross was presented instead of the task screen. The events were pseudo-randomized across the two sessions that only differed in their event randomization. The task was tuned to stimulate an even distribution of choices across all risk levels by varying the reward value with the size of the assumed risk so that the expected value (i.e., the sum of probabilistically weighted wins and losses) would match across all possible choices. The experimental design enabled us to associate neural activity related to negative or positive outcomes to the riskiness of choice behavior. In particular, we were able to assess differential outcome related activity depending on whether the decision preceding it was risk-

averse (i.e., playing it safe but being punished for it) or risk-taking (i.e., taking a risk and being punished for it).

MRI aquistion

All subjects were scanned on a clinical approved 1.5 T Siemens Magnetom Avanto scanner (Siemens Medical Solutions, Erlangen, Germany) using a12-channel receive-only head array coil was used. The same acquisition protocol was used in all participants and included, among others, the following acquisitions: Siemens Auto Align scout protocol in order to minimize alterations in head positioning; high-resolution whole-brain 3D T1-weighted Magnetization Prepared Rapid Gradient Echo (MPRAGE) acquisition with 176 sagittal slices (repetition time (TR) = 2730 ms, echo time (TE) = 3.5 ms, field of view (FoV) = 256 x 256 mm2, flip angle (FA) = 7°, in-plane resolution = 1 x 1 mm2 and slice thickness of 1 mm); T2* weighted echo-planar imaging (EPI) acquisition (38 interleaved axial slices, TR = 2500 ms, TE = 30 ms, FoV = 250 x 250 mm2 , FA = 90°, in-plane resolution = 3 x 3 mm 2, slice thickness = 3 mm, between-slice gap = 0.9 mm). 315 volumes with BOLD contrast were acquired using the T2* acquisition in two separate runs, making a total of 630 volumes acquired during the gambling task. The task stimuli were presented using the fully integrated fMIR system IFIS-SA (Invivo Corporation, Orlando, FL, USA) and the same system was used to record the subject key-press responses.

Volumetric Analysis

Estimation of gray and white matter structures' volumes from the T1-weighted structural MRI data was performed using the freely available Freesurfer toolkit version 5.1 (http://surfer.nmr.mgh.harvard.edu). The pipeline and procedures employed were improved over the last decade and have been validated across sessions, scanner platforms, updates and field strengths. The data was initially converted to to Freesurfer's MGZ (compressed Massachusetts General Hospital file) file format and then processed with the standard pipeline. Briefly, the pipeline involves the following steps: pre-processing of MRI images; non-uniform intensity normalization; normalization to the standard Talairach space using a twelve degrees of freedom affine transformation; intensity normalization with corrections of fluctuations in scan intensity; skull stripping; linear and non-linear registrations of the patient volume to the FreeSurfer atlas when applying segmentation labels cortical and subcortical structures; reconstruction of cortical

surfaces and tessellation of the GM and WM boundary and pial surfaces; inflation of each tessellated cortical surf and registration to a spherical atlas; parcellation according to gyri-sulci folding patterns.

Manual adjustments and visual inspection in the normalization procedure, skull stripping, WM segmentation and pial surface boundary, were performed whenever necessary. Estimated intracranial volume (ICV) was used to correct the volumetric data.

Functional Analysis

Before statistical analysis, functional data from all participants was preprocessed using the Statistical Parametric Mapping version 8 (SPM8) software (http://www.fil.ion.ucl.ac.uk/spm). The preprocessing procedures included: slice-timing correction using the first volume as reference; field-map reconstruction in order to obtain the corresponding voxel displacement maps (VDMs); realignment to the first volume of the acquisition and unwarping using the corresponding VDM to correct for geometric distortions; spatial normalization to Montreal Neurologic Institute (MNI) standard space and resampling to 2x2x2 mm3 voxel size; spatial smoothing with a 8 mm full-width at half-maximum (FWHM) Gaussian kernel; high pass temporal filtering at 128 s.

Each subject's dataset was then analyzed in the context of the General Linear Model (GLM). For the first level GLMs, the 6 risk-levels were grouped: odds of 1/7 and 2/7 formed the high-risk group, odds of 3/7 and 4/7 were modeled as medium-risk and odds of 5/7 and 6/7 formed the low-risk group. These risk-groups were modeled in 3 conditions: during the decision-making stage, when receiving negative outcomes and when the bets resulted in positive outcomes. As so, 9 regressors of interest were modeled, one for each combination of risk-level and condition. Moreover, 8 additional regressors were included (1 for the information phase, 1 for the missed bets and 6 for the motion parameters). The two runs were analyzed in the same GLM, modeling the 17 regressors for each run and 2 additional regressors, one for each session. In total 36 regressors were included for each participant.

For the second level (group level) random-effects analysis, the average contrasts of the first level across both runs were considered for the 9 regressors of interest. These contrasts were analyzed in three different group (2: controls vs OCD) x condition (3: high vs medium vs low risk) ANOVA models: one for the decision phase, one for negative outcomes and one for positive outcomes. For each model overall effect (one-sample t-test), group effect, risk effect and group by risk

interaction were analyzed. These models were implemented with GLMFlex (http://nmr.mgh.harvard.edu/harvardagingbrain/People/AaronSchultz/GLM_Flex.html), which uses partitioned error terms for within-group and between-group comparisons, thus enabling the estimation of all the effects of interest with a single model.

The one-sample t-test results were considered significant at a height threshold of $p < 0.05$ after Family wise Error (correction). For the remaining comparisons, all results were considered significant at $p<0.05$ corrected for multiple comparisons using a combination of an uncorrected height threshold of $p<0.005$ with a minimum cluster size. The cluster size was determined over 1000 Monte Carlo simulations using AlphaSim program distributed with REST software tool (http://resting-fmri.sourceforge.net/). This resulted in a minimum cluster size of 952 mm3 for between-group comparisons and 1040 mm3 for within-group comparisons. The different cluster size requirements result from differences on the estimated smoothness of the residuals of those models.

Results

Behavioral correlates of indecisiveness in risky decision-making

Data shows that patients with OCD take longer time to decide their options in a risky decision-making paradigm, particularly when high risky is involved (High Risk: $P < 0.05$, Intermediate Risk: P=0.08; Low Risk: P=0.12 Figure 2; Panel B). Moreover, the differential response times from the first to the last block of options decreased less in OCD patients that in controls (High Risk: P=0.25, Intermediate Risk: P=0.52; Low Risk: P<0.05, Figure 2, Panel C). This pattern of altered temporal dynamics reveals an impairment in deciding in OCD patients.

Interestingly, no differences in choice preferences between OCD patients and controls (Odd 1/7: P=0.84; Odd 2/7: P=0.36; Odd 3/7: P=0.86; Odd 4/7: P=0.73; Odd 5/7: 0.43; Odd 6/7: 0.55, Figure 2, Panel A).

OCD patients display abnormal amygdala activation during risky options and are more sensitive to losses

As shown in Table 2, the task used herein triggered in all subjects activations in regions in the (orbito)fronto-striato-thalamic circuit as well as areas outside this loop, such as anterior cingulate cortex, when choosing high risk options. Additionally, we also show that receiving a positive outcome after a high risk choice (versus a low risk choice) was associated with higher activation of insular, orbitofrontal and anterior cingulate cortical areas (Table 3).

In OCD patients, the decision phase of the paradigm triggered a significant lower activity on the caudate nucleus, a striatal area known to be disrupted in the disorder [t(19)=4.89, P<0.005, Figure 4, panel A] and critical for goal-directed actions. Additionally, when compared with controls, OCD subjects displayed an inverse pattern of amygdalar activation: on one hand, there was a significant deactivation of the amygdala before high risk choices and on the other hand, an increased activation of this brain region before low risk choices [F(1,19)=15.42, Figure 4, panel B).

Importantly, upon receiving a negative outcome, OCD patients showed an increased activation of (orbito)fronto-striatal regions and the anterior cingulate cortex (Table 4) (Figure 5). No significant differences were found between OCD subjects and controls in positive outcomes.

Finally, the a volumetric analysis revealed that OCD patients have a significant atrophy on relevant brain areas for decision-making such as the left insula, the right pars triangularis and the right pars opercularis ($P<0.05$, Figure 3).

Discussion

Doubt is a central feature in OCD. The underlying mechanisms for this difficulty to decide at still being unraveled. Conceptually, decision situations which provide explicit rules for rewards and punishments, and obvious probabilities (decisions under risk conditions), can be differentiated from situations in which information about contingencies and gains and losses is not available (decisions under ambiguity) (see Bechara, 2004 and Brand et al., 2006). Interestingly, OCD patients were described to be affected on the latter, but not to display deficits on the former (REFS). Herein, we show that is only partially true: while OCD patients did not differ from controls in the pattern of risky choices, they reveal signs of indecisiveness, a characteristic of the disease, that does not seem to disappear with the short-term repetition of the task. Interestingly, our finding of decreased caudate activation in OCD patients points to a hypoactivation of the associative network that rules goal-directed behaviors, which hints to the possibility that these individual develop a bias for habitual actions.

This bias to habits has been shown to be influence by several factors, including stress exposure (Soares et al., 2013). Importantly, we have previously demonstrated that OCD patients display traces of increased stress and anxiety that correlate with behavioral symptoms. The stress-induced deficits in instrumental decision-making have been proved to be dependent on alterations in corticostriatal networks that are also implicated in OCD. This is probably not a mere coincidence but rather the confirmation that the same neuronal networks are involved and that the decision impairments are central in the ethipathogenesis of this disorder. Interestingly, this study has also revealed a pattern of cortical atrophy in the left insula, the right pars triangularis and pars opercularis.

The insula is known to be involved in both early and late effects of subject-specific risk preferences, suggestive of a role in both risk assessment and risk anticipation during choice (Symmonds et al., 2013). While the early effect indicates a possible role in a risk-processing network, the later insula response is consistent with an affective component in risky choice, particularly as it follows rather than precedes choice-sensitive premotor activity. Thus, the finding of structural abnormalities in this brain region is likely to produce different risk preference profiles in OCD. In addition, the structural changes in pars opercularis and triangularis are probably related to the inhibition of responses that are critical for response selection (Mostofsky and Simmonds, 2008; Picard and Strick, 2001; Rizzolatti et al., 2002); interestingly, this may be

particularly relevant if the emotional processing is disturbed. Nowadays a growing interest in the mechanisms by which the limbic substrates for emotion perception influence the inferior frontal inhibitory circuits, has converged on the amygdala, which receives extensive sensory input (Price, 2003), and in turn, has bidirectional functional connections with the prefrontal cortex (Hampton et al., 2007; Herwig et al., 2007). The amygdala is believed to encode the emotional value of stimuli (Dolan, 2007; Pego and Sousa, 2013) and is consistently engaged by affective stimuli (Costafreda et al., 2008; Phan et al., 2002). Noticeable, the present study reveals abnormal amygdalar activation in OCD patients that probably influences the substantial differences in the sensitivity to losses, but not to gains, displayed by our OCD patients. The differential sensitivity to the outcome is relevant, in as much as it helps understanding the genesis of the conflict in the decision-making process – a fear to loose - and points for future areas of therapeutic interventions.

We hypothesize that the indecisiveness displayed by OCD patients might be correlated with the differential activation pattern of the amygdala, a key-region for decision-making as it pre-emptively signals good and bad choices. In addition, the increased activation of the orbito-fronto-striatal loop upon receiving a negative outcome suggests that OCD patients are more sensible to losses, which may mediate their previously described risk-aversion. These results contribute for the comprehension of decision-making impairments among OCD patients, although more studies are needed to detail the brain circuits involved.

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Tables

Table 1. Socio-demographic and clinical characteristics of patients with obsessive-compulsive disorder and healthy comparison subjects.

Characteristics	Subjects with OCD (n=20)	Healthy comparison subjects (n=20)	Statistics
Age, years [mean ± S.D. (range)]	28.05 ± 7.22 (19-42)	26.60 ± 4.90 (20-40)	*P* = 0.86
Male/female	6/14	6/14	
Education, years [mean ± S.D. (range)]	13.15 ± 3.76 (6-24)	14.60 ± 3.75 (9-24)	*P* = 0.158
Age of onset [mean ± S.D. (range)]	21.65 ± 7.26 (9-35)		
Y-BOCS (total score)	24.55 ± 8.03 (12-30)		
HDRS (global score)	6.90 ± 2.82 (4-15)		
HARS (global score)	4.75 ± 3.50 (0-18)		
PSS-10	22.05 ± 7.53 (6-33)	12.55 ± 4.10 (7-20)	*P* < 0.01*
Medication	Only SSRI – 16 (80%) SSRI with TCA – 4 (20%)		

OCD = obsessive–compulsive disorder, Y-BOCS = Yale–Brown Obsessive–Compulsive Scale, HDRS = Hamilton Depression Rating Scale, HARS = Hamilton Anxiety Rating Scale, SSRI = Serotonin Selective Reuptake Inhibitors, TCA = Tricyclic Antidepressant.

Table 2. Response rate during the decision phase of the experimental paradigm. Comparison between high and low risk choices (significance: p<0.05 corrected for multiple comparisons with Monte Carlo (ppeak<0.005 cluster size>130).

Condition	Regions	Peak MNI coordinates	Cluster size (voxels)	Maximum Z score
Decision phase	Putamen (left)	-16, 14, -2	462	6.41
(high>low)	Ant Cingulum (right)	10, 28, 26	2037	5.84
	Ant Cingulum (left)	-2, 26, 28	2037	5.04
	Caudate (right)	12, 10, 6	441	5.00
	Frontal Sup Medial (left)	2, 28, 48	2037	4.73
	Pallidum (left)	-10, 0, 0	462	4.11
	Supplementary Motor Area (left)	0,10, 62	2037	4.53
	Thalamus (right)	14, -10, 16	441	4.51
	Caudate (right)	16, -4, 2	441	4.28
	Precentral (left)	-56, 4 , 18	229	4.26
	Med Cingulum (right)	14, 20, 36	2037	4.23
	Frontal Inf Tri (right)	50,24, 12	221	4.29
	Parietal Superior (left)	-24, -70, 58	229	4.05
	Parietal Inferior (right)	40, -46, 50	401	4.04
	Insula (left)	-24, 20, -8	172	3.94
	Parietal Inferior (left)	-48, -58, 44	504	3.91
	Parietal Superior (right)	38, -48, 62	401	3.91
	Med Cingulum (left)	0, 16, 48	2037	3.70
	Frontal Inf Opercularis (left)	-48, 6, 28	229	3.28
	Orbitofrontal Inferior (left)	-44, 18, -6	172	3.27
Decision phase	Calcarine (left)	-12, -66, 16	157	3.64
(high<low)	Precuneus (left)	0, -64, 24	157	3.30

Table 3. Response rate during the outcome phase of the experimental paradigm. Comparison between high and low risk choices with positive (A) or negative (B) outcomes (significance: p<0.05 corrected for multiple comparisons with Monte Carlo (ppeak<0.005 cluster size>130).

Condition	Regions	Peak MNI coordinates	Cluster size (voxels)	Maximum Z score
Positive Outcomes	Med Cingulum (right)	6, 32, 36	279	5.39
(high > low)	Ant Cingulum (right)	8, 32, -4	198	4.72
	Insula (left)	-26, 16, -14	151	4.52
	Olfactory (right)	4, 24, -4	198	4.44
	Frontal Sup Medial (left)	4, 54, 26	325	4.01
	Orbitofrontal Inferior (right)	28, 30, -18	159	3.67
	Ant Cingulum (right)	8, 52, 8	325	3.63
	Orbitofrontal Inferior (left)	-20, 8, -22	151	3.12
Positive Outcomes	Postcentral (left)	-18, -30, 64	253	-4.77
(high < low)	Postcentral (right)	12, -40,68	203	-4.25
	Precentral (right)	34, -22, 66	162	-4.24
	Precuneus (left)	-12, -42, 62	253	-4.04
Negative Outcomes	Temporal Superior (right)	48, -24, -2	134	5.09
(high > low)	Occipital Superior (left)	-20, -66, 36	283	4.91
	Precuneus (left)	-14, -56, 40	283	4.31
	Temporal Medium (right)	58, -28, 0	134	3.63
	Temporal Inferior (right)	50, -64, -8	362	3.49
Negative Outcomes	-	-	-	-
(high < low)	-	-	-	-

Table 4. Response rate during negative outcomes. Comparison between OCD patients and controls. [significance: p<0.05 corrected for multiple comparisons with Monte Carlo (ppeak<0.005 cluster size>119)].

Condition	Regions	Peak MNI coordinates	Cluster size (voxels)	Maximum Z score
Negative Outcomes	-	-	-	-
(Controls > OCD)	-	-	-	-
Negative Outcomes	Postcentral (left)	-18, -30, 64	253	-4.77
(Controls < OCD)	Postcentral (right)	12, -40,68	203	-4.25
	Precentral (right)	34, -22, 66	162	-4.24
	Precuneus (left)	-12, -42, 62	253	-4.04
	Temporal Inferior (right)	64, -28, -20	244	-5.43
	Temporal Mid (right)	66, -28, -6	244	-5.32
	Frontal Mid (left)	-24, 22, 34	293	-4.89
	Frontal Inferior Opercularis (left)	-58, 6, 6	167	-4.60
	Parietal Superior (right)	20, -56, 64	384	-4.36
	Frontal Superior (left)	-16, 64, 26	220	-4.21
	Putamen (left)	-20, 6, 10	135	-4.18
	Frontal Mid (right)	38, 33, 54	133	-4.10
	Frontal Sup Medial (left)	2, 50, 34	203	-4.09
	Putamen (left)	-18, 6, 12	135	-3.96
	Occipital Inf (right)	26, -96, -8	149	-3.58
	Frontal Superior (right)	30, 32, 54	133	-3.56
	Cingulum Mid (left)	-2, 6, 38	337	-3.49
	Cingulum Mid (right)	12, 4, 42	337	-3.48
	Frontal Sup Medial (right)	12, 54, 40	203	-3.46
	Parietal Sup (left)	-32, -48, 64	187	-3.42
	Obritofrontal Mid (left)	-4, 42, -12	146	-3.36
	Pallidum (left)	-18, 4, 0	135	-3.16
	Temporal Sup (left)	-60, 2, -6	167	-2.81

Figures

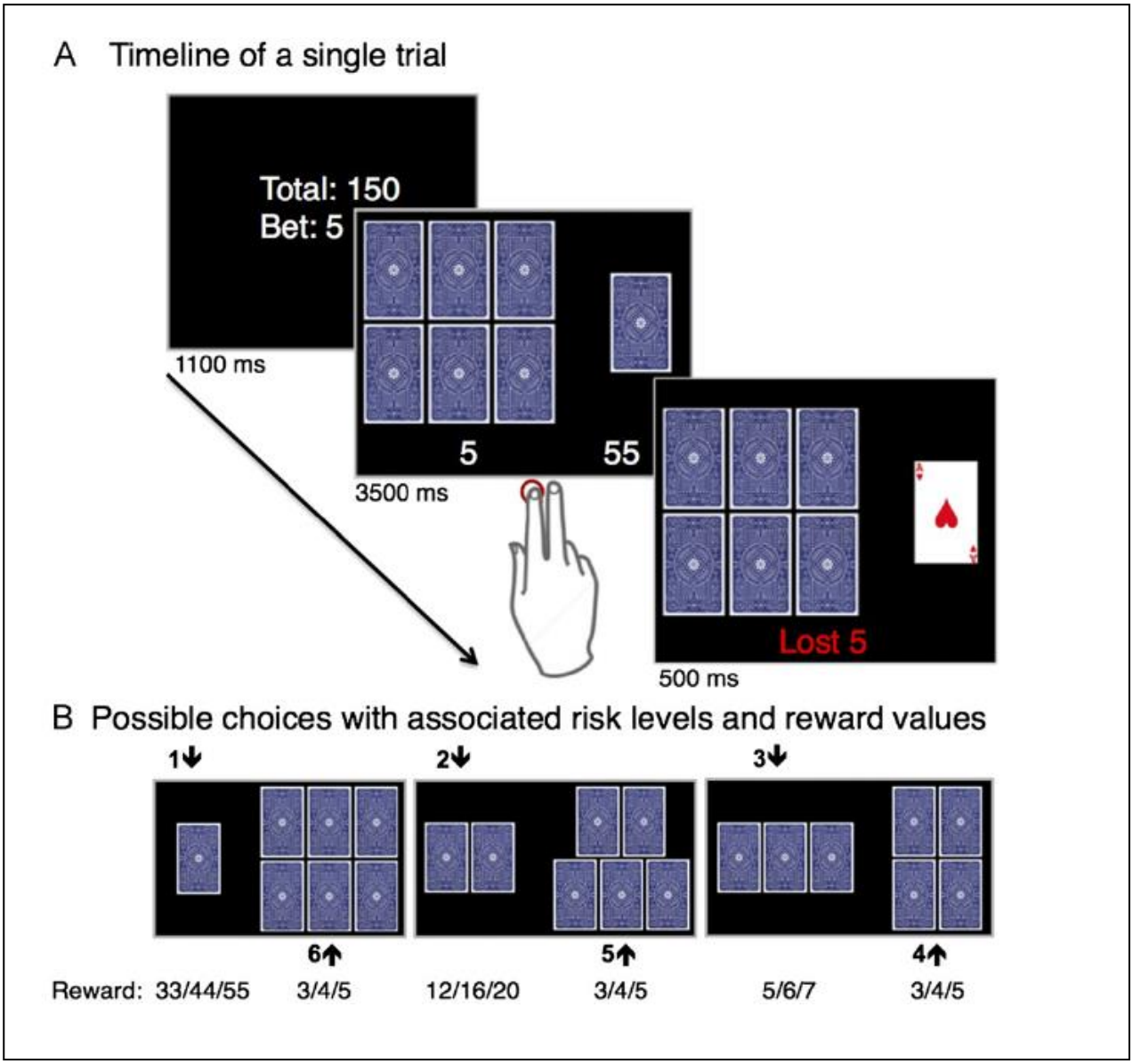

Figure 1. Gambling task. (A) Temporal structure of a single gambling trial. Each trial was divided into three phases: information, decision and outcome. **(B)** Possible choices with associated risk levels and rewards. Choices 1 and 2 were categorized as high-risk, 3 and 4 as medium-risk, 5 and 6 as low-risk. [from Macoveanu et al. (2013) with permission].

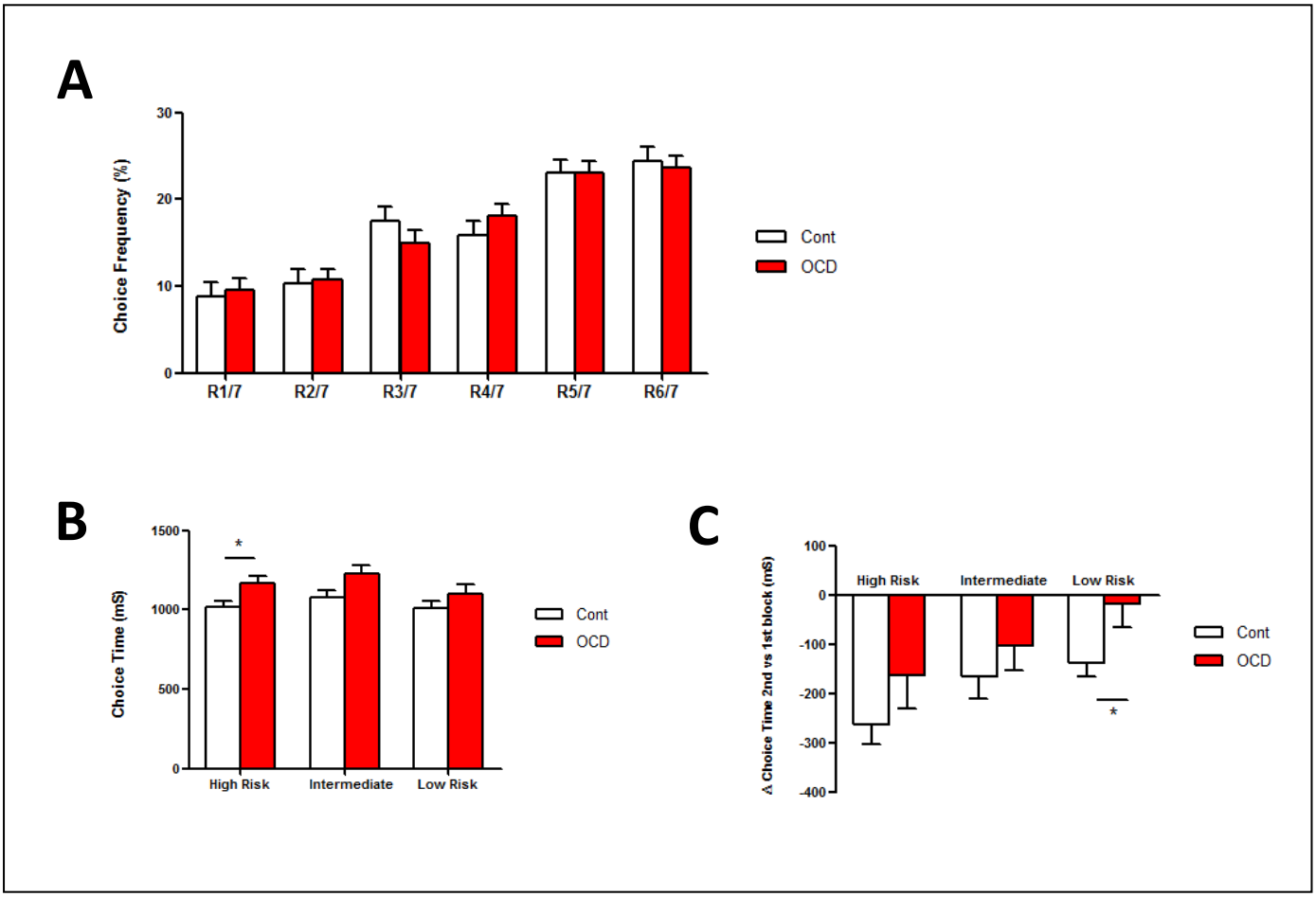

Figure 2. Pattern of responses. (A) Distribution of choices across the six risk levels (odds). No significant differences were found among groups. **(B)** Distribution of response times across the three risk levels in the last block (High Risk: 1/7 and 2/7 odds; Intermediate risk: 3/7 and 4/7 odds; Low Risk: 5/7 and 6/7 odds); OCD patients spend more time to make choices in all different odds, with significantly higher times in high risk choices **(C)** Variation of response times between last and first block; OCD patients displayed smaller variations in all different odds, mainly in the low risk ones. Results are present as mean + SEM (n=20, per group). *, $P < 0.05$.

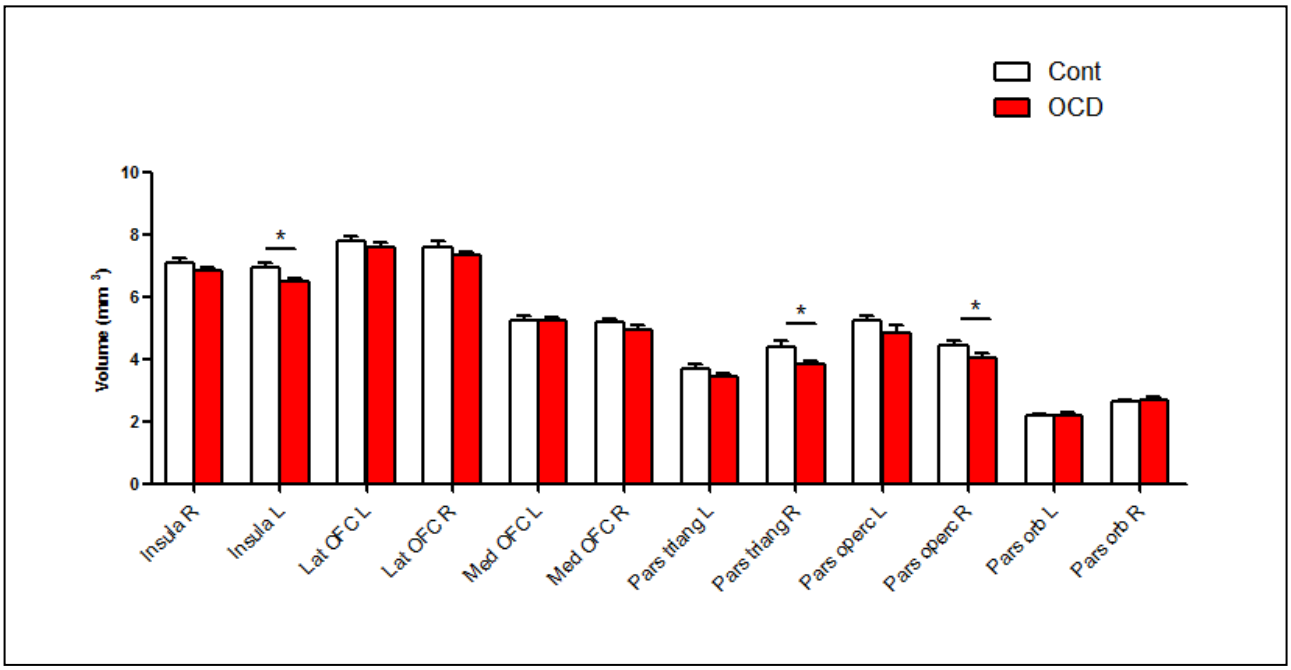

Figure 3. Volumetric data. OCD patients display atrophy of left insular cortex and areas of inferior frontal gyrus (pars triangularis and pars opercularis). Results are present as mean + SEM (n=20, per group). *, $P < 0.05$.

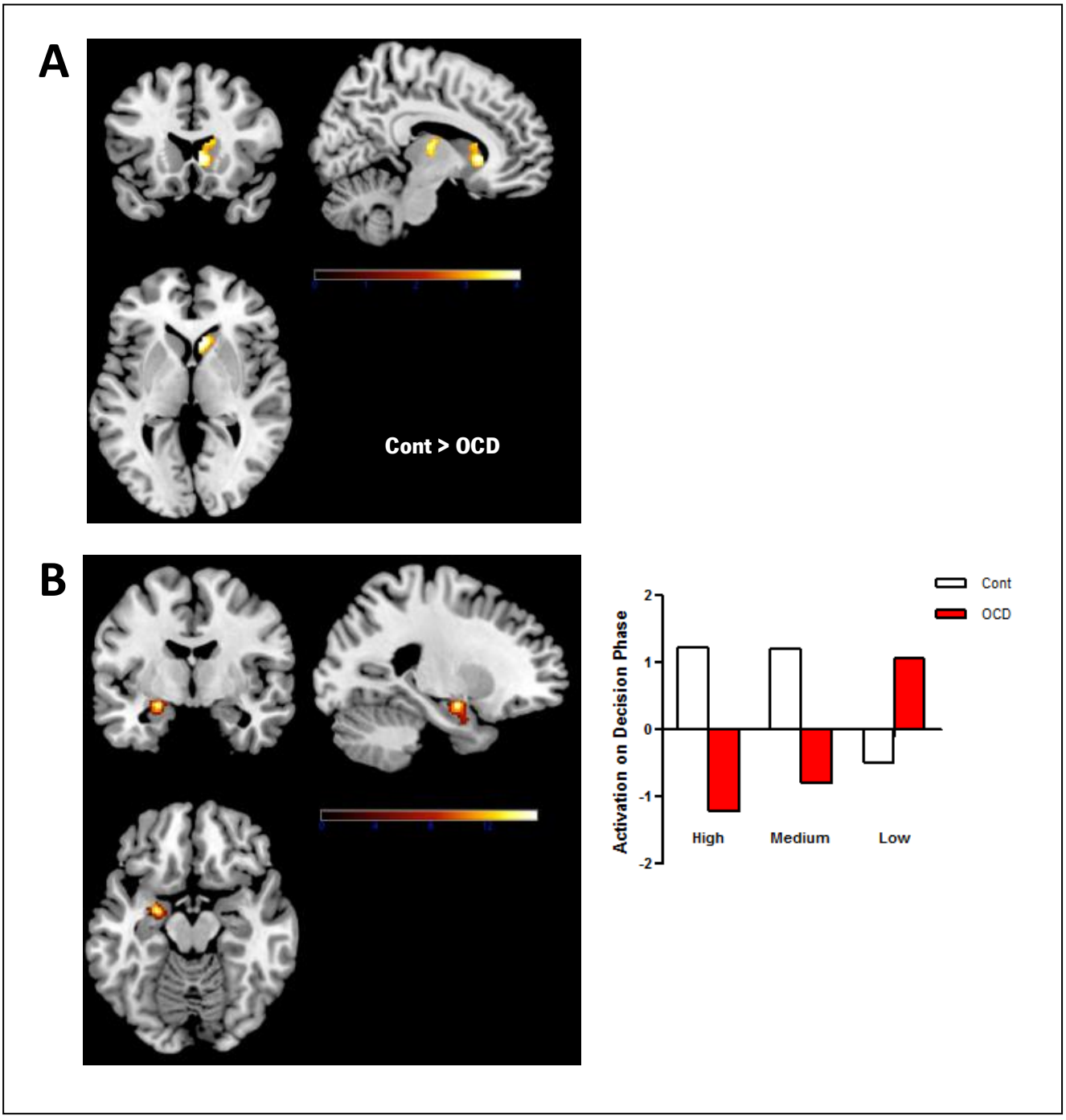

Figure 4. Pattern of activation during the decision phase. (A) Controls display a higher activation in the right caudate nuclei [significance: p<0.05 corrected for multiple comparisons with Monte Carlo (ppeak<0.005 cluster size>119)]. **(B)** Paradoxical pattern of amygdalar activation among three different odds were found between OCD patients and healthy controls. [significance: p<0.05 corrected for multiple comparisons with Monte Carlo (ppeak<0.005 cluster size>130)].

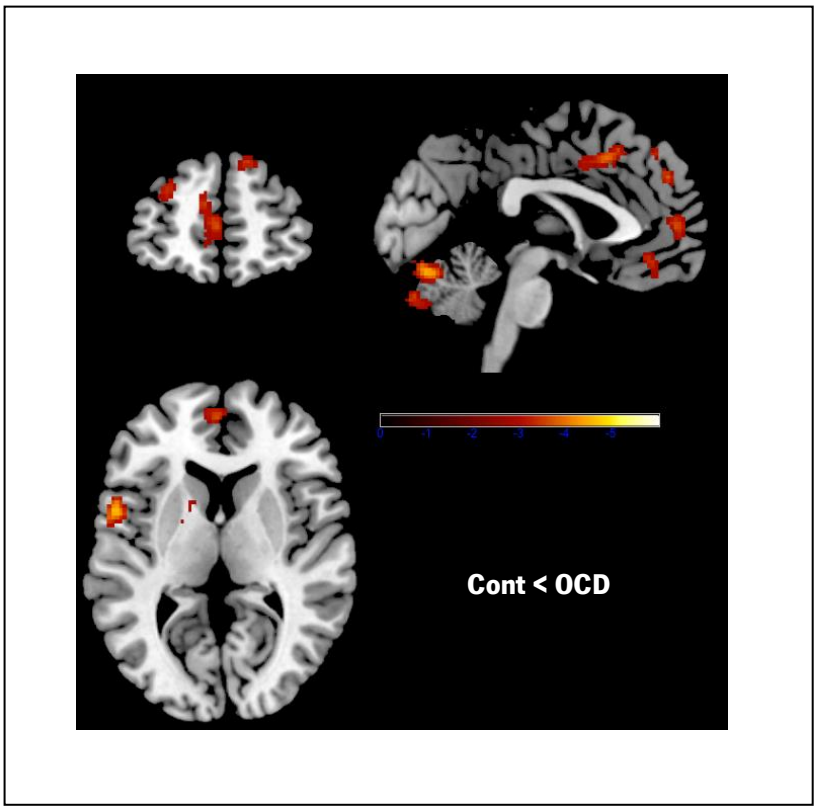

Figure 5. Pattern of activation during negative outcomes. OCD patients display a higher activation in several brain areas [significance: $p<0.05$ corrected for multiple comparisons with Monte Carlo ($p_{peak}<0.005$ cluster size>119)].

Chapter 3

Discussion

3. DISCUSSION

Decisions based in the probability of future events (e.g. assuming risks) are routine in our lives. Importantly, the algorithm of decision-making processes in adaptive organisms is dynamic as the subject, based on previous experiences, is able to weigh the risks and benefits of each option, selecting the alternative that is most valuable. Exposure to stress, which is known to affect brain structure and function and have important consequences in our behaviour, is also a feature of our lives that can influence, positively and/or negatively, decisions. Chronic stress was found to bias behavior from goal-directed to habit-based in tasks where outcomes are easily predicted (Dias-Ferreira, 2009). However, an ongoing challenge lays in the exploration of how chronic stress influences choice processes when consequences are unknown, that is, decision-making under risk.

The primary aim of the present work was to analyze the impact of chronic stress on decision-making processes, particularly those involving risk, using both a variety of animal paradigms and exploring specific features of psychiatric conditions in which patients characteristically present impaired decision-making abilities. We first explored the impact of chronic stress on two paradigms of decision-making in rats: the pavlovian to instrumental transfer (PIT - presented in Chapter 2.1.) and a newly developed task to assess decisions involving uncertainty and risk (presented in Chapter 2.2). We then assessed the relationship between the stress response and decision-making in patients with OCD (presented in Chapter 2.3.) and started to explore the neural networks involved (Chapter 2.4.).

3.1. Animal decision-making paradigms

Throughout this work we aimed at further characterizing the impact of chronic stress in decision-making, analyzing underlying cerebral mechanisms that might explain the behavioral changes encountered. In order to pursue this goal, the use of decision-making animal models, allowing a detailed dissection of the different decision-components and a complete analysis of the brain mechanisms involved, was of the utmost importance. Indeed, the use of animal models in the field of neuroeconomics has been extensively validated (Balleine and O'Doherty, 2010), driven

mainly by methodological considerations, since several research techniques leading to important physiological and biochemical insights cannot be used in humans, for ethical or practical reasons. Importantly, with the recent developments in non-invasive techniques, particularly in the field of MRI and EEG/MEG, this is a changing scenario, as can be gleaned from the preliminary results presented in Chapter 2.4.

The first animal paradigm to be tested was PIT (Chapter 2.1.), a well characterized task that evaluates the ability of environmental cues (conditioned stimuli) to modulate instrumental actions. The influence of cues on instrumental behavior underlies many aspects of everyday life and could guide decisions in many adaptive situations, such as cues that guide behavior to obtain food or water when hungry or thirsty (Perks and Clifton, 1997). Additionally, this mechanism has been implicated in the genesis of pathological behaviors in some psychiatric disorders such as addiction (Robinson and Berridge, 2008) and compulsive over-eating (Volkow et al., 2008).

Performance on instrumental decision-making tasks is dependent on two different learning processes: one, responsible for goal-directed actions, encodes the relationship between actions (response, R) and its consequences (outcome, O); other, responsible for habit learning, is governed by stimulus-response (S-R) associations, not incorporating changes in outcome value (tested by outcome devaluation tasks) neither changes in the casual relationship between an action and its consequences (tested by contingency degradation tasks) (Balleine and O'Doherty, 2010). In addition, instrumental behavior is also influenced by environmental cues (stimuli, S) that, by means of a pavlovian associative mechanism, signal the presence or absence of a reward (outcome, O) (Doya, 2008). It is the influence of such associative learning (S→O) on instrumental actions (mainly R→O) that is the focus of PIT tasks (Holmes et al., 2010).

PIT was firstly described in several animal species, including rodents (Estes, 1943), and only more recently has been demonstrated in humans (Paredes-Olay et al., 2002). Using a natural reward, such as food or water, animal models of PIT were shown to be highly reliable as a model of cue-controlled behavior, in which decisions are highly influenced by environmental or internal cues (Holmes et al., 2010). This is of relevance for the study of addictive and/or compulsive disorders, of which these behaviors are a hallmark (Hogarth et al., 2013).

The neuronal networks that mediate the PIT effect are diverse and include several brain regions such as amygdala, nucleus accumbens, striatum and prefrontal cortex (reviewed by Holmes et al., 2010). Importantly, Basolateral Nucleus of Amygdala (BLA), rather than the Central Nucleus (CN), is critically involved in the assignment of each cue to a particular outcome (Schoenbaum et al., 1998; Holland et al., 2002; Pickens et al., 2003) whereas the CN appears to be more involved in appetitive arousal and general motivation (Wallace et al., 1992; Balleine and Killcross, 2006; Kaufling et al., 2009). It has been also demonstrated that, while the NAcore mediates the general excitatory effects of reward-related cues, the NAshell mediates the effect of outcome-specific reward predictions on instrumental performance. These areas interact with cortical regions, such as the mPFC and the OFC, integrating affective stimuli with executive commands (Christakou et al., 2004; Kelley, 2004; Pasupathy and Miller, 2005; Saddoris et al., 2005; Stalnaker et al., 2007). Indeed, Homayoun and Moghaddam (2009) demonstrated that OFC and mPFC orchestrate the integration of Pavlovian and instrumental processes during PIT.

Secondly, we used a new decision making paradigm for rodents, designed to explore specific features of risk-based decisions (Chapter 2.2.). Animals, like humans, have to make "economic decisions", adapting their choice behavior to maximize benefits while minimizing resource expenditure (in most animal cases amounting to energy). Indeed, several studies have shown strong similarities between human and animal models of decision-making, including those related with decision under risk (Kalensscher and Wingerden, 2011). However, there are some differences that should be discussed and taken into account when analyzing results of decision-making tasks in rodents: first, humans are usually verbally informed about rules, times and probabilities in a one-shot scenario while animals learn those determinants of decision in multi-trial settings; second, in human gambling tasks the incentive is usually money while rodent models of decision making often use food and/or water as a reinforcer, thus requiring previous food and/or water deprivation; third, animals cannot finish the session with less than what they had at beginning and, as a consequence, they cannot work to restore the initial budget as it could happen in human gambling tasks (Champbell-Meiklejohn et al., 2007). Despite all of these limitations, however, behavior observed in animal models of decision-making tends to mimic the behavior observed in human subjects (Wallis, 2011).

Given the interest in neuroeconomics, the dissection of the neural substrate of economic decisions, and the possibility of studying such behaviors in animals, including rodents, several

experimental paradigms of gambling and/or risky decision-making in rats have been put forward in the past years. One of the most popular is the rodent equivalent of the Iowa Gambling Task (IGT), developed for humans by Bechara and collaborators (1994) and adapted for rodents by van den Bos (2006), Pais Vieira (2007), Rivalan (2009) and Zeeb (2009). In IGT, the subject has to choose between four options (cards in humans; levers, maze arms or nose poke apertures in rodents), two of which yield higher rewards but also, randomly presented, higher losses than the other two, resulting in an overall net loss when choosing the former (disadvantageous options) comparing with an overall net gain when choosing the latter (advantageous options). Choices in this paradigm depend on the factoring of value, uncertainty and, particularly, time-discount, with near sighted subjects more sensitive to immediate gains that to long-term losses, constituting an interesting model of complex economic decisions. Extensive research has shown that performance of the IGT depends on the activity of several brain areas including the ventro-medial prefrontal cortex (Bechara et al., 1999; Fellows and Farah, 2005), the dorsolateral prefrontal cortex (Manes et al., 2002; Bolla et al., 2004; Fellows and Farah, 2005), the orbitofrontal cortex (Manes et al., 2002, Bolla et al., 2004; Hsu et al., 2005), anterior cingulate cortex (Tucker et al., 2004), the amygdala (Hsu et al., 2005) and the striatum (Hsu et al., 2005). Additionally, IGT performance was shown to be negatively affected by stress both in humans (van den Bos et al., 2009) and rodents (Koot et al., 2013) and highly vulnerable to dopaminergic manipulations (Zeeb et al., 2009).

Besides IGT, other paradigms have been developed. These include: (1) risk-discounting task (Cardinal and Howes, 2005; Floresco et al., 2008), where subjects have to choose between small certain rewards and large probabilistically delivered rewards presented in a crescent and/or descendent manner which allows the evaluation of preference for risky vs. certain rewards (in the equal rewards condition), and preference for large vs. small rewards (in the equal probabilities condition); (2) delay-discounting task, supported by the observation that the value of a reward is discounted over time (Mazur, 1987), is characterized by choice between smaller rewards available immediately versus larger rewards available after a varying delay and has frequently been used for study impulsive choice both in humans (Dixon et al., 2003; Johnson and Bickel, 2002) and in rodents (Green and Estle, 2003; Ito and Asaki, 1982; Kobayashi and Schultz, 2008); (3) risk punishment decision task where rats choose between a small safe reward and a large reward advocated with punishment (Simon et al., 2007; Simon et al., 2009); (4) effort-discounting tasks (Floresco et al., 2008; Cocker et al., 2012) that evaluates cost/benefit

decision-making and where animals choose between a small reward obtainable after a low amount of physical effort and a larger reward after considerably more work. Although these tasks were developed to assess decision-making under uncertainty and risk, none isolates this factor from value and/or time-discounting, which makes interpretation of the results difficult. In order to overcome this difficulty, and contribute to the dissection of the neural substrates factoring uncertainty in the process of decision-making, we developed a novel risk-based decision-making task, presented in Chapter 2.2.. In each trial of this task animals have to choose between making a nose poke in a hole (which randomly varies from trial to trial and where no light is turned on) that always triggers the delivery of a reward (certain/safe option), or in one of four holes (where a light is turned on) that trigger the delivery of a 4 times bigger reward only 1 in 4 times (uncertain/risky options). Importantly, due to their design, both choices yield, on the long run, the same amount of reward, thus isolating uncertainty from both value and time-discounting. In addition, our newly developed task has other important differences when compared with previously described ones (Cardinal and Howes 2005, van den Bos, 2006; Floresco and Whelan, 2009; Boulougouris et al., 2009; Zeeb et al., 2009; Simon et al., 2009): i) different options randomly vary across different holes, thus attenuating the interference of spatial reference memory in the choice processes; ii) in basal conditions, animals have a similar preference for the safe and each of the risk options, making analysis of behavior more informative.

In the optimization of our protocol, we realized that small manipulations could have a profound impact in the behavioral pattern of choice displayed by rats. As an example, we found that when risk and safe options remain in the same position during the entire session, rats significantly increased their preference for safe choices (Annex I). This can just reflect the acquisition of an habitual behavior (always doing the nose poke in the same hole) or suggest that spatial reference memory (regarding the position of the different options) can strongly bias behavior, with either mechanism playing a relevant role in the performance of risk-based decision-making tasks and confounding the interpretation of results. To solve this limitation, we randomly changed the position of the "safe" hole from trial to trial and signalized it by turning on a small light inside. However, by testing this design, we found that the association between light and safe option also biased choices to safe holes (Annex I). Interestingly, this was an example of the modeling of operant actions by environmental cues, as discussed above, where the appetitive value of the light stimulus is transferred to the association between doing the nose poke in the safe hole and receiving a reward, promoting this decision in detriment of doing the nose poke in any of the risky

(non illuminated) holes. We dealt with this effect by keeping the random allocation of the safe hole in each trial, but turning on the light in each of the risky hole while keeping the safe hole in the dark. As expected, this, although it did not completely eliminate the impact of the environmental cues in the decision process, made animals evenly choose certain and uncertain options, without biases towards any of the holes, thus providing a neutral baseline from where variations in each way could be easily detected and analyzed (Chapter 2.2). In order to test the ability of our paradigm to discriminate differences in preference between risk and safe options, we decided to manipulate the value of each option, and found that animals were able to recognize such changes and shift their preference accordingly, as revealed by an increased preference to risky options when risk profit was doubled and to safe options when amount of reward was increased (Chapter 2.2). Importantly, the above-mentioned observations support the task design adopted in the subsequent studies of this thesis.

Having optimized the protocol, we decided to analyze its neuroanatomical substrate by analyzing, using c-fos expression data, the regions whose activation was triggered by performance of the task. C-fos is an immediate early gene, whose expression and subsequent translation are triggered by neuronal activity, in a time dependent manner. In most brain regions, where the c-fos protein is barely detected in basal conditions, neuronal activation is accompanied by a rapid and transient expression of the gene, detectable by an increased expression of its mRNA 30 to 60 min latter and/or the presence of its protein product 60 to 90 min latter (Bisler et al., 2002). C-fos expression is widely used to map the brain regions whose activation is triggered by a stimulus or performance of a task, being particularly useful for cortical regions, where its expression is more abundant (delta-fos being more relevant when analyzing the activation of subcortical regions) (Bisler et al., 2002; Solinas et al., 2009). Our results demonstrate that our task recruits several brain regions known to be crucial for decision-making behaviors, including the medial prefrontal cortex (mPFC), the orbitofrontal cortex (OFC), the insular cortex, the nucleus accumbens (NAcc) and the dorsal striatum. In this regard, although the processing of uncertainty has been attributed to the loop between the NAcc and the OFC (Doya, 2008), it does not differ from similar tasks such as the ones previously described.

An interesting extension of these studies would be the analysis of inter-subjects variability. As already mentioned, in basal conditions, animals showed a similar preference for risk and safe options. However, analysis of their individual performance revealed a high between-subjects

variability in risk or safe preference that, importantly, was relatively stable among sessions (Annex II). Of relevance, these inter-individual differences in preference for risk were also described in human risk-based decision-making tasks (DeVito et al., 2008; Gianotti et al., 2009; Parasuraman and Jiang, 2011), which additionally backs the validity of our behavioral model and can provide useful insights for future studies on behavioral and neurobiological correlates of decision-making.

3.2. Stress induced behavioral impairments on decision making

Several animal models of stress have been described in the literature. Differences between them are related with duration of treatment (acute or chronic), frequency of exposure to stressors (continuously, one-shot per day, two-shots per day), type of stressor (physical or psychological) and variability of stressor (only one stressor or series of several stressors). In this work, we used the chronic unpredictable stress (CUS) protocol in the rat, an animal model of stress extensively used in our lab that mimics the persistency and variability of stressful daily life-events. In CUS, the chronic and unpredictable nature of the stressful stimuli induces a persistent hyper-activation of the physiological stress response that in turn leads to a disruption of the coping mechanisms usually triggered by stress (Sousa and Almeida, 2002). Over the last years, our lab has shown that this disruption contributes to disturbed anxiety responses (Pêgo et al., 2006), impairments in spatial reference and working memory and behavioral flexibility (Cerqueira et al., 2007) and habit-based instead of goal directed decision-making (Dias-Ferreira et al., 2009), and has explored its neurobiological substrates. In the work presented in this thesis, we complement these earlier works by showing that CUS further impairs decision-making, having an impact in PIT and risk-taking behavior.

In Chapter 2.1 we showed that chronic stress impairs PIT and that this effect is reversible after six weeks of recovery from stress. The PIT task is composed of three phases: a pavlovian phase, in which stimulus-outcome (S-O) associations are established; an instrumental phase, in which actions to obtain reward (R-O) are trained and a test phase, in which the impact of the conditioned stimuli on instrumental actions is assessed (Holmes et al., 2010). As consistently demonstrated in the literature, impairments of PIT can arise from a disruption of each of the three phases (Dickinson and Balleine, 2002; Holland, 2004; Yin and Knowlton, 2006). However,

in our experiments, both pavlovian and instrumental learning were unaltered, which led us to postulate that the chronic stress-induced PIT impairment reported in chapter 2.2 results most probably from a deficit in the transfer between the two learning processes. Importantly, as already mentioned, this is critically dependent on the function of the prefrontal cortex, a key target of chronic stress, in both animals and humans.

During decades, the enhancing effect of the conditioned stimulus on the instrumental response observed in PIT was attributed to the general motivating role of the conditioned stimulus (Estes, 1943; Rescorla and Solomon, 1967; Holland and Gallagher, 2003). However, although this reasoning fits with data from a generalized form of PIT, it does not account for the response observed in specific outcome PIT protocols, such as the one employed in our experiments, in which a conditioned stimulus only enhances a specific action (that associated with the same outcome) and not any other. Taking into account this difficulty, Balleine and Ostlund (2007) recently proposed that PIT elicits a stimulus-outcome-response (S-O-R) associative chain that is the basis of the observed behavior. According to this hypothesis, while the pavlovian learning period promotes S-O associations, two different outcome representations are established during the instrumental training: the outcome as the consequence of a response (R-O) and the outcome as a stimulus preceding the (next) response (O-R). When these conditions occur in series, as in the final PIT period, the stimulus activates an S-O association which elicits the corresponding O-R representation, thus promoting the respective action. This interpretation highlighted the fact that, besides motivation, which is crucial for both types of PIT (general and specific), outcome value is also indispensable for specific outcome PIT. Importantly, since outcome valuation is a hallmark of decision-making processes, this fact brings specific PIT under influence of the same networks and into the realm of goal-directed actions. As a consequence, stress-induced impairments on specific outcome PIT could be due to a relative inability to upgrade outcome values, an effect that could be related with the previously reported bias to habit-based actions promoted by chronic stress (Dias-Ferreira, 2009). Interestingly, similar impairments of specific PIT were found after treatment with dexamethasone (DEX), a selective glucocorticoid receptor (GR) agonist that mimics aspects of the normal arousal and/or stress response of animals (Zorawski and Killcross, 2003). Importantly, we showed that stress-induced effects on PIT are transient which is in accordance with several observations on structural, functional and behavioral recuperation after stress recovery (Sousa et al., 1998).

Along with described impairments in PIT, we demonstrated, for the first time, that chronic stress induces a risk-aversive pattern of choices in all three different conditions studied (basal, risk favorable and safe favorable) (Chapter 2.2.). Although it is has been clearly shown that chronic stress has a strong impact in decision-making abilities, impairing behavioral flexibility (Cerqueira et al., 2007) and goal directed behaviors (Dias-Ferreira et al., 2009), no study had already explored its effect on risk-based decision-making. Our results are in line with two previous studies in which it was found that acute stress exposure could induce a risk-aversive behavior in water foraging choice task (Graham et al., 2010) and decrease preference for rats to work harder to obtain a larger reward (Shafiei et al., 2012). On the contrary, a recent human decision-making study found that acute stress exposure can increase the preference for risk options, which seems to be related with higher levels of cortisol (Pabst et al., 2013) and a study in a rat gambling task has shown that an acute injection of corticosterone, an endogenous GR/mineralocorticoid receptor (MR) agonist which is one of the key players of the stress response, induced a preference for risky options (Koot et al., 2013). In comparison with our experimental approach, it is important to note that these studies: i) focused only on the effects of acute stress, which seems to be critical, since opposing effects of acute and chronic stress have been described in several other behavioral domains, including cognition (Lupien, 2009) and ii) assessed choices between advantageous and disadvantageous options, which made the evaluation of the risk preference more complex, since other factors such as potential gains and losses had also to be factored in. On the contrary, our task is mainly dependent on risk preference, since the expectations (balance between value and effort) and the predictability (time until reward delivery) associated with the different options are leveled off (in the neutral condition) or tightly controlled. In addition, as already discussed, our task highlighted the fact that, despite their risk aversion, animals submitted to chronic stress could keep their ability to flexibly adapt their behavior according to the reward associated with each option, further supporting the identification of the observed behavioral changes with “willingness to risk” and not any kind memory-based process.

Despite these considerations, more studies are necessary to clarify whether the reported risk-aversion is due to continuous responding to the previously reward reinforced option (“habit based behavior”) or avoidance of the previously unrewarded choice (“learned avoidance”). Interestingly, human studies have shown that acute stress could enhance learning of positive outcomes and weaken learning of negative outcomes of choices (Petzold et al., 2010; Lighthall et al., 2013) but

the underlying processes that may explain these behavioral effects of stress were not addressed in these works.

In summary, we have shown that chronic stress impairs the ability to incorporate relevant environmental cues in guiding instrumental behavior and biases risk-based decision making to safe/certain options. Since relevant decisions are often made under stress, these findings could have a profound impact, which led us to further analyze and discuss possible mechanisms underlying these changes.

3.3. Reorganization of neural systems of decision making

After characterizing stress induced impairments induced by chronic stress in PIT (Chapter 2.1.) and risk-based decision making (Chapter 2.2.) we searched for functional, anatomical and neurochemical alterations that could explain reported behavioral biases. Having previously shown which areas were activated by performance our risk-based decision-making paradigm, as discussed above, we used c-fos labeling to further identify those that were differentially activated in chronically stressed and control rats. Our main finding was a significant overactivation, in the former, of the lateral OFC and the insular cortex (Chapter 2.2.), which is in accordance with observations by Koot and colleagues (2013) in a rat gambling task performed under an acute corticosterone injection and strongly suggests these areas to be key players in risk-based decision-making under stress.

The OFC is known to be involved in the representation of stimulus-reward value (Izquierdo et al., 2004; Schoenbaum and Roesch, 2005), the update of relative values of selected and non-selected outcomes (Wallis 2007), the mounting of appropriate responses to motivationally salient stimulus (Osteund and Balleine, 2007), the factoring of efforts associated to each option (Roesch and Olson, 2005, Kennerley et al., 2009) and the processing of confidence in the decision (Kepecs et al., 2008). Rodent lesion studies have highlighted that the OFC encodes specific information about the outcome rather than its general affective value (Burke et al., 2008). Importantly, this region integrates an OFC-striatal-amygdala circuitry that could be affected by peripheral states of arousal, such as stress, and that competes with a more cognitive network, dependent on the medial PFC (Ongur and Price, 2000; Barbas and Zikopoulos, 2007). Indeed,

the findings we present in this thesis, including the hyperactivation of the OFC and a tendency, albeit non significant, for a hypoactivation of the mPFC (Chapter 2.2) in stressed individuals performing the risk-based task, are in accordance with previous observations from our laboratory suggesting that chronic stress promotes a shift from a prefrontal loop, depicting atrophy after CUS exposure, to a hypertrophied OFC loop that controls choice behaviors and biases decisions to habits (Yin et al., 2004, Dias-Ferreira et al., 2009). In addition, although it was not addressed by this thesis, the shift between these two cortico-striatal loops could also be implicated in the observed PIT impairments. In fact, a recent study demonstrates that OFC and mPFC orchestrate the integration of Pavlovian and instrumental processes during PIT (Homayoun and Moghaddam, 2009) backing previous observations that the mPFC and OFC encode distinct components of both Pavlovian and instrumental processes (Gallagher et al., 1999; Chudasama and Robbins, 2003; Ostlund and Balleine, 2007; Homayoun and Moghaddam, 2008).

Similarly to the OFC, the insular cortex seems to be critically involved in decision-making. Several studies, mainly in humans and primates, implicated the insular cortex in the processing of representations of bodily internal states and needs (Naqvi and Bechara 2009) and signaling risk-aversion (Clark et al., 2008; Preuschoff et al., 2008). Interestingly, the magnitude of insular activation as a correlate of risk avoidance was found to increase with age (Paulsen et al., 2011) and lesion studies have shown that insula shut-down is associated with risk-taking behaviors (Clark et al., 2008) which is in accordance with our observation that insular cortex over-activation in the adult stressed rodent is related with a risk-aversive pattern of choice (Chapter 2.2.).

Obviously these changes in the activity of distinct brain regions under stress are underlied by changes in neurotransmission. Amongst others, stress is known to induce a dopaminergic dysfunction in several brain areas that correlates with behavioral impairments (Mizoguchi K et al., 2000; Tseng and O'Donnell 2004, Gruber et al., 2010; Rodrigues et al., 2012), and dopamine transmission has been involved in several decision-making tasks (Rogers, 2011). Bearing this in mind, we characterized the dopaminergic system in the OFC and the insular cortex and found that, in chronically stressed animals in which these regions are overactivated upon performance of the task, dopamine levels are reduced in the former and present a trend towards an increase in the latter region (Chapter 2.2.), whereas expression of D2 receptors mRNA is significantly increased in the OFC (Chapter 2.2.).

Dopaminergic activity in the OFC is known to be crucial to decision-making, being implicated in incentive motivation (Schultz, 2002; Cetin et al., 2004, Kheramin et al., 2004) and in the stabilization of internal representations of the S-O associations (Robbins and Roberts, 2007). Indeed, dopaminergic depletions in the OFC had been associated with insensitiveness to conditioned reinforcers and persistent responding in the absence of reward in extinction, a pattern of deficits that may reflect basic deficits in the associative processing of reward (Walker et al., 2009). Moreover, a loss of OFC dopamine may disrupt prefrontal control over the striatum, resulting in the potentiation of habitual responses, an effect that seems to be specifically modulated by an over-responding dopamine-depleted OFC (Walker et al., 2009). In further support of our view of a stress-induced hypodopaminergic overactivated OFC being crucial for the observed risk-aversive behavior, dopaminoceptive neurons were found crucial for social aversion induced by chronic stress (Barik et al., 2013).

As already mentioned, our study also included a morphological analysis of pyramidal neurons of lateral part of OFC and insular cortex (Chapter 2.2.). We found that chronic stress induces a hypertrophy of apical dendrites of lateral OFC neurons that, importantly, is also present in the neurons activated during the risk-based task, but does not seem to affect insular pyramidal neurons. These findings are in accordance with previous published data (Dias-Ferreira et al., 2009) and, since dendrites are targets for ingrowing axonal fibers derived from cortical and subcortical regions, may reflect the stress-induced structural rearrangement of neural circuitry involving OFC.

Summing all the previous findings, the present studies on neurochemical and structural effects of chronic stress add relevant insights on the relevance of dopaminergic dysfunction for the reported impairments on decision-making processes. Driven by these observations, we proposed a pharmacological intervention with a specific D2/D3 agonist quinpirole to ameliorate decision-making impairments induced by stress. This intervention provided one of the most surprising observations of our studies: quinpirole reverted stress-induced risk-aversion, making behavior of rats indistinguishable from non-stressed controls (Chapter 2.2.). As previous studies have described that dopaminergic agents could, by themselves, increase risk choices in gambling tasks (Onge and Floresco, 2009; Riba et al., 2008; Onge et al., 2010) it could be argued that the observed effect was related with a generalized increase in risk choices induced by quinpirole, and not a specific reversal of the stress-induced risk-aversive behavior. However, the latter seems not

to be the case, as quinpirole was shown to have no effect on non-stressed animals, at least in the doses used in our study. Of notice, this pharmacological reversion of risk-aversion induced by stress could pave the way for new therapeutical approaches to disorders, such as obsessive compulsive disorder (OCD), gambling disorders or schizophrenia, in which patients are known to display decision-making dysfunctions,

3.4. Obsessive-compulsive disorder and decision-making: insights from stress response dysfunction

Obsessive-compulsive disorder (OCD) is a psychiatric disorder characterized by obsessions (persistent, intrusive and inappropriate thoughts, as well as impulses or images that cause anxiety) and compulsions (repetitive behaviors or thoughts performed in order to decrease the anxiety caused by the obsessions). Importantly, this disorder is also characterized by severe impairments in decision-making processes (Gillan et al., 2011) and several reports implicate environmental influences, including stressful events, in the onset and exacerbations of disease (Lochner et al., 2002; Forray et al., 2007; Cromer et al., 2007; Gershuny et al., 2003; Real et al., 2011; Jordan et al., 1991). Thus, we decided to explore the specific features of the stress response and decision-making in a cohort of OCD patients.

In the work presented in this thesis, we found that OCD patients report significantly higher levels of perceived stress than healthy controls, and that these are accompanied by higher serum cortisol levels (Chapter 2.3.). Additionally, we found that perceived stress levels were positively correlated with the severity of obsessive symptoms but not with the severity of the compulsive component of disease (Chapter 2.3.). These observations are in accordance with previous studies (Coles et al., 2005; Cougle et al., 2011, Gehris et al., 1990; Kluge et al., 2007) and support the theory that animal models of chronic stress could provide relevant information over mechanisms underlying some OCD features. Indeed, dysfunction in orbitofronto-striatal circuits, whose implication in the pathophysiology of OCD has been extensively documented, can be easily induced by chronic stress exposure in humans (Soares et al., 2012), highlighting the relevance of our findings. Importantly, stress-induced shift in decision making behaviors from goal directed to habits might be of interest for the knowledge of underpinning mechanisms involved in compulsive symptoms of OCD (Gillan et al., 2011) whereas the impaired ability to

associate environmental cues to goal-directed behaviors (Chapter 2.1.) and the risk-aversion associated with orbitofrontal and insular over-activation (Chapter 2.2.) described in this thesis might be of interest for further exploration of clinical features often described in OCD patients such as risk avoidance (Admon et al., 2012) and impaired sensibility to environmental cues (Ristvedt et al., 1993).

Risk-based decision-making was also assessed on OCD patients using an fMRI paradigm. Behavioral results did not show differences in the frequency of risk and safe choices, but significant differences were found in decision-making strategies and in the time used to take risky decisions (Chapter 2.4.). Additionally, increased activation of (orbito)fronto-striatal regions and the anterior cingulate cortex after negative outcomes were found among OCD patients when compared with healthy volunteers (Chapter 2.4.). This finding is in accordance with reported risk-aversion (Lagemann et al., 2012; Admon et al., 2012) and helps understanding the genesis of the conflict in the decision-making process, pointing for future areas of therapeutic interventions.

3.5. Chronic stress and obsessive related disorders: a new translational approach

The idea that Obsessive Compulsive Spectrum Disorders can be viewed along a dimension of compulsivity *versus* impulsivity is widely accepted. The compulsive end, represented by OCD, body dysmorphic disorder and anorexia nervosa, is characterized by risk avoidance related with an exaggerated estimation of harm and a tendency to avoid harm or reduce anxiety by performing compulsive behaviors. In contrast, the impulsive end, represented by pathological gambling and sexual compulsivity, is characterized by an underestimation of risk, seeking of pleasure, arousal or gratifications and actions can be aggressive and out of control (Hollander, 1995). Impulsive-like disorders are usually considered as “addictive disorders”.

Summing up our findings with previous reports in the literature, it is possible to characterize CUS exposed animals as risk-aversive (Chapter 2.2.), habit-based (Dias-Ferreira et al., 2009), PIT-impaired (Chapter 2.1.) and less addictive prone (Kabbaj and Isgor, 2007; Kabbaj et al., 2002). This stress-induced phenotype shares several nuclear features with compulsive-end disorders (including OCD) highlighting the role of stress in the pathophysiology of OCD and making rodent CUS paradigm a putative animal model for the study of such disorders. On the contrary, animals

submitted to chronic stress on developmental periods of life (pre-natal to adolescence) displayed a phenotype characterized as risk-prone (Toledo-Rodriguez and Sandi, 2011), goal-directed (Rodrigues et al, 2012), impulsive (Rodrigues et al, 2012) and addictive prone (Rodrigues et al., 2012; Hollis et al., 2013), a phenotype similar to that described for impulsive-end disorders. These observations suggest that the detrimental effects of chronic stress vary according to the lifetime period of its exposure. Interestingly, as observed in humans, early life stress seems to bias behavior to impulsive-like disorders while stress later in life seems to favor the establishment of a compulsive-like behavior (Chapter 2.1.; Chapter 2.2.; Dias-Ferreira et al., 2009; Rodrigues et al., 2012). Intriguingly, low cortical dopamine levels were found in both stress models which suggests that other mechanisms may underlie this changes. Early disruption of the dopaminergic system was shown to affect brain maturation (Lauder, 1988; Lauder, 1993), playing an important role in division, migration and differentiation processes of cortical neurons, namely in prefrontal cortex (Lewis et al., 1998). Thus, depletions in dopamine during neurodevelopment could impair the development of mechanisms of behavior control (Nieoullon, 2002).

In the last decades, theories focused on the role of serotonin on compulsive-impulsive spectrum disorders and proposed a parallel pathophysiological scheme with compulsive disorders associated with increased frontal activity and impulsive disorders associated with decreased frontal lobe activity. Importantly, we have shown that a pharmacological intervention with dopaminergic agents could also be useful for the restoration of detrimental effects of stress on behavior, which, in light of the above discussed relationship between stress and OCD spectrum disorders, brings dopamine to the centre of discussions on such disorders. In line with this, aripiprazole, a D2 specific agonist, has been recently shown to be effective in the treatment of OCD (Sayyah et al., 2012; Abdel-Ahad and Kazour, 2013), despite the lack of a clear neurobiological hypothesis underlying its utility.

In conclusion, the studies presented in this thesis provided evidence that chronic stress disrupts decision-making behaviors, in particular those associated with the processing of risk, and that these behavioral changes, which seem to be related with rearrangements of the neural circuitry and low dopamine levels in brain regions targeted by stress, can be completely reverted by treatment with a D2/D3 dopamine receptor agonist, quinpirole. Additionally, we provided new insights on OCD as a stress-related disorder and, in light of the previous findings, suggested a role for dopamine dysfunction in the ethiopathogenesis of this disorder. This new

conceptualization could be of interest not only to the comprehension of mechanisms underlying OCD but also to the research on new therapeutical approaches directed to this chronic and, frequently, incapacitating disorder.

3.6. References

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Chapter 4

Conclusions

4. CONCLUSIONS

The present work has characterized the impact of chronic stress on decision-making, exploring its neural substrates, and analyzed the stress response and decision-making in obsessive compulsive disorder, proposing potential pharmacological interventions that can be translated in the clinical settings. By doing so, we are able to conclude that:

1) Chronic stress transiently disrupts the ability of conditioned cues to influence instrumental behaviors, as assessed by pavlovian to instrumental transfer;

2) Chronic stress induces a risk-aversive pattern of choice in a new rodent risk-based decision-making paradigm, which is associated with over-activation of the orbitofrontal and insular cortices and low dopamine levels and high expression of D2 dopamine receptor mRNA in the orbitofrontal cortex;

3) Treatment with the D2/D3 selective agonist quinpirole reverts the stress-induced risk-aversion;

4) Obsessive compulsive patients display higher levels of perceived stress which are positively correlated with the severity of obsessive symptoms; this suggests that OCD can be considered a stress-related disorder and opens avenues for further research in the field, including on the use of dopaminergic treatments.

5) Obsessive compulsive patients have difficulties in risk-based decision-making which are associated with decreased activity in the caudate when deciding, hypoactivation of the amygdala before making high-risk choices and increased activity in several areas of the (orbito)fronto-striato-thalamic circuit implicated in decision upon loosing.

Chapter 5

Future Perspectives

5. FUTURE PERSPECTIVES

Despite the increasing relevance decision-making processes, their underlying mechanisms are largely unknown. By crossing evidence from neurosciences and clinical psychiatry, the present work aimed to answer some questions in this field but raised a significantly higher number of questions that should be further investigated.

First, future work should be directed to clarify neuroanatomical and functional correlations of the stress-induced behavioral changes described. To achieve this goal, techniques that assess brain activation in real-time, such as electrophysiology studies, should be used to better characterize the involvement of brain regions in the described behavioral tasks. In addition, the role of the increased orbitofrontal and insular cortices activation in the genesis of stress-induced risk-aversion could be studied by specifically inactivating each of these regions, either with a local injection of drugs or, more elegantly with the use of optogenetic approaches; the latter, could even allow a better characterization, by transiently silencing or activating only certain neuronal types (such as dopaminergic or glutamatergic cell, for example) in specific regions of the brain.

Decisions are not only modulated by reward quantity. Thus, the stress induced risk-aversive behavior should be further detailed using behavioral tasks, in the same decision-making paradigm, that evaluate how stress impacts on probability and on reward quality changes, since as we only focused on changes on reward quantity.

Third, mechanisms underlying quinpirole treatment could be further detailed by direct injection of a D2/D3 agonist on orbitofrontal cortex and, eventually, on insular cortex, to avoid the non-specificity of system administration. While other dopaminergic agents, with higher D2 receptors specificity, could be tested, another approach could be the use of viral gene delivery to selectively drive an increased expression of D2 receptors or enhance its activity.

On the clinical grounds, an interesting question that rose from our experiments with OCD patients was related with characterization of additional biological markers of stress response, such as ACTH and NK cells activation. Certainly, the dopaminergic insights on OCD should be further explored using PET/MRI techniques that allow *in vivo* quantification of dopamine levels in

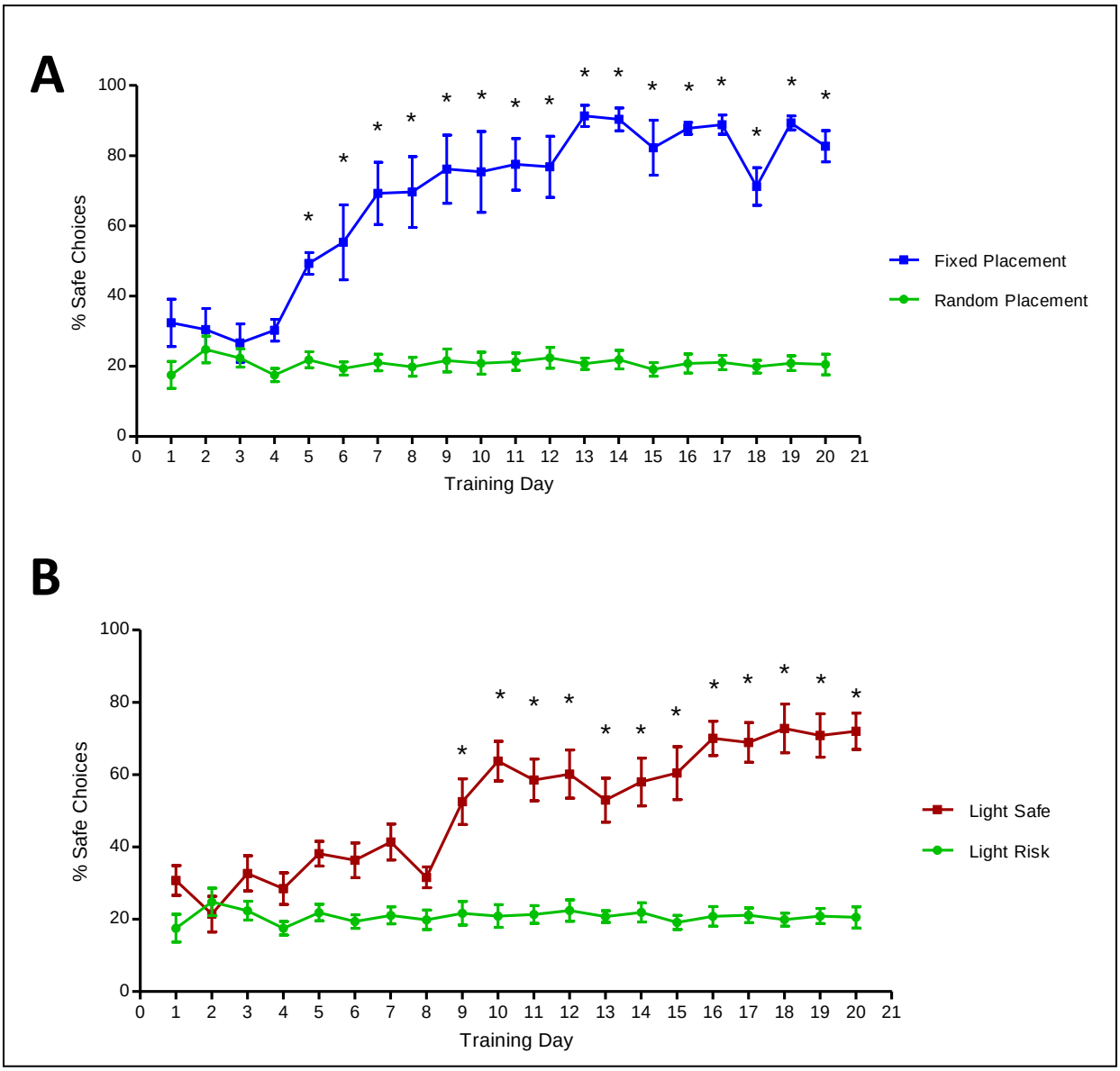
different brain regions and characterizing, using pharmacological MRI, the brain response to approved dopaminergic agents.

And, finally, the characterization of other pathologies of the OCD spectrum regarding their relationship with stress and their decision-making profile would be fundamental.

Annexes

Annex 1

Optimization of the risk-based decision-making task described in Chapter 2.2



Optimization of the risk-based decision-making task described in Chapter 2.2

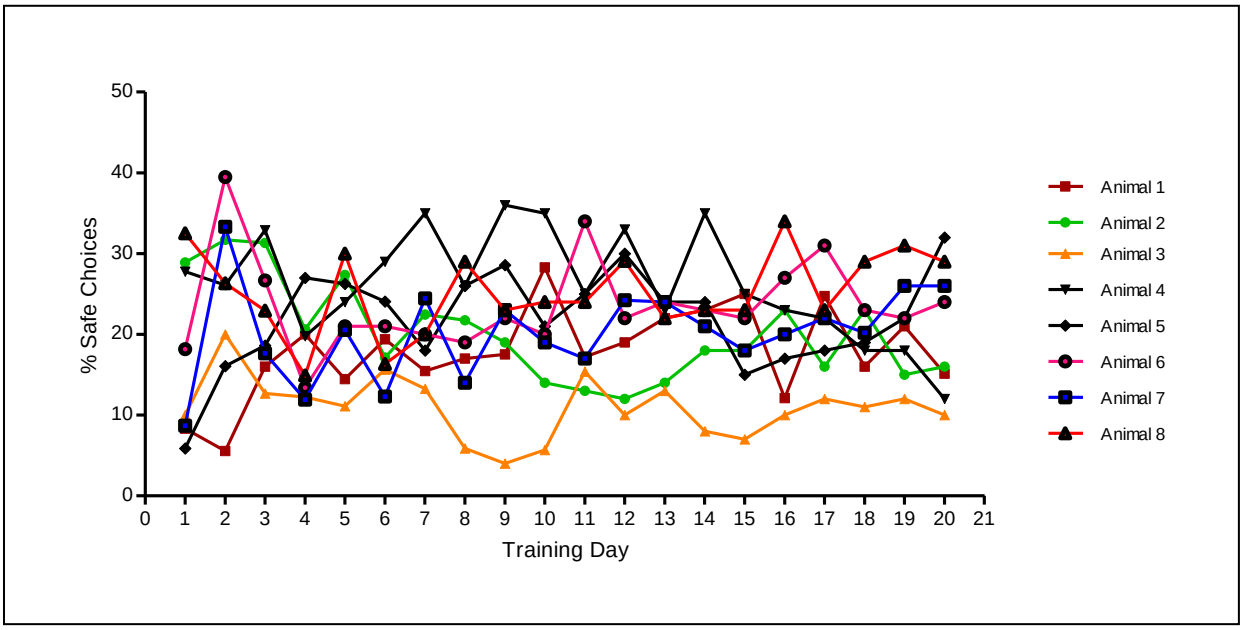
(A) Comparison between fixed and a random placement of the safe nose-poke hole. Using a fixed placement design, animals increase their preference for safe choices to more than 80%, whereas with a random placement design their preference remains rather stable at around 20% (chance levels). The latter was the design adopted in the final version of the task. *p<0.05. **(B)** Comparison between the use of light to signal safe or risky choices. When the only illuminated nose-poke hole was the safe/certain option - light signaled safe - animals consistently increased

their preference for these option to more than 60%. On the contrary, when the nose-poke holes corresponding to risk/uncertain options were illuminated (and safe option hole was left in the dark) – light signaled risk – performance remained stable at around 20% (chance levels). The latter was the design adopted in the final version of the task. *p<0,05.

Annex 2

Inter-individual variation in the risk-based decision-making task described in Chapter 2.2.

ANNEX 2



Inter-individual variation in the risk-based decision-making task described in Chapter 2.2.

Rats display different, individual, preferences for safe/certain and risk/uncertain options. All animals are controls and were tested at the same time, under the neutral condition, on the final protocol.

Annex 3

Dias-Ferreira E, Sousa JC, Melo I, Morgado P, Mesquita AR, Cerqueira JJ, Costa RM, Sousa N. (2009).

Chronic stress causes frontostriatal reorganization and affects decision-making.

Science. 325(5940): 621-5

Chronic Stress Causes Frontostriatal Reorganization and Affects Decision-Making

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The ability to shift between different behavioral strategies is necessary for appropriate decision-making. Here, we show that chronic stress biases decision-making strategies, affecting the ability of stressed animals to perform actions on the basis of their consequences. Using two different operant tasks, we revealed that, in making choices, rats subjected to chronic stress became insensitive to changes in outcome value and resistant to changes in action-outcome contingency. Furthermore, chronic stress caused opposing structural changes in the associative and sensorimotor corticostriatal circuits underlying these different behavioral strategies, with atrophy of medial prefrontal cortex and the associative striatum and hypertrophy of the sensorimotor striatum. These data suggest that the relative advantage of circuits coursing through sensorimotor striatum observed after chronic stress leads to a bias in behavioral strategies toward habit.

In everyday life, we constantly have to select the appropriate actions to obtain specific outcomes. These actions can be selected on the basis of their consequences (*1*, *2*), e.g., when we press the elevator button to get to the particular floor of our new apartment. This goal-directed behavior is crucial to face the ever-changing environment, but demands an effortful control and monitoring of the response. One way to balance the need for flexibility and efficiency is through automatization of recurring decision processes as a rule or a habit (*3*). Habitual responses no longer need the evaluation of their consequences and can be elicited by particular situations or stimuli (*1*, *2*), e.g., after living for some time in that apartment, we automatically press the button of our home floor when we enter the elevator. The ability to shift between these two types of strategies is necessary for appropriate decision-making (*2*), and in some situations, it may be crucial to be able to inhibit a habit and use a goal-directed strategy, e.g., if we are visiting a new building, we should not press the button for our home floor.

Chronic stress, mainly through the release of corticosteroids, affects executive behavior through sequential structural modulation of brain networks (*4*, *5*). Stress-induced deficits in spatial reference and working memory (*6*) and behavioral flexibility (*7*) are associated with synaptic and/or dendritic reorganization in both the hippocampus (*8*) and the medial prefrontal cortex (mPFC) (*9*). However, the effects of chronic stress on action-selection strategies have not been investigated. Here, we examined whether previous exposure to chronic stress would affect the ability of animals to select the appropriate actions, based on the consequences of their choice. Because associative corticostriatal circuits involving the prelimbic (PL) cortex (*10*) and the dorsomedial striatum (DMS) (*11*) have been implicated in the acquisition and execution of goal-directed actions, whereas sensorimotor circuits, namely, the dorsolateral striatum (DLS) (*12*), are necessary for habit formation, we examined the effects of chronic stress on these brain areas.

In an attempt to mimic the variability of stressors encountered in daily life, adult rats assigned to the stress group were exposed to a well-established stress paradigm (*13*) that combines different stressors in an unpredictable manner to

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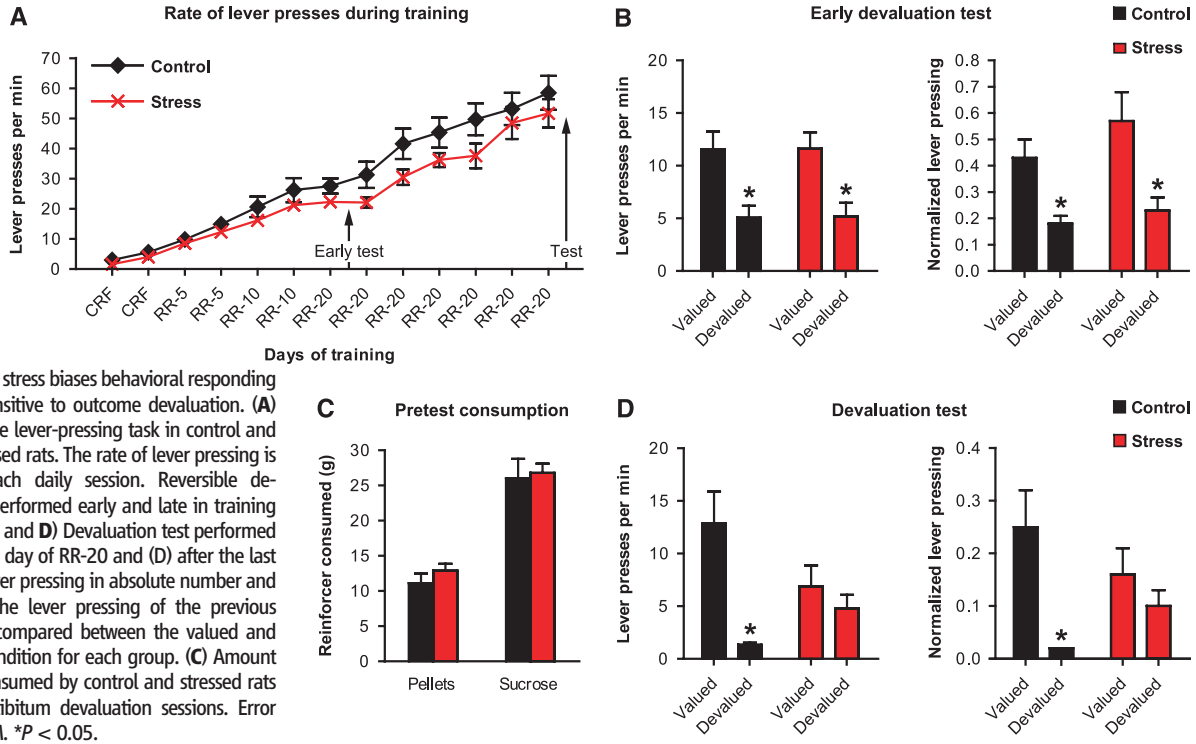

Fig. 1. Chronic stress biases behavioral responding to become insensitive to outcome devaluation. (**A**) Acquisition of the lever-pressing task in control and chronically stressed rats. The rate of lever pressing is depicted for each daily session. Reversible devaluation tests performed early and late in training are indicated. (**B** and **D**) Devaluation test performed (B) after the first day of RR-20 and (D) after the last training day. Lever pressing in absolute number and normalized to the lever pressing of the previous training day is compared between the valued and the devalued condition for each group. (**C**) Amount of reinforcer consumed by control and stressed rats during the ad libitum devaluation sessions. Error bars denote SEM. *$P < 0.05$.

avoid the resilient effect of behavioral control over stressors (*14*). Twenty-one days of stress exposure decreased body-weight gain (fig. S1A), reduced the thymus/body-weight ratio (fig. S1B), and resulted in persistently raised serum corticosterone levels (fig. S1C), when compared with attributes of handled controls. After stress exposure, we tested whether chronic stress affected the ability of animals to perform actions, based on the consequences of their behavior, using two different instrumental tasks.

We first examined whether previous exposure to chronic stress affected the ability of animals to perform actions based on the expected value of predicted outcomes (*1*, *15*). Rats ($n = 8$ per group) were trained to press a lever for a particular outcome (pellets or sucrose, counterbalanced) under a random ratio schedule that was previously shown to bias for goal-directed behavior (*3*, *15*, *16*). Training started with 2 days of continuous reinforcement (CRF) and progressed under increasing random ratio (RR) schedules of reinforcement to RR-20 (on average one reinforcer every 20 lever presses). Both groups increased lever pressing across training days ($F_{12,168} = 95.489$, $P < 0.001$), and there was no interaction with ($F_{12,3} = 1.089$, $P = 0.372$) or main effect of ($F_{1,14} = 3.094$, $P = 0.100$) stress treatment (Fig. 1A). To evaluate whether animals could learn to press for the specific outcome delivered contingent on lever pressing, we performed an early devaluation test after the first day of RR-20 (Fig. 1B). Both stressed animals and controls significantly reduced their responses after the outcome they pressed for during training was devalued by sensory-specific satiety (devalued condition), when compared with the situation when a different outcome was devalued (valued condition) (*13*) (lever presses per min: control, $t_7 = 3.197$, $P = 0.015$; stress, $t_7 = 2.931$, $P = 0.022$; normalized lever pressing: control, $t_7 = 3.106$, $P = 0.017$; stress, $t_7 = 2.694$, $P = 0.031$). With increased training and in accordance with previous studies (*3*, *15*, *16*), the actions of control animals became highly sensitive to sensory-specific satiety [(Fig. 1D) lever presses per min: $t_7 = 3.672$, $P = 0.008$; normalized lever pressing: $t_7 = 3.042$, $P = 0.019$]. In contrast, the actions of stressed animals became insensitive to the expected value of the outcome, as indicated by the lack of a devaluation effect [(Fig. 1D) lever presses per min: $t_7 = 0.984$, $P = 0.358$; normalized lever pressing: $t_7 = 1.095$, $P = 0.310$]. It is noteworthy that the early devaluation test demonstrates that this insensitivity did not arise from an inability of the stressed animals to learn the relation between the action and the outcome or from changes in motivation, food valuation, or hedonics (*17*), but rather because stressed animals rapidly shift to a habitual strategy as training progresses. The amount of reinforcer consumed during the ad libitum devaluation sessions was similar in stressed and control animals [(Fig. 1C) pellets: $t_{14} = -1.072$, $P = 0.302$; sucrose: $t_{14} = -0.252$, $P = 0.805$].

Although it seems unlikely that the results obtained in the test above were due to differences in hedonics or value processing, we used a different task to confirm whether animals previously exposed to chronic stress really had impairments performing actions on the basis of the consequences of their behavior. We therefore investigated whether the behavior of chronically stressed animals would depend on the contingency between getting the outcome and the previous execution of the action (*1*, *18*). We trained a separate group of rats ($n = 15$ per group) in a task in which one action (pressing the left lever) would lead to a particular outcome (i.e., pellets), and another action (pressing the right lever) would lead to a different outcome (i.e., sucrose). Every day animals had two training sessions, one for each action-outcome pair (counterbalanced). Both groups increased lever pressing as training progressed across days under increasing ratio schedules of reinforcement (pellets: $F_{11,308} = 138.213$, $P < 0.001$; sucrose: $F_{11,308} = 88.578$, $P < 0.001$), and there was no interaction with stress (pellets: $F_{11,18} = 0.419$, $P = 0.947$; sucrose: $F_{11,18} = 0.831$, $P = 0.609$), or main effect of stress (pellets: $F_{1,28} = 2.742$, $P = 0.109$; sucrose: $F_{1,28} = 0.781$, $P = 0.384$) on acquisition (Fig. 2A). Similar to the previous task, both controls and stressed animals were able to learn the action-outcome relation as shown by their clear preference toward the valued lever in an early devaluation test after the first day of RR-20 (lever presses per min: control valued, 15.73 ± 2.24; devalued, 4.88 ± 0.95; $t_{14} = 4.150$, $P = 0.001$; stress valued, 11.19 ± 1.40; devalued, 5.33 ± 0.77; $t_{14} = 4.262$, $P = 0.001$; normalized lever pressing: control valued, 0.41 ± 0.04; devalued, 0.14 ± 0.03; $t_{14} = 5.167$, $P < 0.001$; stress valued, 0.34 ± 0.04; devalued, 0.18 ± 0.03; $t_{14} = 4.133$, $P = 0.001$; results are means ± SEM). After the last day of acquisition, we tested whether stressed animals were performing actions because they were necessary to obtain the outcome or not. For each animal, we degraded the contingency between one of the actions and the respective outcome (degraded condition: to get this outcome, the animals no longer needed to press the lever), but not between the other action-outcome pair (non-degraded: to obtain this outcome, the animals needed to press the lever) (*13*). After 2 days of forced-choice degradation training in which

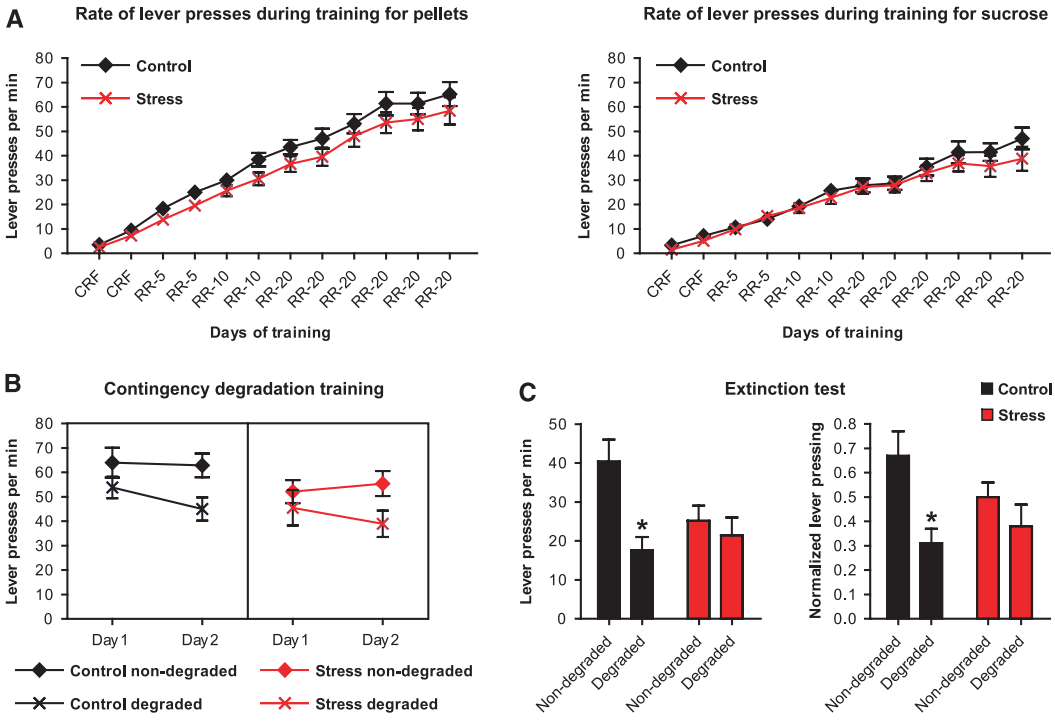

Fig. 2. Chronic stress predisposes choices to be insensitive to changes in action-outcome contingency. (**A**) Acquisition of the lever-pressing task in control and chronically stressed rats. The rate of lever pressing is depicted for each daily session for pellets and for sucrose. (**B**) Performance for each group during forced-choice sessions in which one instrumental outcome continued to be obtained in a RR-20 schedule (non-degraded) and the other outcome was delivered noncontiguously or freely (degraded). (**C**) Critical choice test between the lever for which the action-outcome contingency was preserved and the lever that had the contingency degraded. Lever pressing in absolute numbers and normalized to the lever pressing of the last acquisition training day is compared between levers for each group. Error bars denote SEM. *$P < 0.05$.

both groups changed their behavior [(Fig. 2B) degradation effect: control, $F_{1,28}$ = 4.342, P = 0.046; stress, $F_{1,28}$ = 2.189, P = 0.150; training × degradation interaction: control, $F_{1,28}$ = 2.396, P = 0.133; stress, $F_{1,28}$ = 5.580, P = 0.025], animals were given a free-choice test between the degraded and non-degraded lever, in extinction [to avoid the confounding effects of consumption and reinforcement (*11*)] (Fig. 2C). Control animals significantly reduced their responses on the degraded lever compared with the non-degraded (lever presses per min: t_{14} = 2.552, P = 0.023; normalized lever pressing: t_{14} = 2.645, P = 0.019). However, stressed animals pressed both levers similarly (lever presses per min: t_{14} = 0.808, P = 0.433; normalized lever pressing: t_{14} = 1.330, P = 0.205), which indicated that they failed to choose the action that was necessary to obtain the outcome and that their behavior was habitual.

These data indicate that previous exposure to chronic stress biases decision-making and pre-

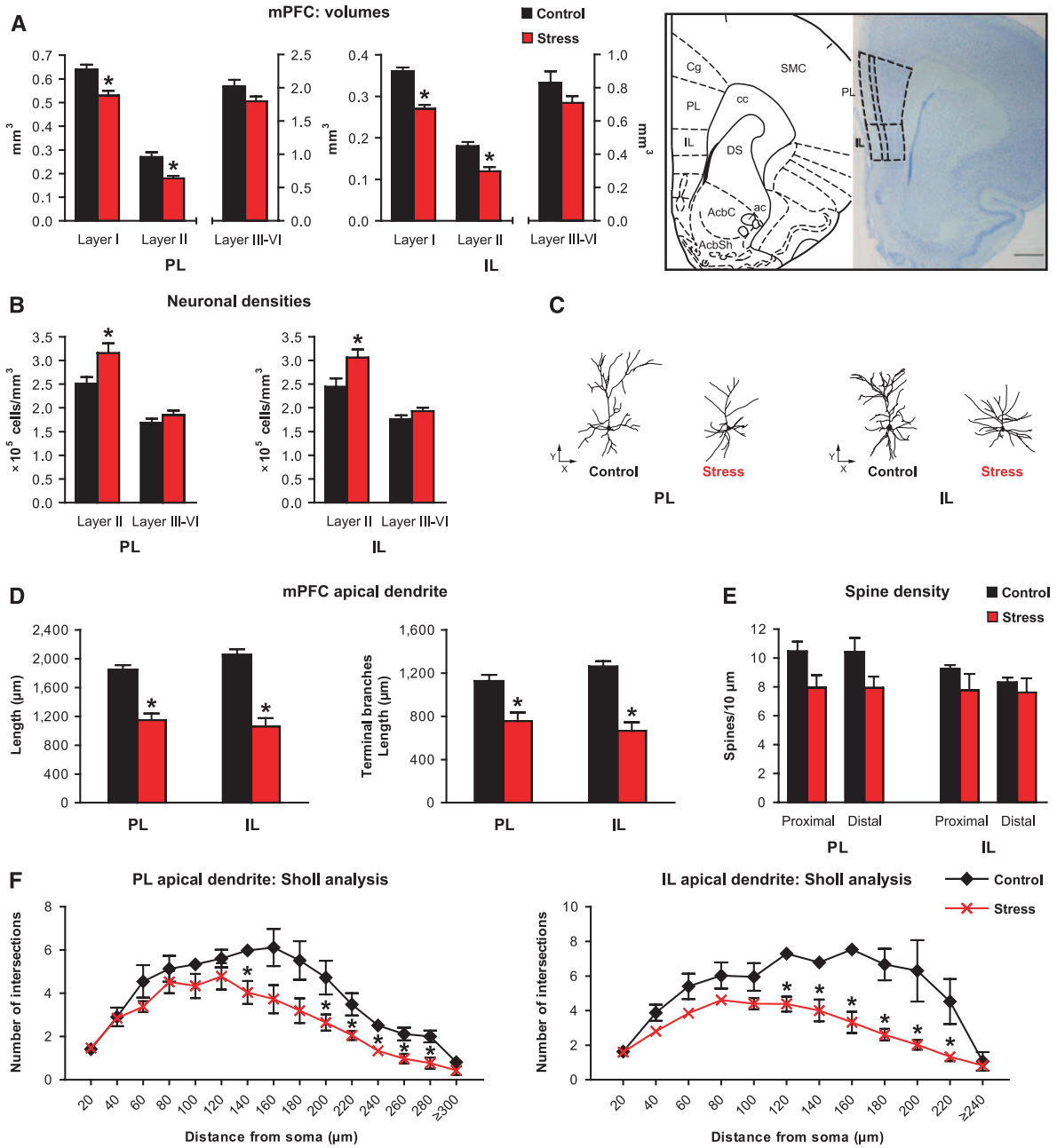

Fig. 3. Chronic stress results in selective atrophy within the external layers of both PL and IL mPFC subregions. Several structural measurements of control and chronically stressed rats are compared. (**A** and **B**) Stereological estimations of (A) volumes and (B) neuronal densities. (A, right) Outlining between regions and layers is represented; diagram was adapted from (*31*) and corresponding brain slice stained with Giemsa (2.20 mm from bregma). Cg, cingulate cortex; SMC, sensorimotor cortices; cc, corpus callosum; DS, dorsal striatum; AcbC, core, and AcbSh, shell, of nucleus accumbens; ac, anterior commissure. Scale bar, 800 μm. (**C** to **F**) Morphometric analysis in 3D of Golgi-stained pyramidal neurons of superficial layers (II/III). (C) Computer-assisted reconstructions of representative neurons depicted in the XY orthogonal plane. (D) Length, (E) spine density, and (F) differential rearrangement of apical dendrites. Error bars denote SEM. *P < 0.05.

disposes animals to more readily shift between goal-directed and habitual behavioral strategies as training progresses, similar to the effects observed after manipulations of the associative (*10*, *11*) or sensorimotor (*12*, *16*) corticostriatal circuits (*19*–*21*). Therefore, in a separate cohort of animals ($n = 5$ per group, submitted to chronic stress or handling but not submitted to instrumental training), we investigated the effects of chronic stress on the structure of cortical and striatal circuits known to be required for goal-directed actions and habits. Within the mPFC, the PL and infralimbic (IL) subregions have been implicated in instrumental behavior (*10*, *19*). Volumetric estimations showed a selective atrophy of external cortical layers in both mPFC subregions of stressed animals [(Fig. 3A) PL: layer I, $t_8 = 4.066$, $P = 0.004$; layer II, $t_8 = 3.697$, $P = 0.006$; layer III-VI, $t_8 = 1.725$, $P = 0.123$; IL: layer I, $t_8 = 6.225$, $P < 0.001$; layer II, $t_8 = 4.743$, $P = 0.001$; layer III-VI, $t_8 = 1.411$, $P = 0.196$]. Consistently, we observed an increase in neuronal density in these layers in the same animals [(Fig. 3B) PL: layer II, $t_8 = -2.602$, $P = 0.032$; layer III-VI, $t_8 = -1.383$, $P = 0.204$; IL: layer II, $t_8 = -2.488$, $P = 0.038$; layer III-VI, $t_8 = -1.688$, $P = 0.130$]. Three-dimensional (3D) morphometric analysis of dendritic arbors of layer II/III pyramidal cells in the mPFC indicated that these changes in volume and density could be ascribed to dendritic atrophy (PL: $t_8 = 6.457$, $P < 0.001$; IL: $t_8 = 7.021$, $P < 0.001$), particularly in terminal branches (PL: $t_8 = 3.851$, $P = 0.005$; IL: $t_8 = 6.389$, $P < 0.001$) of the apical tree (Fig. 3, C and D). These effects suggest a loss of neuronal connectivity that does not seem to result from spine loss [(Fig. 3E) PL: proximal, $t_8 = 2.290$, $P = 0.051$; distal, $t_8 = 1.960$, $P = 0.086$; IL: proximal, $t_8 = 1.270$, $P = 0.240$; distal, $t_8 = 0.669$, $P = 0.522$] or maturation (fig. S2A), but rather to an impoverished arborization confined to distal portions [(Fig. 3F) PL: stress effect, $F_{1,8} = 12.150$, $P = 0.008$; post hoc 140, 200 to 280 μm, $P < 0.05$; IL: stress effect, $F_{1,8} = 17.117$, $P = 0.003$; post hoc 120 to 220 μm, $P < 0.05$] of the apical tree. No consequences were observed in basal dendrites (fig. S3). Note that this atrophy was not generalized to all the regions of the frontal cortex. The orbitofrontal cortex (OFC), which is also a target of stress (*22*) and has been implicated in decision-making (*23*), showed a different pattern of change, with the most medial portions (medial orbital, MO) showing no alteration, whereas the most lateral regions (lateral orbital, LO) displayed a clear structural hypertrophy (fig. S4). In addition, no differences were found in the motor and somatosensory cortices (fig. S5).

We next examined the effects of chronic stress on the projection areas of these cortices into the dorsal striatum (DS), which has been previously implicated in controlling goal-directed and habitual strategies. We investigated more specifically the DMS, which receives input from the PL cortex (*24*) and has been implicated in goal-directed

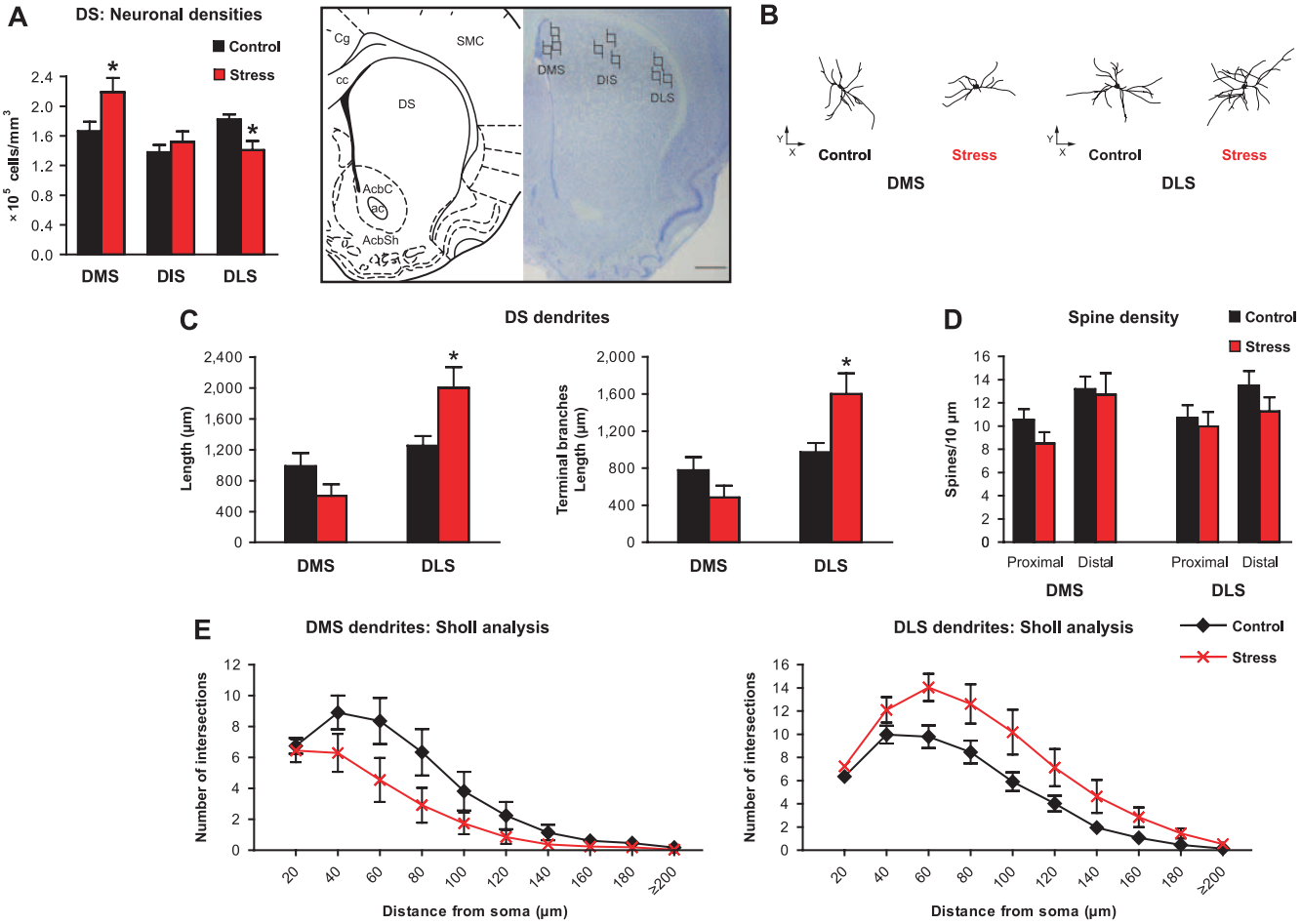

Fig. 4. Chronic stress induces opposing modulating effects in DMS and DLS networks. Several structural measurements of control and chronically stressed rats are compared. (**A**) (Left) Stereological estimation of neuronal densities. (Right) Sampling of the DMS, DIS, and DLS regions is illustrated; diagram was adapted from (*31*) and corresponding brain slice stained with Giemsa (1.00 mm from bregma). Abbreviations are as in Fig. 3. Scale bar, 800 μm. (**B** to **E**) Morphometric analysis in 3D of Golgi-stained MSNs [sampling following the same approach as for neuronal densities; for illustration, see (A)]. (B) Computer-assisted reconstructions of representative neurons depicted in the XY orthogonal plane. (C) Length, (D) spine density, and (E) differential rearrangement of dendrites. Error bars denote SEM. *$P < 0.05$.

actions (*11*), and the DLS or sensorimotor striatum, which is critical for habit formation (*12*) and receives input from the sensorimotor cortices (*24*) and, more laterally, from the LO cortex (*25*). Given the lack of precise anatomical landmarks delimiting these subregions in the DS, which could bias volumetric measures, we measured neuronal densities within the areas previously shown to be important for goal-directed and habitual behavior (Fig. 4A) (*11*–*13*) and found opposing effects of chronic stress in DMS and DLS. Neuronal density decreased in the DLS ($t_8 = 2.970$, $P = 0.018$) and increased in the DMS ($t_8 = -2.343$, $P = 0.047$) (Fig. 4A); these findings indicate atrophy of DMS and hypertrophy of DLS after stress exposure. These differences were not the result of generalized changes in the DS, because no differences in neuronal density were found in the intermediate area between medial and lateral regions (DIS: $t_8 = -0.802$, $P = 0.446$). To determine whether these changes in density were due to changes in dendritic arborization, we performed a 3D morphometric analysis of the medium spiny neurons (MSNs) within the same conservative limits for these DS subregions (Fig. 4, B, C, and E). We found a significant increase in dendritic arbors of DLS neurons [(Fig. 4C) length, $t_8 = -2.527$, $P = 0.035$; terminal branches length, $t_8 = -2.563$, $P = 0.033$; (Fig. 4E) $F_{1,8} = 5.016$, $P = 0.055$] and a nonsignificant trend toward a reduction in the dendrites in DMS neurons [(Fig. 4C) length, $t_8 = 1.682$, $P = 0.131$; terminal branches length, $t_8 = 1.550$, $P = 0.160$; (Fig. 4E) $F_{1,8} = 2.820$, $P = 0.132$] of stressed animals. No significant effects of stress were observed in spine density [(Fig. 4D) DMS: proximal, $t_8 = 1.504$, $P = 0.171$; distal, $t_8 = 0.221$, $P = 0.831$; DLS: proximal, $t_8 = 0.451$, $P = 0.664$; distal, $t_8 = 1.267$, $P = 0.241$] or morphology (fig. S2B). Taken together, the neuronal density and dendritic measures suggest a bidirectional modulation of neuronal connectivity in the DS expressed by a global hypertrophy of the DLS and shrinkage of the DMS.

The present results show a divergent structural reorganization of corticostriatal circuits after chronic stress, with atrophy of the associative corticostriatal circuits and hypertrophy of the circuits coursing through the sensorimotor striatum. This frontostriatal reorganization is accompanied by a shift toward habitual strategies, affecting the ability of stressed animals to perform actions based on their consequences. These data are consistent with previous studies showing that lesions of the PL cortex (*10*) and the DMS (*11*) can bias behavior to be more habitual, whereas inactivation of the DLS (*12*) can render the behavior of habitual animals goal-directed again, which suggest that competing corticostriatal circuits underlie the ability of animals to switch between these two modes of responding (*1*). Our results, using a natural model, indicate that the relative advantage of the sensorimotor network after chronic stress biases behavioral strategies toward habit and offer further insight into how chronic stress can lead to dysfunctional decision-making.

In addition to the role of the PL cortex (*10*), DMS (*11*), and DLS (*12*), the role of other brain regions affected by chronic stress in the behavioral bias herein described should be further investigated. For example, we did not observe changes in the sensorimotor cortices projecting to DLS but did find that the LO cortex, which also projects to the more lateral parts of the dorsal striatum (*25*), presents a clear hypertrophy. [The MO that projects to more medial striatal areas (*25*) does not.] Therefore, the role of the different subregions of the OFC in instrumental conditioning should be further explored, especially because although the atrophy of the PL cortex could contribute to the observed effects, the atrophy of IL cortex does not easily explain the bias toward habitual strategies, because lesions of this region have been shown to impair habit formation (*19*). Another possibility is that changes in the sensorimotor striatum relative to the associative striatum without parallel changes in the projecting cortices are sufficient to readily shift the behavioral strategies as training progresses. This is an interesting possibility given that more ventral striatal areas like the nucleus accumbens seem to have a more prominent role in appetitive Pavlovian responses than in control of instrumental behavior (*26*, *27*). Furthermore, a potential role of thalamic inputs to the sensorimotor striatum in mediating habitual strategies should not be discarded. Finally, the effects of chronic stress on the hippocampus (*8*) and amygdala (*28*) cannot easily explain the behavioral bias observed, because the early devaluation tests revealed that chronically stressed animals can learn action-outcome relations, and their behavior becomes biased as training progresses.

Optimization of decision-making processes confers an important advantage in response to a constantly changing environment. The ability to select the appropriate actions on the basis of their consequences and on our needs at the time of the decision allows us to respond in an efficient way to changing situations. However, the continuous control and attention that this process demands can result in an unnecessary expenditure of resources and can be inefficient in many situations. For instance, when behavior is repeated regularly for extensive periods without major changes in outcome value or contingency, or under uncertain situations where we cannot manipulate the probability of obtaining an outcome, general rules and habits can be advantageous (*3*). Thus, the more rapid shift to habits after chronic stress could be a coping mechanism to improve performance of well-trained behaviors, while increasing the bioavailability to acquire and process new information, which seems essential for adaptation to complex environments (*4*, *5*). However, when objectives need to be re-updated in order to make the most appropriate choice, the inability of stressed subjects to shift from habitual strategies to goal-directed behavior might be highly detrimental. Such impairment might be of relevance to understand the high comorbidity between stress-related disorders and addictive behavior or compulsivity (*29*, *30*), but certainly has a broader impact spanning activities from everyday life decisions to economics.

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32. We thank M. Carlos, L. Martins, and L. G. Pinto for technical assistance and T. Gremel, X. Jin, and P. Fitzgerald for comments on the manuscript. E.D.-F., J.C.S., and A.R.M. received fellowships from the Portuguese Foundation for Science and Technology. This work was supported by the Bial Foundation (134/06), the ICVS, and the Division of Intramural Clinical and Basic Research, NIAAA, NIH. The authors declare that they have no conflicts of interest.

Supporting Online Material

www.sciencemag.org/cgi/content/full/325/5940/621/DC1
Materials and Methods
Figs. S1 to S5
References

21 January 2009; accepted 24 June 2009
10.1126/science.1171203

Annex 4

Rodrigues AJ, Leão P, Pêgo JM, Cardona D, Carvalho MM, Oliveira M, Costa BM, Carvalho AF, Morgado P, Araújo D, Palha JA, Almeida OF, Sousa N (2011).

Mechanisms of initiation and reversal of drug-seeking behavior induced by prenatal exposure to glucocorticoids

Mol Psychiatry. 17(12): 1295-305.

Molecular Psychiatry (2011), 1–11

www.nature.com/mp

ORIGINAL ARTICLE

Mechanisms of initiation and reversal of drug-seeking behavior induced by prenatal exposure to glucocorticoids

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Stress and exposure to glucocorticoids (GC) during early life render individuals vulnerable to brain disorders by inducing structural and chemical alterations in specific neural substrates. Here we show that adult rats that had been exposed to *in utero* GCs (iuGC) display increased preference for opiates and ethanol, and are more responsive to the psychostimulatory actions of morphine. These animals presented prominent changes in the nucleus accumbens (NAcc), a key component of the mesolimbic reward circuitry; specifically, cell numbers and dopamine (DA) levels were significantly reduced, whereas *DA receptor 2* (*Drd2*) mRNA expression levels were markedly upregulated in the NAcc. Interestingly, repeated morphine exposure significantly downregulated *Drd2* expression in iuGC-exposed animals, in parallel with increased DNA methylation of the *Drd2* gene. Administration of a therapeutic dose of L-dopa reverted the hypodopaminergic state in the NAcc of iuGC animals, normalized *Drd2* expression and prevented morphine-induced hypermethylation of the *Drd2* promoter. In addition, L-dopa treatment promoted dendritic and synaptic plasticity in the NAcc and, importantly, reversed drug-seeking behavior. These results reveal a new mechanism through which drug-seeking behaviors may emerge and suggest that a brief and simple pharmacological intervention can restrain these behaviors in vulnerable individuals.
Molecular Psychiatry advance online publication, 4 October 2011; doi:10.1038/mp.2011.126

Keywords: DNA methylation; dopamine receptor 2; levodopa; nucleus accumbens; mesolimbic circuit; prenatal glucocorticoids

Introduction

Stressful events during critical developmental periods have long been considered as etiological factors in psychiatric disorders such as schizophrenia, depression and drug-seeking behavior.[1–4] The programming effects of stress are most likely mediated by endogenous glucocorticoids (GC), whose ability to produce structural re-organization and dysfunction of the neural substrates that underpin these stress-related pathologies are well known.[1,5–7] Although administration of prenatal GC does not mimic prenatal stress, synthetic GC such as dexamethasone (DEX) are widely used in obstetrics, for example, to ensure fetal lung maturation during late pregnancy in humans.[8] DEX is not biodegraded in the same way as its naturally occurring congeners, and crosses the maternal-placental barrier to a greater extent than endogenous GC;[9,10] it can thus pose additional risk for the developing brain.

We previously demonstrated that fetal exposure to GC leads to hyper-emotionality in adulthood.[11] In addition, we showed that prenatal DEX/GC targets the mesolimbic dopaminergic system;[12] this system comprises projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc) and is strongly implicated in motivational and reward aspects of addictive behaviors.[13–15] Specifically, the NAcc of adult rats exposed to GC *in utero* (iuGC) display reduced neuronal numbers and fewer dopamine (DA) inputs from the VTA.[12] Further, early life stress is known to influence DA receptor expression in the adult NAcc[16,17] and changes associated with increased behavioral responses to stress and cocaine.[1,4,18,19] Together, these observations suggest that prenatal exposure to elevated levels of GC can program the mesolimbic circuit. In the present study, a multimodal analysis was used to further define the molecular neurobiological mechanisms that underlie the initiation and reversibility of drug-seeking behavior by prenatal exposure to GC.

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Received 13 April 2011; revised 1 August 2011; accepted 30 August 2011

Materials and methods

Animals and behavioral tests
Pregnant Wistar rats were individually housed under standard laboratory conditions (light/dark cycle of 12/12 h with lights on at 08:00 h; 22 °C); food and water were provided *ad libitum*. Subcutaneous (s.c.) injections of DEX at 1 mg kg^{-1} (DEX; iuGC animals) or saline (control) were administered on gestation days 18 and 19. All manipulations were done in accordance with the local regulations (European Union Directive 2010/63/EU) and NIH guidelines on animal care and experimentation.

Male offspring ($n \geqslant 8$) derived from four different litters were subjected to behavioral tests when they were 3–4 months old.

Open field
Locomotor behavior was investigated using the open-field test. Briefly, rats were placed in the center of an arena (MedAssociates, St Albans, VT, USA) and their ambulation was monitored online over a period of 15 min. Total distances traveled were used as indicators of locomotor activity. Animals were injected with saline or morphine and tested 30 min after injection.

Conditioned place preference (CPP)
The place preference apparatus consisted of two compartments with different patterns on floors and walls, separated by a neutral area (MedAssociates). Animals were placed in the central neutral area and allowed to explore both compartments, allowing definition of the preferred compartment (day 1). During the conditioning phase (day 2–4), rats were confined to the pre-test preferred compartment for 20 min after saline injection (1 ml kg^{-1}, s.c.) and, after a 6-h gap, to the other compartment for 20 min after injection of morphine (10 mg kg^{-1}, s.c.). CPP was assessed on day 5 (20 min) when all compartments were accessible to the animal. Results are expressed as the difference of time spent in the drug-paired to saline-paired side.

Ethanol consumption
The two-bottle choice protocol was carried out for 15 days as described previously.[20] Briefly, after 3 days of taste habituation (one bottle with 10% ethanol and other with 5% sucrose), rats were offered both bottles. Each bottle was weighted daily; bottle positions were changed every day to control for position preference. Corrections were made for daily evaporation and spillage.

Cross-fostering and maternal behavior
For cross-fostering experiments, litters from five control and five DEX-treated mothers were exchanged on postnatal day 1. Maternal behavior was assessed every second day, over a period of 30 min. Both, pup-directed (nursing, non-nutritive contact, licking and nest building) and self-directed (self-grooming, resting, vertical activity and carrying) behaviors were registered.

Drugs
Morphine hydrochloride (Labesfal Pharmaceutical, Campo de Besteiros, Portugal) was administered s.c. at a dose of 10 mg kg^{-1}; sesame oil was used as the vehicle. L-dopa/carbidopa (Sinemet, Merck, NJ, USA) at a dose of 36.0/9.0 mg/kg (in water) was administered daily by oral gavage.

Tyrosine hydroxylase (TH) immunohistochemistry
Animals were deeply anesthetized and transcardially perfused with 4% paraformaldehyde. Cerebral hemispheres were separated by a longitudinal cut in the midsagittal plane. Sections of 30 μm were treated with 3% H_2O_2 and blocked with 4% bovine serum albumin in phosphate-buffered saline. Sections were then incubated overnight at 4 °C with rabbit anti-TH serum (1:2000; Affinity Reagents, CO, USA). Antigen visualization was carried out by sequentially incubating with biotinylated goat anti-rabbit antibody, ABC1 (Vector, Burlingame, CA, USA) and diaminobenzidine (DAB, Sigma, St Louis, MO, USA). The density of TH-positive fibers impinging upon the NAcc was estimated as previously described.[12]

Structural analysis
Rats were transcardially perfused with 0.9% saline under deep pentobarbital anesthesia and processed as described previously.[21] Briefly, brains were removed and immersed in Golgi–Cox solution[22] for 14 days; brains were then transferred to a 30% sucrose solution (7 days), before being cut on a vibratome. Coronal sections (200 μm thick) were collected and blotted dry onto cleaned, gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Rochester, NY, USA), fixed in Kodak Rapid Fix (prepared as per package instructions with solution B omitted), dehydrated through a graded series of ethanols, cleared in xylene, mounted and coverslipped. For each selected neuron, all branches of the dendritic tree were reconstructed at × 600 magnification, using a motorized microscope with oil objectives (Axioplan 2, Carl Zeiss, Thornwood, NY, USA) that was attached to a camera (DXC-390, Sony, Tokyo, Japan) and Neurolucida software (Microbrightfield, Williston, VT, USA). A 3D analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield). Twenty neurons were studied in each animal, and results from the same animal were averaged. To assess differences in the arrangement of dendritic material, a 3D version of a Sholl analysis[23,24] was performed. For this, we counted the number of intersections of dendrites with concentric spheres positioned at radial intervals of 20 μm; in addition, we also measured dendritic tree lengths located between two consecutive spheres. The method for sampling dendritic branches for spine density was designed as follows: only branches that (1) were either parallel or at acute angles to the coronal surface of the section and (2) did not show overlap with other branches that would obscure visualization of spines

were considered. Because treatment-induced changes in the apical dendritic branches varied with distance to soma, segments were randomly selected in the proximal parts of the tree; selection of basal dendrite was done at radial distances between 50 and 100 μm. To assess treatment-induced changes in spine morphology, spines in the selected segments were classified according to Harris *et al.*[25] in mushroom, thin, wide and ramified categories. Thin spines were considered immature, whereas the other spine types were considered to be mature spines.

Macrodissection
Animals were anesthetized, decapitated, and heads were immediately snap-frozen in liquid nitrogen. Brain areas of interest were rapidly dissected on ice under a stereomicroscope, observing anatomical landmarks. Samples were snap-frozen (dry ice) and stored at −80 °C until use.

Neurochemical evaluation
Levels of catecholamines were assayed by high-performance liquid chromatography, combined with electrochemical detection (HPLC/EC) using a Gilson instrument (Gilson, Middleton, WI, USA), fitted with an analytical column (Supleco Supelcosil LC-18 3 μM, Bellefonte, PA, USA; flow rate: 1.0 ml min^{-1}). Samples were stored overnight in 0.2 N perchloric acid at −20 °C, sonicated (5 min on ice) and centrifuged at 5000 *g*. The resulting supernatant was filtered through a Spin-X HPLC column (Costar, Lowell, MA, USA) to remove debris and 150 μl aliquots were injected into the HPLC system, using a mobile phase of 0.7 M aqueous potassium phosphate (pH 3.0) in 10% methanol, 1-heptanesulfonic acid (222 mg l^{-1}) and Na-EDTA (40 mg l^{-1}). A standard curve using known concentrations of all catecholamines was run each day.

Molecular analysis
For real-time PCR analysis, total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) and DNase treated (Fermentas, Burlington, Canada) following recommended protocols. Two μg of RNA was converted into cDNA using the iSCRIPT kit (Biorad, Hercules, CA, USA). Reverse transcription PCR was performed using Quantitec SyberGreen (Qiagen, Venlo, The Netherlands) and the Biorad q-PCR CFX96 apparatus. *Hprt* was used as a housekeeping gene. Relative quantification was used to determine fold changes (control vs iuGC), using the ΔΔCT method. Primer sequences are shown in Supplementary Table 1.

For western blotting procedures, ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, complete protease inhibitors (Roche, Basel, Switzerland)) was added to each frozen area. After disruption of the tissue using a 23G needle, 0.1% SDS and 1% Triton X-100 was added to each sample. After incubation on ice for 1 h, samples were centrifuged at 13 000 r.p.m. for 10 min at 4 °C; the supernatant was quantified using the Bradford method. Forty μg of total protein was loaded into SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. After incubation with the primary antibodies: rabbit anti-Dopamine receptor D1 (1:2500, ab20066, Abcam, Cambridge, UK), rabbit anti-Dopamine receptor D2 (1:2000, ab21218, Abcam) and mouse anti-alpha-tubulin (1:200, DSHB, Iowa, USA); the secondary antibodies were incubated at a 1:10 000 dilution (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Detection was done using ECL kit (Pierce, Rockford, IL, USA). Band quantification was performed using ImageJ (http://rsbweb.nih.gov/ij/) as advised by the software manufacturers, using α-tubulin as the loading control. At least six animals per group were analyzed.

For epigenetic analysis, four animals per group were analyzed. Genomic DNA of 2 μg were bisulfite-converted (EZDNA Methylation Kit, Zymo Research, Irvine, CA, USA) and amplified with primers CpG-Drd2_F and CpG-Drd2_R (designed using Methprimer), using AmplitaQ Gold (Applied Biosystems, Carlsbad, CA, USA). Bands were purified using innuPREP Gel extraction kit (Analytik Jena, Jena, Germany). After elution, 2 μl of product were used in a TOPO cloning reaction (Invitrogen) following recommended procedures. XL1-blue competent cells were transformed with the TOPO reaction and plated onto LB–50 μg ml^{-1} kanamycin plates, suplemented with X-GAL (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside). A total of 10 clones were isolated per animal; plasmid DNA was purified using innuPREP Plasmid Mini Kit. Plasmids were sequenced using standard M13 primers.

Results

In utero *GC exposure triggers increased drug-seeking behavior in adulthood*
To test the hypothesis that prenatal GC exposure would increase drug preference, we compared all experimental groups in a CPP paradigm. As compared with controls, iuGC-treated animals developed a stronger preference for morphine, spending more time in the compartment previously associated with morphine reward (Figure 1a; $t = 4.623$, $P = 0.0036$). Whereas control and iuGC animals did not differ in their intake of sucrose solution (Supplementary Figure S1), iuGC animals demonstrated an approximately two-fold greater preference than controls for ethanol in a two-bottle free-choice paradigm over a period of 2 weeks (Figure 1b; $t = 3.523$, $P = 0.0048$). As locomotor activity is considered to predict susceptibility to drug abuse,[1,26] it was interesting to note that morphine stimulated locomotor activity (open-field arena) to a greater extent in iuGC animals than in controls (~160% vs ~35%; $F_{(3,15)} = 67.94$, $P < 0.0001$; Figure 1c). To exclude the potentially confounding effects of inadequate maternal care, itself a suspected etiological factor in stress-related psychiatric disorders,[27–29] we analyzed the maternal behavior of control and GC-treated dams, and also performed a

cross-fostering experiment. Neither self- nor pup-directed behaviors were significantly influenced by GC treatment (Supplementary Figure S2). Identical behaviors were observed when iuGC offspring raised by natural and fostered mothers were compared in the CPP (Figure 1a; $t=6.877$, $P<0.0001$) or ethanol consumption (Figure 1b; $t=12.58$, $P<0.0001$) tests. Although the hypolocomotor profile observed in non-fostered iuGC animals in the open field test was not seen in cross-fostered iuGC rats (Figure 1c), morphine elicited a hyperlocomotor response in both cross-fostered and non-fostered iuGC animals as compared with control rats raised by foster mothers (Figure 1c; $t=2.737$, $P=0.021$). Collectively, these findings indicate that exposure to prenatal GC increases vulnerability to drug-seeking behavior.

Morphological and neurochemical changes in the NAcc after in utero *GC exposure*

Increased sensitivity to the psychomotor-stimulatory actions of drugs such as morphine reflects increased DA release into the NAcc.[1,26] Furthermore, the dopaminergic system seems particularly sensitive to the effects of GCs.[5,12,30] Thus, we next assessed the impact of prenatal GC upon the number of TH-positive fibers, DA and DA metabolite levels, as well as DA turnover in the NAcc (Figure 2). The number of TH-positive fibers in both the core and shell divisions of the NAcc were significantly reduced in iuGC animals (Figure 2a, shell: $t=2.827$, $P=0.022$; Figure; core: $t=10.48$, $P<0.0001$; Supplementary Figure S3), in parallel with markedly reduced NAcc levels of DA ($t=2.567$, $P=0.0247$) and the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC; $t=2.362$, $P=0.0376$; Figure 2c); interestingly, the levels of norepinephrine and epinephrine, two other catecholamine transmitters whose synthesis indirectly depends on TH, as well as of the unrelated monoamine serotonin (5-HT), were not affected by prenatal GC exposure. Importantly, besides the reduced availability of DA in the NAcc, iuGC-treated animals also displayed increased DA turnover (Figure 2d; $t=2.835$, $P=0.0196$). Moreover, as no remarkable neurochemical changes were observed in the VTA or other DA projection fields (prefrontal cortex, hippocampus; data not shown), the

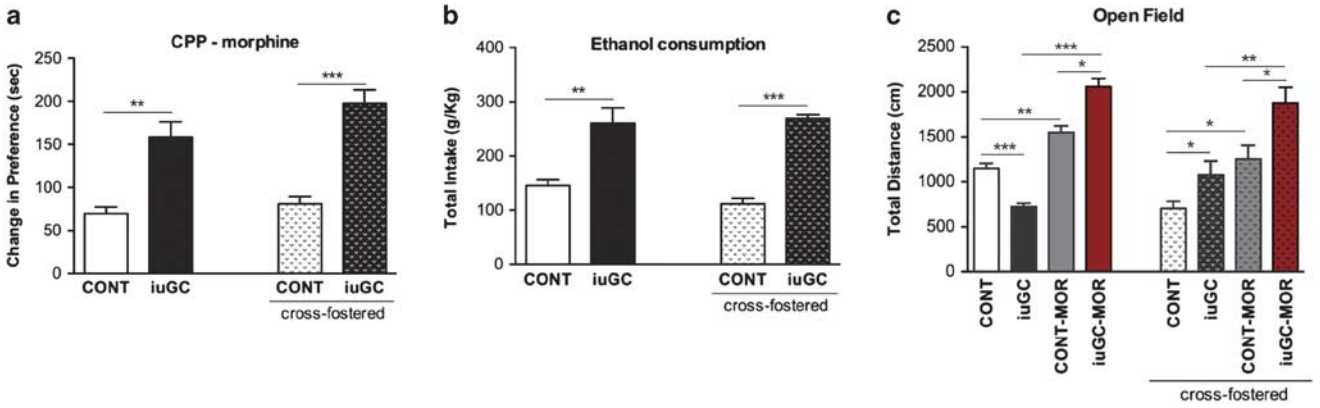
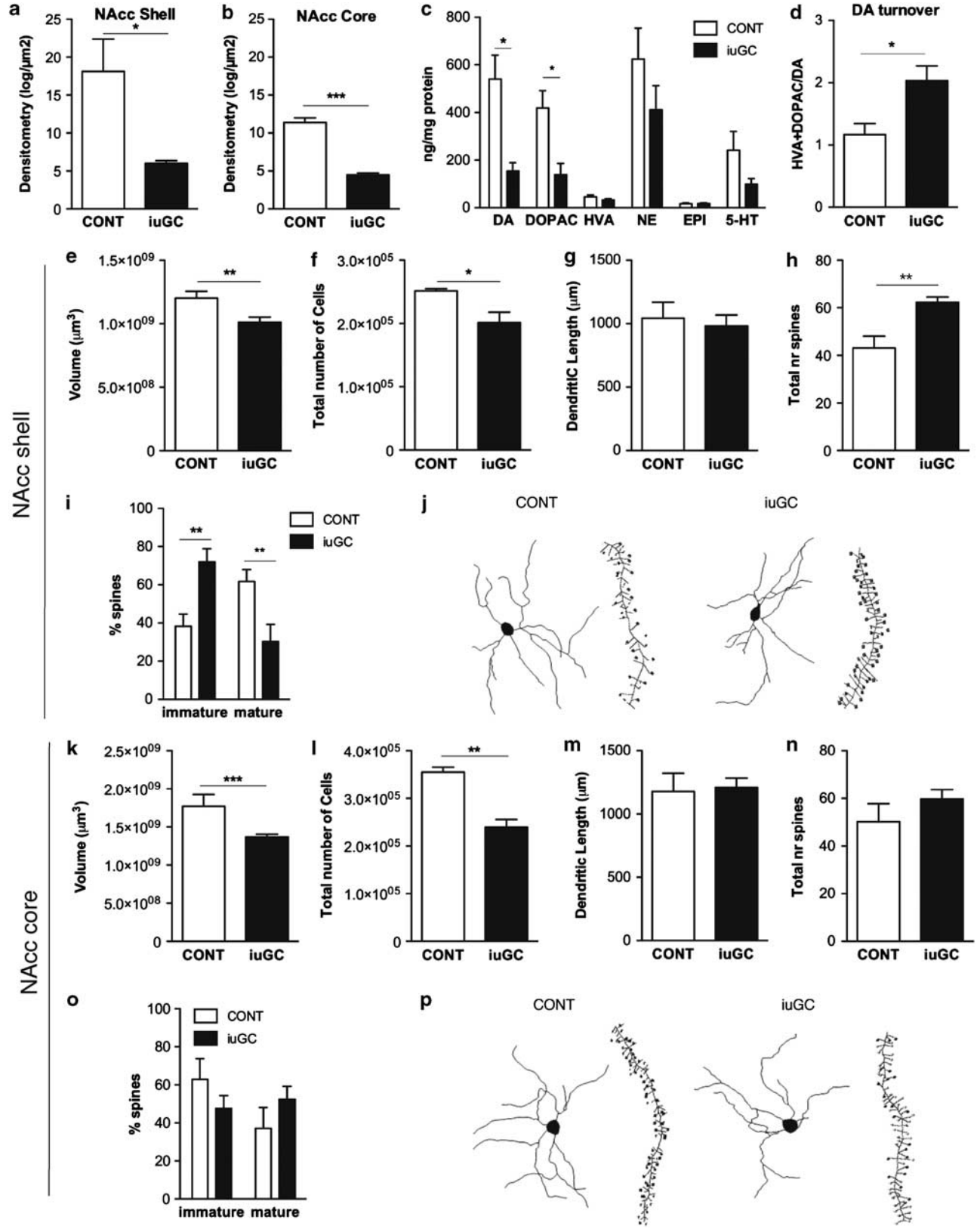


Figure 1 Prenatal *in utero* glucocorticoid (iuGC) exposure enhances drug-seeking behaviors. (**a**) In the contingent conditioned place preference paradigm (CPP), iuGC animals spend significantly more time in the morphine-associated compartment than controls. (**b**) In the non-contingent two-bottle preference paradigm, total ethanol consumption was higher in iuGC animals than in controls. Similar results were obtained for cross-fostered animals in both paradigms. (**c**) Locomotor activity was assessed in the open field. Although in basal conditions, iuGC animals presented reduced locomotor activity, after morphine administration (MOR), iuGC rats displayed increased locomotor activity when compared with controls. Cross-fostered iuGC-animals no longer present the basal hypolocomotor phenotype, but after MOR, they still presented increased locomotor activity. Data is presented as mean ± s.e.m. CONT, controls; MOR, morphine (10 mg kg^{-1}) s.c. injection. $^{*}P<0.05$, $^{**}P<0.01$,*** $P<0.001$.

Figure 2 Prenatal glucocorticoid (GC) reorganizes dopaminergic innervation and dendritic structure in the nucleus accumbens (NAcc). *In utero* GC-exposed (iuGC) animals presented reduced tyrosine hydroxylase (TH)-positive fibers in the shell (**a**) and core (**b**) subdivisions of the NAcc when adults. (**c**) High-performance liquid chromatography (HPLC) measurements confirmed reduced levels of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA) in the NAcc of iuGC animals in comparison with controls in parallel with increased turnover of DA in this brain region (**d**). Stereological assessment revealed a volumetric atrophy (**e**) in the NAcc shell in iuGC animals together with reduced number of cells (**f**). We observed no changes in dendritic length (**g**), but there was an increase in the total number of spines in the medium spiny neurons of iuGC animals when compared with controls (**h**), as a result of increased number of immature spines (**i**). (**j**) Representative reconstruction of medium spiny neurons of NAcc shell in control and iuGC animals. The NAcc core of iuGC animals also presented volumetric atrophy (**k**) and reduced number of cells (**l**), but preserved dendritic length; spine numbers and mature/immature spine ratio (**m–o**). (**p**) Representative reconstruction of a medium spiny neuron from NAcc core in control and iuGC animals. Data is presented as mean ± s.e.m. CONT, controls; NE, norepinephrine; EPI, epinephrine; 5-HT, serotonin. $^{*}P<0.05$, $^{**}P<0.01$,*** $P<0.001$.

NAcc is seemingly most sensitive to the effects of prenatal GC.

Extending our previous finding that prenatal GC treatment leads to reduced neuronal proliferation in the NAcc,[12] we now report that iuGC results in volumetric atrophy (Figure 2e, shell: $t=4.340$, $P=0.0025$; Figure 2k, core: $t=5.906$, $P=0.0004$) and a reduction of total cell numbers in both the shell and



core divisions of the NAcc in iuGC adult animals (Figure 2f, shell: $t=3.018$, $P=0.0166$; Figure 2l, core: $t=3.760$, $P=0.0055$). Subsequent 3D morphological analysis of dendrites and spines showed that whereas prenatal GC did not influence dendritic lengths of neurons in the NAcc (Figure 2g and m), the treatment produced significant increases in the number of spines within the shell (Figure 2h; $t=3.775$, $P=0.0069$), but not the core division (Figure 2n). The increase in spine number was accompanied by a significant increase in the relative number of immature spines in the shell (Figure 2i; $t=3.108$, $P=0.017$), which, presumably, serve to compensate for the loss of cells in the NAcc and for the reduced amounts of DA reaching the NAcc from the VTA. Notably, although iuGC treatment was associated with increased total spine numbers in the VTA, the treatment did not alter the ratio of immature to mature spines in this region (Supplementary Figure S4). These morphological data, together with the neurochemical data described above, suggest a link between a hypodopaminergic state in the NAcc and the behavioral phenotype observed in animals exposed to prenatal GC.

Altered expression of DA receptor 2 *(*Drd2*) is associated with differential methylation of* Drd2 *gene in iuGC-treated animals*

We next used quantitative reverse-transcription PCR and immunoblotting to identify molecules that might be responsible for the observed behavioral, morphological and neurochemical phenotypes. Expression levels of the mRNAs encoding the GC receptor and corticotropin releasing factor receptors 1 and 2 (all implicated in the neuroendocrine adaptation to stress as well as in drug-seeking behavior[1]), did not differ between controls and iuGC subjects (Supplementary Figure S5). Likewise, no significant differences were found in the expression levels of the synaptic plasticity-related genes *Bdnf*, *synapsin-1*, *Cdk5*, *Creb* and *NCAM* (Supplementary Figure S5). However, there was a significant upregulation of *Drd2* mRNA (Figure 3a; $t=2.764$, $P=0.028$) and DRD2 protein (Figures 3b and c; 35 kDa precursor, $t=3.740$, $P=0.0028$; 47kDa isoform, $t=3.372$, $P=0.005$; 72 kDa glycosylated DRD2, $t=2.177$, $P=0.050$) in the NAcc of iuGC animals. Prenatal GC exposure did not influence either *Drd1* or *Drd3-5* mRNA expression levels (Figure 3a) or the levels of DRD1 protein (50 kDa and glycosylated 74 kDa isoforms; Supplementary Figure S5). In the VTA of iuGC animals, *Drd5* levels were downregulated (Supplementary Figure S5), but the expression of other DA receptors was unchanged (data not shown).

Strikingly, repeated exposure to morphine and ethanol in prenatal GC-treated adult rats led to a significant decrease in the expression of *Drd2* mRNA in the NAcc (Figure 3d; morphine: $t=2.346$, $P=0.043$; ethanol: $t=3.330$, $P=0.0021$). As recent studies reported that psychostimulant treatment induces epigenetic changes in the NAcc,[31–33] we next analyzed the pattern of methylation (strongly correlated with transcriptional repression) in a conserved (human and rodent) CpG island within the *Drd2* gene, covering part of the promoter region and exon 1 (Figure 3e). Our analysis shows that whereas the general DNA methylation profile did not differ between controls and iuGC subjects under basal conditions, overall methylation of the CpG island was significantly increased after chronic morphine administration in adult iuGC-treated animals (Figure 3f–h; $t=3.085$, $P=0.0215$). These changes in DNA methylation are consistent with the finding that *Drd2* expression is downregulated after morphine treatment (Figure 3d). Further, the observation that voluntary ethanol consumption (Figure 3d) also downregulates *Drd2* suggests *Drd2* DNA methylation as a potentially important mechanism in response to substances of abuse.

Restoration of DA levels reverts the molecular, cellular and behavioral phenotype of iuGC animals

The results presented up to this point indicate a strong association between the hypodopaminergic state that prevails in the NAcc of iuGC-exposed subjects and their likelihood to seek drugs of abuse. We next examined whether the phenotype produced by iuGC could be rescued using a simple pharmacological approach. To this end, we administered the DA precursor L-dopa (together with carbidopa to prevent peripheral degradation) for 3 days. This treatment regimen resulted in concomitant increases in DA levels (Figure 4a; $F_{(3,21)}=23.79$, $P<0.0001$) and correspondingly, decreases in *Drd2* expression (Figure 4c; $t=2.982$, $P=0.038$) in the NAcc of controls and iuGC-treated animals. Interestingly, the dynamic *Drd2* response to morphine was normalized after restoration of DA in the NAcc by L-dopa treatment, with iuGC-treated and control animals showing similar patterns of *Drd2* mRNA expression (Figure 4c) and *Drd2* promoter methylation (Figure 4d–f). Interestingly, the neurochemical adjustments induced by L-dopa were accompanied by signs of structural plasticity in the NAcc. These were particularly marked in the core division of the NAcc, where L-dopa-treated animals displayed increased dendritic lengths (more pronounced in iuGC-exposed animals; Figure 4j; $F_{(3,12)}=4.587$, $P=0.023$) and spine numbers (Figure 4k; $F_{(3,12)}=10.01$, $P=0.0014$), though the type of spines were similar between the two groups (Figure 4l). In contrast, increased spine numbers was the only noticeable morphological change observed in the NAcc shell (Figure 4h; $F_{(3,10)}=14.86$, $P=0.0005$).

Remarkably, acute (3 days) L-dopa treatment also reversed the vulnerability of iuGC-exposed animals to drug-seeking behaviors, in both contingent ($t=1.851$, $P=0.101$) and non-contingent ($t=0.0192$, $P=0.985$) paradigms (Figures 4m and n), and rescued the hyperlocomotor phenotype displayed by iuGC-treated animals after morphine administration (Figure 4o; $t=2.292$, $P=0.05$). Reversal of these behaviors by

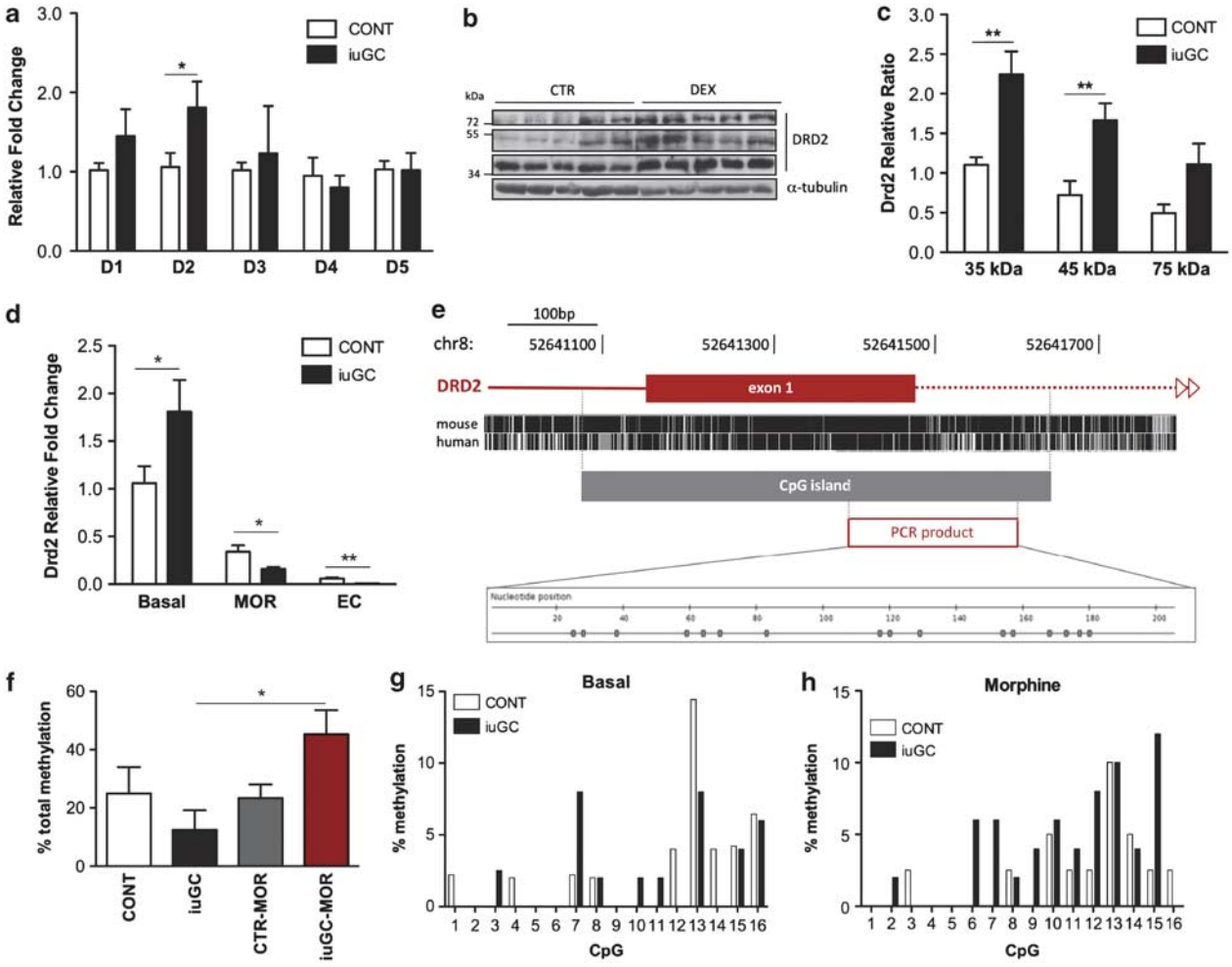


Figure 3 Impaired *dopamine receptor 2* (*Drd2*) response in *in utero* glucocorticoid-exposed (iuGC) animals under basal conditions and after exposure to substances of abuse. (**a**) *Drd2* mRNA expression was augmented in iuGC animals when compared with controls, but no changes were found in the expression of other dopamine receptors. (**b**) Representative immunoblot of DRD2 in five control and five iuGC animals. The levels of the putative DRD2 percursor (35 kDa), the non-glycosylated form (~50 kDa) and the glycosylated receptor (74 kDa) were higher in iuGC animals (**c**). (**d**) Although in a basal situation, *Drd2* was upregulated in iuGC animals, after four injections of morphine (MOR) or 15 days of ethanol consumption (EC), the levels of this receptor were significantly lower in iuGC animals when compared with controls. (**e**) Scheme of the rat *Drd2* CpG island that covers part of the promoter, and exon 1 and respective amplicon with the 16 potential methylation sites are marked (small squares). Also shown is the sequence conservation in humans and mouse (chr8: rat chromosome 8; bp: base pairs). (**f**) Percentage of total *Drd2* CpG methylation in the NAcc of control and iuGC animals revealed a trend for a reduction in the methylation pattern of *Drd2* CpG island in basal conditions, but in opposite pattern after exposure to morphine. (**g**) Percentage of methylation of each dinucleotide in the *Drd2* CpG island in a basal situation. (**h**) After drug exposure, iuGC animals presented an increase in the methylation status of several dinucleotides. Data is presented as mean ± s.d. CONT, controls; $*P<0.05$, $**P<0.01$.

acute L-dopa administration however proved to be only transient; the reversal was not sustained when animals were tested 3 weeks after the last dose of L-dopa (Supplementary Figure S6). On the other hand, when the L-dopa treatment regimen was extended to 3 weeks, reversal of the behavioral, morphological and molecular anomalies associated with a hypodopaminergic state was observable for at least 3 weeks after discontinuation of the drug (Supplementary Figure S6).

Discussion

Work over the last two decades has identified the dopaminergic mesolimbic 'reward pathway,' of which the NAcc is a crucial component, as essential for drug-seeking behaviors.[13,14,34,35] The central role of DA released into the NAcc in the generation of enhanced feelings of pleasure and satisfaction[15] and, thus, in the timing of the initiation of response patterns (e.g., drug-seeking behavior) within the frontocortico-

striatal loop,[36] is well established. Current views suggest that repetitive exposure to drugs of abuse evolve from goal-directed behaviors into habit-based actions.[37,38] We previously demonstrated that stress, associated with increased GC secretion, alters the structure of the corticostriatal loops and steers the development of instrumental behavior into habitual behavior.[39] The present demonstration of GC-induced programming of the structure and function of the NAcc provide, on the other hand, new insights into the mechanisms that underlie the transfer of conditioned behavior to instrumental behavior. Notably, the NAcc (the core in particular) is a crucial determinant of the efficiency of response-outcome associative learning[40] and thus, of the rewarding effects of drugs of abuse;[34] the NAcc modulates motivational drive ('wanting of a reward') and thus, drug-craving. In all these processes, DA seems to have an essential role.

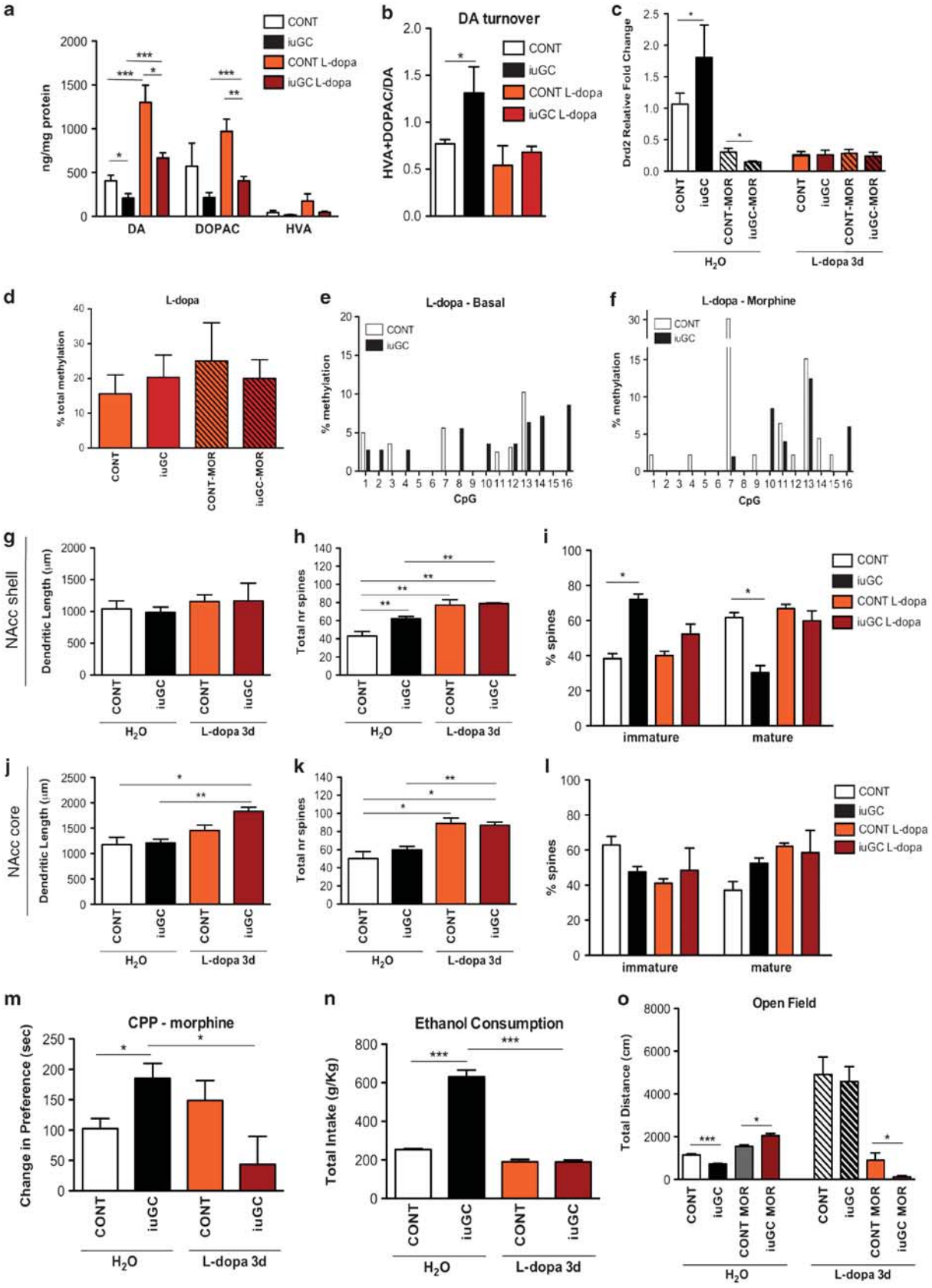
An intricate relationship between stress, the GC released in response to stress, and dopaminergic tone in the regulation of vulnerability to drug and substance abuse has been suggested.[1,5,14,26,41] Stress and drugs of abuse appear to activate dopaminergic synapses in a similar manner,[41] culminating in DA release in the NAcc.[1,4,42] Stress induces sensitization to the psychomotor effects of a number of drugs of abuse and GC have been shown to have an essential role in this process.[1] Specifically, GC are known to modulate the reinforcing properties of drugs and, in fact, have positive reinforcing properties of their own.[43] Adding a new perspective, the present study demonstrates that iuGC triggers an impoverishment in dopaminergic inputs and DA levels in the NAcc, leading to increased drug-seeking behavior in adulthood; notably, hypodopaminergic status is a hallmark of the 'addicted brain.'[44,45] Associated with their lower intra-NAcc levels of DA, animals exposed to prenatal GC expressed more *Drd2* in the NAcc, potentially indicating a compensatory mechanism in this structure. The finding that morphine and ethanol downregulated *Drd2* expression is consistent with the DA-releasing abilities of these substances. The fact that this downregulation is more pronounced in iuGC subjects most likely reflects receptor hypersensitivity due to the hypodopaminergic state previously induced by iuGC.

The regulation of *Drd2*, implicated in different phases of addiction, is seemingly complex;[44] although the short DRD2 isoform interacts with DA transporters and functions as a presynaptic autoreceptor to regulate dopaminergic tone, the long DRD2 isoform is largely localized in postsynaptic targets and mediates the effects of psychostimulants.[46] The present study reveals that vulnerability to substance abuse depends on the dynamic range response of *Drd2* to increased DA release in the NAcc, rather than simply on the expression of *Drd2* at a given time point. Such dynamic regulation is likely to depend on different levels of transcriptional control.

Epigenetic mechanisms are being increasingly implicated in the stable programming by early life events of a spectrum of psychopathological states, including anxiety and depression,[29] impaired cognition[47] and drug abuse,[31–33,48,49] and transient epigenetic modifications have been shown to underlie neural processes such as learning and memory.[50] Such epigenetic changes could imprint dynamic environmental experiences on the unchanging genome, resulting in stable and adaptive alterations in the phenotype. Our results demonstrate that exposure to high GC levels during uterine development increase the risk of drug-seeking behavior in association with altered methylation status of a conserved CpG island in *Drd2* gene and therefore, interfering with the dynamics of *Drd2* expression. Further, they show that repeated administration of morphine to iuGC animals results in marked epigenetic modifications of the *Drd2* gene promoter. These modifications, together with the induced hypodopaminergic state in iuGC-exposed animals, may be considered as key mechanisms that underpin increased susceptibility to drug abuse on one hand, and the dysregulated *Drd2* response to drugs of abuse on the other.

Intriguingly, we found that reduced levels of *Drd2* expression are not necessarily coupled to hypermethylation of *Drd2* gene. Although *Drd2* expression was downregulated after morphine administration in

Figure 4 Restoration of dopamine (DA) levels by L-dopa reverts the molecular, structural and behavioral phenotypes of *in utero* glucocorticoid (iuGC) animals. (**a**) Acute (3 days) treatment with L-dopa increased DA levels in the nucleus accumbens (NAcc) of both experimental groups; although iuGC animals still exhibited less DA than controls. In fact, iuGC animals given L-dopa presented DA levels similar to those of controls without treatment. (**b**) No differences were found in DA turnover after L-dopa treatment in iuGC animals. (**c**) *Dopamine receptor 2* (*Drd2*) expression was diminished after L-dopa treatment both in a basal situation and after morphine exposure (values normalized to controls given water). (**d**) L-dopa treatment did not change *Drd2* methylation status in a basal situation (**e**), but was able to revert the increased methylation in iuGC animals after morphine exposure (**f**). L-dopa supplementation had no significant effect on NAcc shell dendritic length (**g**), but triggered an increase in the number of spines, albeit similarly in control and iuGC animals, and reverted the altered ratio of mature to immature spines observed in iuGC animals (**h** and **i**). (**j**) In contrast, L-dopa treatment increased dendritic length in the NAcc core of both groups. An increase in the number of spines was also observed in both groups with no changes in the type of spines (**k** and **l**). (**m**) L-dopa treatment reverted the higher vulnerability of iuGC animals to morphine-induced CPP and also reverted the ethanol preference displayed by these animals (**n**). (**o**) In agreement, the higher locomotor pattern after morphine displayed by iuGC rats was completely reverted by L-dopa treatment. No differences were found in the locomotion between L-dopa treated control and iuGC animals in a basal situation. Data is presented as mean ± s.e.m. CONT, controls; MOR, after morphine injection 10 mg kg^{-1}; 3d: 3 days; $^{*}P<0.05$, $^{**}P<0.01$,$^{***}P<0.001$.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both control and iuGC animals, *Drd2* methylation was observed to a greater extent in the iuGC group. This observation suggests that DNA methylation is not the sole mechanism involved in transcriptional repression of *Drd2* gene. Consistent with this, recent studies have demonstrated interdependence and cooperation between DNA methylation and histone modifications in the regulation of gene silencing and activation.[51] More extensive studies are needed to decipher the precise mechanisms underlying the 'epigenetic potential' of iuGC animals, namely the complex regulation of *Drd2* gene expression, which facilitates adaptation to specific physiological states and demands.

In exploring whether the dynamic epigenetic mechanisms that regulate susceptibility to drug-seeking behavior can be exploited in a therapeutic context, we found that systemic administration of L-dopa reverts drug-seeking behavior in iuGC-treated animals. The latter occured in association with morphological plasticity and significant decreases in *Drdr2* expression levels in the NAcc. Accordingly, we suggest that susceptibility to drug-seeking behavior by iuGC exposure results from the sequential depletion of DA, upregulation of *Drd2* and synaptic impoverishment of dopaminoceptive neurons in the NAcc (Supplementary Figure S7). In this scenario, when DA levels are stimulated by substances of abuse, increased methylation of the *Drd2* gene results in downregulation of *Drd2* expression albeit only in iuGC animals. Strikingly, restoration of DA in the NAcc of iuGC-treated animals also normalizes their *Drd2* responses to subsequent morphine and ethanol exposure, a finding that most likely underlies the above-mentioned reversion of drug-seeking behavior. If translatable to humans, our findings suggest that a simple reinstatement of dopaminergic homeostasis may be sufficient to control addictive behaviors in vulnerable individuals.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We would like to thank the members of the Neuroscience Research Domain at ICVS for all the helpful discussions and suggestions. We are especially thankful to the animal facility caretakers, and to Drs Sara Silva, António Melo and Ana Paula Silva and Dieter Fischer for their help. This work was supported by the Institute for the Study of Affective Neuroscience (ISAN). AJR, BC and MC were supported by Fundação para a Ciência e Tecnologia (FCT) fellowships.

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