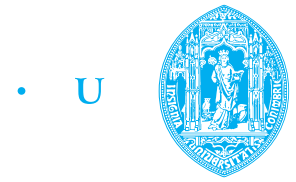

UNIVERSIDADE DE COIMBRA

DEPARTAMENTO DE CIÊNCIAS DA VIDA • FACULDADE DE CIÊNCIAS E TECNOLOGIA

STRESSING OUT DECISIONS

THE ROLE OF STRESS IN CORTICO-BASAL GANGLIA LOOP PROCESSING AND INSTRUMENTAL CONDITIONING

Eduardo Dias Ferreira

Dissertation presented in partial fulfillment of the requirements for a Doctoral degree in Biology, specialty of Physiology.

August 2012

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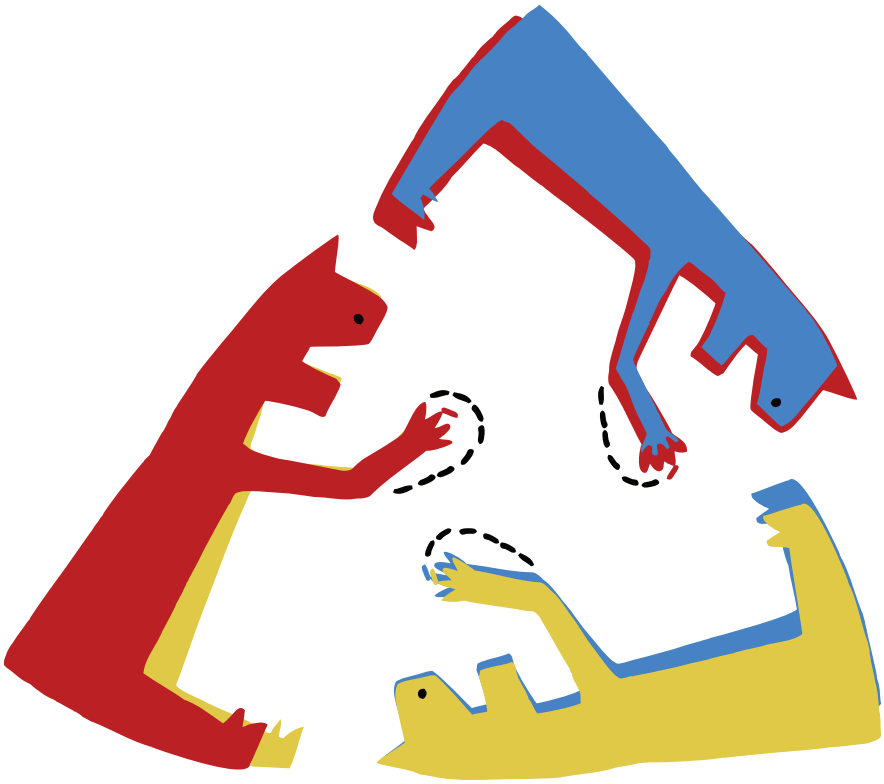

Illustration by Maria Remédio

Eduardo Miguel Gonçalves Dias Ferreira

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RESUMO

A incerteza, mais do que um problema relacionado com a tomada de decisões, é uma característica predominante dos ambientes naturais. Em contextos de incerteza, e perante as contínuas modificações do ambiente que nos rodeia e das nossas necessidades internas, a natureza "equipou-nos" com a capacidade de adaptar as nossas ações e regular parâmetros fisiológicos – um processo conhecido como a resposta de stress. Para além de toda a complexidade inerente à interação com um mundo incerto, as ações, desde as mais simples às mais complexas, podem ser despoletadas por elementos que as antecedem (estímulos), ou executadas com base nas suas consequências (resultados). No momento em que uma ação é executada, o peso de cada uma destas associações vai definir se é utilizada uma estratégia habitual ou uma estratégia intencional. A capacidade para alternar entre a utilização de uma ou outra estratégia de ação é fundamental para a adaptação a um ambiente imprevisível.

Nesta dissertação, demonstramos que a exposição prolongada a um ambiente imprevisível, capaz de gerar uma resposta de stress crónica, promove a utilização de estratégias de ação habitual, ao invés de estratégias de ação intencional. Utilizando duas tarefas instrumentais diferentes, mostramos que em ratos e murganhos sujeitos a protocolos de stress imprevisível crónico, a manipulação de uma alavanca para obter uma recompensa alimentar tornou-se insensível a modificações no valor do seu resultado, e resistente a alterações na contingência ação-resultado. Assim, os animais sujeitos a stress crónico tornaram-se incapazes de adaptar ações com base nas suas consequências; pelo contrário, as ações passaram a ser controladas por regras simples e despoletadas por um estímulo ou estado precedente.

Ao estudar os circuitos associativos e sensório-motores entre o córtex e os gânglios da base (circuitos córtico-basais), que se sabia já mediarem estas diferentes estratégias comportamentais, encontramos um padrão divergente de reorganização estrutural, com atrofia do córtex pré-fron-

tal medial e do estriado associativo, e hipertrofia do estriado sensório-motor. Esta relativa vantagem estrutural dos circuitos sensório-motores levantou a hipótese de que a competição pelo controle da ação entre os diversos circuitos córtico-basais esteja já enviesada quando a aprendizagem e execução destas ações ocorre após exposição crónica a um ambiente imprevisível.

No sentido de explorar esta hipótese com maior profundidade foram executadas, no decorrer do treino de manipulação de alavancas, novas experiências com registo simultâneo da atividade de populações de neurónios nestes circuitos fronto-estriatais. Demonstramos assim que, em animais sujeitos a stress crónico, a estratégia habitual de manipulação de alavancas surge em paralelo com um declínio progressivo nas interações funcionais fronto-estriatais, e com um desvio no padrão da atividade neuronal relacionada com a manipulação da alavanca no estriado dorsal, com um menor envolvimento do estriado associativo em relação ao estriado sensório-motor à medida que o treino progride. Curiosamente, os efeitos do stress crónico na atividade fronto-estriatal não foram aparentes nas fases iniciais de treino, e não modificaram nem a taxa de disparo neuronal em repouso, nem a gama das taxas de disparo, sugerindo que as modificações observadas na atividade neuronal surgiram no decorrer do treino, levando a um desvio na estratégia de ação.

A tendência aqui descrita para a utilização de estratégias de ação habitual após exposição a um ambiente imprevisível poderá ser interpretada como uma resposta de preparação para um contexto de imprevisibilidade, onde não é possível manipular a probabilidade de obter um determinado resultado; e a utilização de uma estratégia em que as ações são controladas por regras simples, tais como um estímulo ou estado particular, pode trazer grandes vantagens. No entanto, num mundo de complexidade crescente, onde decisões quotidianas são continuamente ajustadas a alterações importantes nas circunstâncias externas, assim como a permanentes modulações das nossas necessidades, a incapacida-

de de sujeitos previamente expostos a stress alternarem entre estratégias habituais e intencionais pode ser muito lesiva. Esta incapacidade poderá ser relevante na compreensão dos elevados níveis de comorbilidade entre perturbações relacionadas com stress e a ocorrência de dependências e comportamentos compulsivos, ou até mesmo a manutenção de hábitos antigos com impacto em atividades tão diversas como o nosso quotidiano e a economia.

SUMMARY

Uncertainty is not only a problem in decision-making, but a prevalent quality in natural environments. Nature "equipped" us with the ability to control and coordinate behavioral and physiological adjustments imposed by the continuous reshaping of the surrounding world and of our internal needs – a process that is known as stress response. Beyond the overall complexity of moving and interacting within an uncertain world, single actions or action sequences can be triggered by their antecedents (stimulus) or performed based on their consequences (outcome). The weight of each of these associations at the time of action performance will bias if the action is executed using a habitual or a goal-directed strategy. The ability to shift between these different action strategies is necessary for adaptation to unpredictable environments.

In the present series of studies, we show that a previous chronic exposure to an unpredictable environment, capable of eliciting a sustained stress response, promotes a bias toward the execution of habits versus goal-directed actions. Using two different operant tasks, we uncovered that lever pressing to obtain food rewards in rats and mice submitted to chronic unpredictable stress became insensitive to changes in outcome value and resistant to changes in action-outcome contingency. Therefore, chronic stressed animals were no longer able to adjust their actions based on their consequences, but rather actions were controlled by simple rules, and triggered by an antecedent stimulus or state.

When investigating the associative and sensorimotor cortico-basal ganglia circuits known to mediate these different behavioural strategies, we found a divergent structural reorganization after chronic stress, with atrophy of medial prefrontal cortex and the associative striatum, and hypertrophy of the sensorimotor striatum. This relative structural advantage of the sensorimotor network raised the hypothesis that the competition for action control between these distinct cortico-basal ganglia circuits would already be biased when new actions had to be learned and

performed after a chronic exposure to an unpredictable environment.

In order to further explore this possibility, we recorded the simultaneous activity of neuronal ensembles in these frontostriatal circuits during lever press training. We reveal that habitual lever pressing in chronically stressed animals emerged concomitantly with a progressive decline in functional frontostriatal interactions, and a shift in the pattern of lever press-related activity in dorsal striatum, with the associative striatum becoming less engaged than sensorimotor striatum as training progressed. Interestingly, chronic stress effects on frontostriatal activity were not observed early in training, and did not affect baseline firing rate or the dynamic range of firing rate, suggesting that the observed shift in neuronal activity emerged during lever press training leading to a shift in action mode.

The herein reported bias toward the use of habitual action strategies after exposure to an unpredictable environment could be interpreted as a preparatory response toward a context of uncertainty, where we cannot manipulate the probability of obtaining an outcome, and the use of a strategy in which actions would be controlled by simple rules, a particular stimulus or state, can be highly advantageous. However, in a world of increasing complexity where our everyday life decisions demand a permanent readjustment to major changes in the policies but also to a continuous reshaping of our current needs, 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, or the maintenance of old habits affecting activities spanning from our everyday life to economics.

ABBREVIATIONS LIST

5-HT	Serotonin
ac	Anterior commissure
AcbC	Nucleus accumbens core
AcbSh	Nucleus accumbens shell
ACTH	Adrenocorticotropic hormone
A-O	Action-outcome
cc	Corpus callosum
Cg	Cingulate cortex
Cl	Claustrum
COMT	Catechol-O-methyltransferase
CRF	Continuous reinforcement
CRH	Corticotropin-releasing hormone
DAT	Dopamine transporter
DIS	Dorsal intermediate striatum (intermediate area between medial and lateral regions of the dorsal striatum)
DLS	Dorsolateral striatum
DMS	Dorsomedial striatum
DS	Dorsal striatum
GABA	γ-aminobutyric acid
GR	Glucocorticoid receptors/type II corticosteroid receptors
HPA	Hypothalamic-pituitary-adrenal axis
IC	Insular cortex
IL	Infralimbic cortex
LO	Lateral orbital cortex
LTD	Long-term depression
LTP	Long-term potentiation
MC	Motor cortex
MO	Medial orbital cortex
MR	Mineralocorticoid receptors/type I corticosteroid receptors
mPFC	Medial prefrontal cortex
MSNs	Medium spiny neurons
NMDA	N-methyl-d-aspartate
OFC	Orbitofrontal cortex
PETH	Peri-event time histogram
PFC	Prefrontal cortex
PIT	Pavlovian-to-instrumental transfer

PL	Prelimbic cortex
rf	Rhinal fissure
ri	Rhinal incisura
RI	Random interval
RR	Random ratio
SEM	Standard error of the mean
SMC	Sensorimotor cortices
SNc	Substantia nigra pars compacta
S-R	Stimulus-response
SSC	Somatosensory cortex
VO	Ventral orbital cortex
VTA	Ventral tegmental area

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INTRODUCTION

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After a month of intense work, but also worrying, to accomplish an astonishing amount of unpredictable appointments, you are finally driving home for a long desired family holiday. Although all the work trouble is getting behind, there are still everyday life decisions that need your attention. You prepare yourself by explicitly reviewing some of the simple actions that will take you home fast and safely, like turning left at the second crossroad to avoid the ongoing roadwork on your usual way home. Now you can relax, turn up that Vivaldi concerto, and enjoy your ride home. The problem is that you are not aware of the possible consequences of that stressing month on the way you will perform these simple actions. This dissertation is about the moment you arrive to that second crossroad, and the influence of that last month of unpredictable appointments in the way you will respond, either by promoting the reflex toward the usual right turn (habit), or by considering the ongoing works and taking the alternative route (goal-directed action).

Cannon was the first to borrow the term "stress" from physics to use it in a biomedical sense (Cannon, 1914), but it was Selye that formalized the concept in a letter to Nature in which he described the "General Adaptation Syndrome" (Selye, 1936). According to Selye, stress represents the physiological response to any stimulus perceived as threatening or demanding, being primarily beneficial (or adaptive) for the organism as it promotes homeostasis, but potentially harmful (or maladaptive) if its intensity or duration exceeds a certain individual threshold (Selye, 1936, 1976). Selye recognized behavioral changes as part of the stress response, and since then an extensive literature has implicated stress in adaptive and maladaptive behavioral changes (de Kloet et al., 2005; McEwen, 2007; Sapolsky, 2004). The way organisms interact with the surrounding environment and respond to its challenges will certainly determine their fitness. Behind the overall complexity of this interaction within an uncertain world, single actions or action sequences can be triggered by their antecedents (stimulus) or performed based on their consequences (outcome) (Adams and Dickinson, 1981a; Balleine et al.,

2009; Costa, 2011). In this dissertation we examine the impact of a previous exposure to an unpredictable environment, capable of eliciting a sustained stress response, on the fine balance between these different modes of performing the same action.

GOAL-DIRECTED ACTIONS AND HABITS

The study of how we generate new actions, and why some of them are selected and performed has always been the focus of behavioral science and neuroscience. However, the field has struggled not only with the identification of the circuits and the cellular and molecular bases supporting actions, but also with the definition of the instrumental nature of actions – i.e., the causal relationships between events in the environment and responses or actions[1]. For large portions of the last century, the study of learning was strongly influenced by Hull and his followers, for whom instrumental learning resulted from stimulus-response bonds being strengthened or weakened by subsequent reinforcement. Based on Thorndike's "Law of Effect", the most fundamental assumption of this theory is that learned behavior is elicited by antecedent stimuli. It considers that instrumental actions are acquired reflexes, and that the consequences of behavior merely reinforce or weaken the stimulus-response association by providing satisfaction or dissatisfaction to the organism (Hull, 1943; Thorndike, 1911). Even though researchers like Von Holst proposed alternatives to the dominant view of behavior as a chain of reflexes (Holst, 1973), and Tolman (a rival to Hull's view) proposed that animals could integrate learned information in a flexible way and use cognitive maps (Tolman, 1948, 1949), for a long time behaviorists excluded intentionality, expectation or internal representation of the value of the outcome, which they considered subjective variables given the limitation of their observational methods.

[1]Instrumental behavior is frequently referred to as a response. However, we agree with Skinner that this would imply that instrumental behavior is limited to a reaction to a stimulus (Skinner, 1938). In this sense, and in accordance with the current theoretical framework, along this dissertation instrumental behavior will be more often referred to as an action.

It was in the later part of the 20th century that Dickinson and Rescorla developed experimental tools to investigate if instrumental actions were being performed on the basis of their consequences (Adams and Dickinson, 1981a; Adams and Dickinson, 1981b; Colwill and Rescorla, 1985; Colwill and Rescorla, 1986). To investigate if actions are goal-directed, and consequently establish evidence for stimulus-response habits, two previously neglected variables were elegantly manipulated: the expected value of predicted outcomes, and the causal relationship between the action and the outcome. Based on these manipulations, two types of test have become crucial in the analysis of instrumental learning.

In the first type of test, the value of the outcome is manipulated usually through a devaluation procedure. In this devaluation test, after training animals to perform a particular action in order to get access to food rewards in an operant box, the expected value of the reinforcements is manipulated by decreasing the value of the food, which can be achieved by conditioned taste aversion (food poisoning), or by sensory specific satiety. Conclusions are withdrawn from the comparison of the number of actions performed when the food was devalued versus when it was not devalued during a test in the absence of outcome, to probe the nature of memory for the association (action-outcome or stimulus-response) independently of new learning that can occur during the test. If action performance is sensitive to devaluation (i.e., if the rate of responding decreases after outcome devaluation), then the action is controlled by the anticipation of its consequences (outcome), and is goal-directed. If action performance is insensitive to this manipulation, then the action is controlled by antecedent stimuli, and is habitual (Adams and Dickinson, 1981a; Adams and Dickinson, 1981b; Balleine and Dickinson, 1998; Colwill and Rescorla, 1985; Dickinson and Balleine, 1993).

In the second type of test used to investigate if actions are goal-directed, the contingency between getting the outcome and the previous

execution of the action is manipulated. This is usually achieved either by contingency degradation, through introduction of non-contingent background reinforcers, in order to turn the probability of the reinforcer the same given a particular action or given no action, or by omission, which is a complete reversal of the normal action-outcome contingency – i.e., action prevents the reinforcer, but no action results in reinforcer delivery. Similarly to outcome devaluation, the effects of contingency degradation should also be probed in a test in the absence of the outcome to avoid the confounding effects of consumption and reinforcement. Briefly, if degrading the contingency between one of the actions and the outcome had no effect on the performance of that action specifically, it could be concluded that the performance of that particular action was no longer based on the action-outcome contingency representation, suggesting it was governed by stimulus-response habits (Dickinson and Balleine, 1993; Dickinson et al., 1996; Hammond, 1980).

These two criteria (outcome devaluation and contingency degradation) are essential for any given behavior to be established as a goal-directed action (Dickinson, 1985; Dickinson and Balleine, 1993; Yin et al., 2008). It is important to emphasize that consummatory Pavlovian responses, although differing from instrumental actions since are not necessary to obtain the reward that is paired with a stimulus, can also be sensitive to outcome devaluation (Holland and Rescorla, 1975). Therefore, an impairment in goal-directed action performance should also be supported by manipulations of the action-outcome contingency allowing for the distinction from the stimulus-outcome contingency governing Pavlovian responses (Davis and Bitterman, 1971; Dickinson and Charnock, 1985; Rescorla, 1968; Yin et al., 2008). Because these are essentially tests for features of habits that distinguish them from goal-directed actions, they establish evidence for habits by default, as behaviors that are insensitive to revaluation of the outcome and to changes in the action-outcome contingency, suggesting that they are under control of a stimulus-response association (Balleine et al., 2009;

Dickinson and Balleine, 1993). It is noteworthy that the stimulus-response/reinforcement theory of Thorndike and Hull, according to which the outcome is not part of the stimulus-response association, but merely strengthens or weakens it (Hull, 1943; Thorndike, 1911), has survived the test of time by capturing the nature of habit learning.

It is now generally accepted that an action is driven by both the association with its antecedents (stimulus) and its consequences (outcome). The relative weight of each of these associations at the time of action performance will bias if the action is executed using a habitual or a goal-directed strategy, respectively (Balleine et al., 2009). Adams and Dickinson noticed that the fine balance between these different actions strategies relied on the amount of training and the statistics of reinforcement. Specifically, overtraining on a particular schedule promotes a transition from goal-directed to habitual action performance, but also the use of random interval (RI) schedules of reinforcement, most probably by increasing reward uncertainty (reinforcer is delivered upon the first action after a certain interval had elapsed since the last reinforcer was earned), favors habit formation, while random ratio (RR) schedules (reinforcer is delivered after a certain number of actions) favors goal-directed action performance (Adams, 1982; Adams and Dickinson, 1981a; Derusso et al., 2010; Dickinson, 1985; Dickinson et al., 1983). These instrumental tasks have been very useful to investigate the neural circuits and the cellular and molecular mechanisms involved in goal-directed and habitual action performance, and in the interplay between these different action strategies (Balleine and Dickinson, 1998; Balleine et al., 2009; Hilario and Costa, 2008; Yin and Knowlton, 2006). These are also the definitions and behavioral assays adopted in this study, and similarly to the great majority of the studies cited above, we took advantage of rodent animal models (rats and mice) to go from the behavior into the circuit level of analysis.

CORTICO-BASAL GANGLIA CIRCUITS

The neuroanatomical circuits that support goal-directed and habitual action strategies have been shown to differ, namely at the level of basal ganglia. The basal ganglia constitute a set of nuclei involved in generating and selecting distinct action types (Balleine et al., 2009; Costa, 2011; Doya, 1999; Fee and Goldberg, 2011; Graybiel, 1995; Hikosaka, 1998; Wickens et al., 2003; Yin and Knowlton, 2006). The striatum, being the entry station of the entire basal ganglia, serves as a unique hub for cortico-basal ganglia reentrant loops, capable of integrating cortical, thalamic and midbrain inputs. While the limbic loops that course through the nucleus accumbens seem to mediate consummatory Pavlovian responses or influences on instrumental behavior (stimulus-outcome associations or Pavlovian-to-instrumental transfer), the loops that course through the dorsal striatum seem to be more involved in the control of instrumental actions (Balleine et al., 2009; Yin and Knowlton, 2006; Yin et al., 2008). Although the dorsal striatum in rodents does not present a clear anatomical separation into caudate and putamen, it does have a medial-lateral gradient of connectivity that is similar (but not identical) to the caudate (ventromedial), and putamen (dorsolateral) connectivity in primates. Within the dorsal striatum, the medial region that extends ventrally to the limits of the nucleus accumbens receives most of its inputs from associative cortical areas (similarly to the caudate), while the lateral region receives input from sensorimotor cortical areas (similarly to putamen) (Haber, 2003; Voorn et al., 2004). The associative cortico-basal ganglia circuits involving the dorsomedial striatum (DMS) (Yin et al., 2005a; Yin et al., 2005b), the prelimbic (PL) subregion of medial prefrontal cortex (mPFC) (Balleine and Dickinson, 1998; Corbit and Balleine, 2003; Ostlund and Balleine, 2005), and the mediodorsal thalamus (Corbit et al., 2003) have been shown to support the learning and performance of goal-directed actions. On the other hand, the dorsolateral striatum (DLS) or sensorimotor striatum (Yin et al., 2004), and the infralimbic (IL) cortex (Killcross and

Coutureau, 2003) have been shown to support the formation of habits (Figure 1.1). These studies are in agreement with the differential engagement of associative and sensorimotor striatal circuits respectively during the early and late phases of skill learning (Miyachi et al., 2002; Miyachi et al., 1997; Yin et al., 2009), and indicate that different action modes (even if very similar movements) are learned and executed by different corticostriatal circuits. Furthermore, these parallel corticostriatal circuits dynamically interact with each other (Gremel and Costa, 2012; Kasanetz et al., 2008; Thorn et al., 2010), which suggests that competing corticostriatal circuits underlie the ability of animals to switch between these two modes of performing the same action (Balleine et al., 2009; Daw et al., 2005; Hilario et al., 2012).

The medial-lateral functional gradient in the dorsal striatum does not only reflect a gradient of cortical inputs, but also a gradient of threshold for the induction and expression of synaptic plasticity (Gerdeman et al., 2003; Hilario and Costa, 2008; Partridge et al., 2000; Yin and Knowlton, 2006). Long-term synaptic plasticity, either in the form of long-term potentiation (LTP) and long-term depression (LTD), have been related to learning (Bliss and Lømo, 1973). The vast majority of neurons in the striatum (90-95%) (Kemp and Powell, 1971) are inhibitory, GABA (γ-aminobutyric acid)-containing medium spiny projection neurons (MSNs) (Kita and Kitai, 1988) that receive excitatory, glutamatergic projections from the cortex, but also from the thalamus and amygdala (Voorn

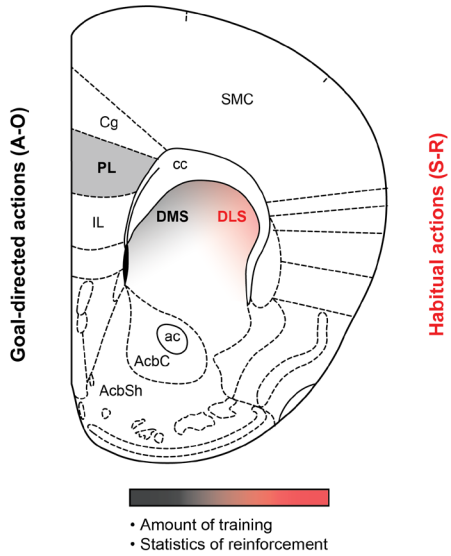

Figure 1.1. Parallel cortico-basal ganglia circuits underlying goal-directed and habitual action strategies.
Depiction of different cortico-basal ganglia circuits according to the medial--lateral functional gradient of dorsal striatum. The sensorimotor striatum (red) and associative corticostriatal circuits (grey) compete for action control, mediating the execution of actions based on their antecedents (stimulus-response – S-R) or taking into account their consequences (action-outcome – A-O) (Balleine et al., 2009). Adams and Dickinson noticed that the fine balance between these different action strategies relied on the amount of training and the statistics of reinforcement (Adams, 1982; Adams and Dickinson, 1981a; Dickinson, 1985).
The diagram illustrating a coronal section of the mouse brain was adapted from (Paxinos and Franklin, 2001). Cg, cingulate cortex; SMC, sensorimotor cortices; cc, corpus callosum; AcbC, core, and AcbSh, shell, of nucleus accumbens; ac, anterior commissure.

et al., 2004). At the synapses between cortical pyramidal neurons and MSNs, LTP was found to occur more easily in the DMS, while LTD has been shown to be easier to induce in the DLS (Partridge et al., 2000), with some of the mechanisms underlying both forms of synaptic plasticity showing regional variation (Gerdeman et al., 2003; Hilario and Costa, 2008; Yin and Knowlton, 2006).

Long-term potentiation in the DMS requires the activation of D1 dopamine receptors and NMDA (N-methyl-d-aspartate) glutamate receptors (Kerr and Wickens, 2001; Partridge et al., 2000; Shen et al., 2008). The blockade of NMDA glutamate receptors in the DMS promotes habit formation, which suggests that action-outcome learning, or even the online maintenance of this association over the course of action learning and performance depends on ongoing plasticity at glutamatergic synapses in the DMS (Yin et al., 2005a). On the other hand, striatal LTD requires dopamine/endocannabinoid signaling that ultimately depends on activation of CB1 receptors (Gerdeman and Lovinger, 2001; Gerdeman et al., 2002; Kreitzer and Malenka, 2005) or, as more recently uncovered, activation of serotonin (5-HT) 5-HT_{1b} receptors (Mathur et al., 2011). The expression of CB1 receptors across the dorsal striatum displays a medial-lateral gradient, with increased expression toward the DLS (Herkenham et al., 1991), turning dopamine-dependent striatal LTD more easy to induce in the DLS (Gerdeman et al., 2003), and most probably underlying the critical role of endocannabinoid signaling through CB1 receptors in habit formation (Hilario et al., 2007).

Dopamine is involved in both forms of striatal plasticity (Gerfen and Surmeier, 2011; Shen et al., 2008). A medial-lateral gradient is also present in the source of dopaminergic input to the dorsal striatum, with the DMS receiving a broader innervation from a region comprising more lateral areas of the ventral tegmental area (VTA) and ventromedial areas of the substantia nigra pars compacta (SNc), and the DLS receiving a broader projection from the dorsolateral SNc (Moore et al., 2001).

This is also the case for dopamine clearance mechanisms, with a higher expression of the dopamine transporter (DAT) toward the DLS and a prevalence of catechol-O-methyltransferase (COMT) in the DMS (Arbuthnott and Wickens, 2007; Matsumoto et al., 2003). In fact, lesions of the SNc input to DLS impair habit formation and favor goal-directed action performance (Faure et al., 2005). Consistently, sensitization with amphetamine, which acts on the DAT, induces divergent changes in spine density in MSNs, with an increase in DLS and a decrease in DMS MSNs (Jedynak et al., 2007), and promotes a bias toward habitual action performance (Nelson and Killcross, 2006).

A different level of functional and anatomical organization in the striatum can also be appreciated from its output perspective. Dorsal striatum MSNs can be divided according to two major output pathways, which comprise separate but approximately equal numbers of neurons (Gerfen, 1992; Gerfen and Young, 1988). The striatonigral pathway provides direct inputs to the substantia nigra, and the striatopallidal pathway provides indirect projections to the substantia nigra through the globus pallidus (Kawaguchi et al., 1990). Striatonigral and striatopallidal MSNs also present a clear-cut dichotomy at the molecular, anatomical, and physiological levels (Gerfen et al., 1990; Gerfen and Surmeier, 2011; Gertler et al., 2008; Shen et al., 2008). The most commonly referred molecular marker is their expression of different dopamine receptor subtypes. The striatonigral MSNs express D1 dopamine receptors and the striatopallidal MSNs express D2 dopamine receptors (Gerfen et al., 1990). Interestingly, D1 and D2 dopamine receptors seem to be differentially expressed throughout the striatum, with D1 dopamine receptor being slightly more prominent in ventrolateral and ventromedial striatum than in DLS, and with D2 dopamine receptor being more abundant in DLS than in DMS (Joyce et al., 1985; Savasta et al., 1986; Yin et al., 2009), which resembles the medial-lateral functional gradient discussed above. Actually, in skill learning, the differential engagement of DMS and DLS as training progresses is paralleled

with a long-lasting potentiation of glutamatergic transmission onto D2 dopamine receptor–expressing striatopallidal MSNs, while the performance of the skill becomes less dependent on the activation of D1 dopamine receptors, which are mainly expressed in striatonigral MSNs (Yin et al., 2009). Furthermore, LTP at the glutamatergic input to the striatopallidal pathway depends on A_{2A} adenosine receptors activation (Shen et al., 2008), and the genetic deletion of A_{2A} adenosine receptors in the striatum selectively impairs habit formation (Yu et al., 2009). Altogether, these studies suggest a role for the striatopallidal pathway in habitual action performance.

The balance between the automatization of recurrent decision processes and the re-evaluation of action consequences is critical for adaptation. These parallel cortico-basal ganglia circuits not only encode habitual and goal-directed action strategies, but dynamically interact to permit switching between these different modes of performing the same action (Figure 1.1).

STRESS RESPONSE

The brain integrates external sensory information from the environment and internal information from the body. This integrative process enables the brain to control and coordinate behavioral and physiological adjustments imposed by external and internal challenges to homeostasis – a process that is known as stress response or allostasis[2] (Dallman, 2003; McEwen, 1998; Sterling and Eyer, 1988). These adjustments are promoted by an intricate interactive network of biological systems highly conserved among vertebrates, including the hypothalamic-pituitary-adrenal (HPA) axis, the autonomic nervous system, and the immune system, among others. This stress response is reflected in the rapid activation of the autonomic sympathetic nervous system,

[2]Because of the paradoxical effects of stress, the term allostasis was introduced by Sterling and Eyer (1988) and extended by McEwen (1998) in order to better characterize the process through which organisms actively adjust to both predictable and unpredictable events in order to maintain homeostasis. Allostasis means "maintaining stability through change", and reappraises the original homeostatic concept by recognizing that any given physiological parameter has more than a single optimal set point (each depending on a specific state), any given optimal set point can be reached through several regulatory mechanisms (each with its consequences and/or side-effects), and regulatory mechanisms can be activated in anticipation of a set point that is likely to be challenged ("pre-occupation") (Sapolsky, 2004).

with release of noradrenaline from widely distributed synapses and adrenaline from the adrenal medulla, and the slower activation of the HPA axis, with secretion of glucocorticoids (cortisol in primates, or corticosterone in most rodents) from the adrenal cortex into the bloodstream. When these mediators of allostasis are released in response to stressors or lifestyle factors such as feeding or exercise, they promote adaptation and are generally beneficial. This is classically envisioned by the "fight-or-flight" response (Cannon, 1929), such as calibrating cardiac output and peripheral vascular resistance to provide hemodynamic and metabolic support for large muscle groups needed for immediate or anticipated action (e.g., escape from a threatening situation). However, when these mediators are not adequately activated, or are overused by excessive or prolonged challenge, the cost of reinstating homeostasis might become too high, leading to the "wear-and-tear" on the body and brain, which is a condition that is termed allostatic load (McEwen, 2007).

When a situation is perceived as stressful several brain circuits are activated. Although the central stress response is not yet completely understood, several of the so-called "stress responsive areas" are known to include the hypothalamus, hippocampus, prefrontal cortex (PFC) and amygdala (Cullinan et al., 1995). Activation of these areas will ultimately lead to the release of corticotropin-releasing hormone (CRH) to the anterior pituitary, in addition to other brain regions (Herman et al., 2003; Holsboer et al., 1992; Owens and Nemeroff, 1991). In the anterior pituitary, CRH released by the paraventricular nucleus of the hypothalamus into the hypophyseal portal system triggers the release of adrenocorticotropic hormone (ACTH) into the general bloodstream, which subsequently induces secretion of corticosteroids, including glucocorticoids by the adrenal cortex (Whitnall, 1993) (Figure 1.2). Interestingly, as for the HPA axis, the activation of the autonomic sympathetic nervous system is under the primary influence of the hypothalamus, placing both of the main peripheral stress response pathways under the in-

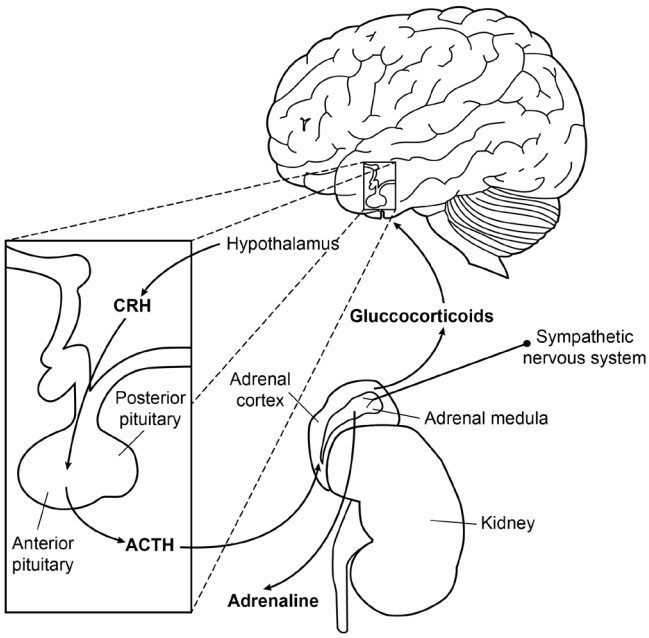

Figure 1.2. Neuroendocrine response to stress.
When a stressor is perceived or anticipated by the brain, the hypothalamus activates the rapid autonomic sympathetic nervous system that triggers the release of adrenaline by the adrenal medulla, and the slower HPA axis. In the latter, the paraventricular nucleus of the hypothalamus releases CRH that reaches the anterior pituitary through the hypophysial portal system causing the release of ACTH into the general bloodstream, which will induce the secretion of glucocorticoids (cortisol in primates, or corticosterone in most rodents) by the adrenal cortex. Note that glucocorticoids, but also CRH, are able to influence several brain regions.

fluence of the same brain regions, such as the hippocampus and the PFC (Herman et al., 2003). However, contrary to the increase in sympathetic activation of the adrenal medulla leading to release of adrenaline, whose effects are confined to the periphery, activation of the HPA axis results in the release of glucocorticoids that are able to cross the blood-brain barrier and act on central nervous system targets (McEwen, 2007). This top-down process of sequential activation of the hypothalamus, anterior pituitary and adrenal cortex – HPA axis – is under the tight control of a negative feedback mechanism by which glucocorticoids themselves inhibit adrenocortical activity, ACTH secretion and CRH release (Jacobson, 2005; Whitnall, 1993). In addition, by activating glucocorticoid receptors (GR) in higher brain centers, such as the hippocampus and the PFC, these hormones also modulate hypothalamic activity and the consequent activity of the HPA axis.

Corticotropin-releasing hormone is a peptide neurotransmitter produced by a variety of neurons and released in several brain regions (Hols-

boer et al., 1992; Owens and Nemeroff, 1991). It binds to two distinct receptors, with a high affinity to type 1 (CRH.R1) and low affinity to type 2 (CRH.R2) (Suda et al., 2004). These two types of CRH receptors are widespread in the brain, with CRH.R1 generally more abundant in cortical regions, but also in the striatum, and CRH.R2 in subcortical regions (Sanchez et al., 1999; Van Pett et al., 2000). The presence of both CRH and its receptors in several brain regions, and the fact that it is released during the stress response implicates this peptide as a key player in mediating some of the central effects of stress. Indeed, it has been shown that CRH may influence brain structure and function (Bayatti and Behl, 2005; Richard et al., 2000; Smagin et al., 2001; Strohle and Holsboer, 2003), and that antagonism of its receptors can elicit behavioral responses [for instance, anxiolytic and antidepressant actions (Gilligan and Li, 2004)] opposite to those seen after chronic stress.

In the brain, glucocorticoids act via two different types of receptors: mineralocorticoid receptors (MR, also known as type I corticosteroid receptors) and GR (also known as type II corticosteroid receptors). The much lower affinity of GR than MR for glucocorticoid ligands implies that GR are more sensitive to hormone secretory bursts, as in the case of a response to a stressor or in the zenith of the circadian rhythm. Similarly to many other physiological processes, activity of the HPA axis displays a circadian rhythm, having a surge just before the animal's active period (daylight for humans and night-time for rodents) after which it rapidly decays to "basal" or "resting" levels. Therefore, the basal physiological condition is that of low GR but high MR occupation throughout most of the day, with the activation of both GR and MR during stress- and circadian-induced increases in the frequency and amplitude of adrenocortical secretory bursts (de Kloet et al., 2005; Reul and de Kloet, 1985; Reul et al., 2000). Interestingly, the distribution pattern of these receptors closely matches the proposed list of "stress responsive areas" (Cullinan et al., 1995). Mineralocorticoid receptors are abundant in the hippocampus (specially in the dentate gyrus) and in the hypothal-

amus, but are also present in the amygdala, septum and cerebral cortex. It is noteworthy that the distribution of MR within these brain regions is not uniform – for instance, in the cortex, MR seem predominantly located in the external layers (Ahima et al., 1991; Kawata et al., 1998; van Eekelen et al., 1991). Glucocorticoid receptors have a more widespread distribution, being abundant in the hippocampus, hypothalamus, amygdala, bed nucleus of stria terminalis, striatum and cerebral cortex, as well as in almost all nuclei of the lower brainstem (Ahima and Harlan, 1990; Cintra et al., 1994; Fuxe et al., 1985; Kawata et al., 1998). This distribution pattern has led to the proposal that the central response to stress is predominantly a consequence of elevated glucocorticoid levels. In fact, systemic high-dose corticosterone treatments are widely used to mimic the situation found after stress-induced activation of the HPA axis. However, although simple experimental models can help unravel some of the pathways and mechanisms that mediate stress effects in the brain, it should be remembered that activation of the stress response has a broader spectrum of consequences, involving a myriad of other mediators, as well as a range of affected targets, which may feedback to regulate the overall stress response (Sousa et al., 2008).

A sustained stress response is not only characterized by increased circulating levels of glucocorticoids, but as Selye noticed almost eighty years ago (Selye, 1936), the exposure to stressful stimuli leads to changes in several body structures, some of which have been implicated along these years in the regulation of stress response. In addition to the classically described decreases in body weight gain and thymus weight, and increases in adrenals weight, also several brain circuits are sculpted by stress, affecting their function and associated behavior (de Kloet et al., 2005; McEwen, 2007; Sapolsky, 2004).

IMPACT OF STRESS ON BRAIN CIRCUITS

The first insight that corticosteroids could influence the brain came from descriptions by Cushing of the syndrome of excessive production and release of adrenal steroids (Cushing, 1932), in which mood and behavioral changes are among the characteristic symptoms (Newell-Price et al., 2006; Starkman and Schteingart, 1981). At about the same time, Selye described the "General Adaptation Syndrome" (Selye, 1936). Selye also recognized behavioral changes as part of the stress response, but the link between the two conditions – stress and Cushing's syndrome – was only possible after the recognition that release of glucocorticoids from the adrenal gland is a major component of the stress response. The decades that followed were marked by numerous discoveries on the pathways of glucocorticoid synthesis and their physiological effects, in particular the description of their anti-inflammatory actions (Hench et al., 1949). From this time onwards, corticosteroids were recognized as therapeutically useful compounds, even though it took only a year after Hench's report for the first description of steroid psychosis (Rome and Braceland, 1950). This observation together with the behavioral changes observed in Cushing's syndrome patients, prompted research into the mechanisms of corticosteroid actions in the brain.

In the late 1960s, McEwen and collaborators showed that corticosterone was taken up and retained by the brain, particularly by regions of the limbic system (Gerlach and McEwen, 1972; McEwen et al., 1968), which together with the first reports of the effects of corticosteroids in the structure (Muhlen and Ockenfels, 1969) and function (Steiner, 1972) of brain circuits, provided a firm basis for understanding the robust behavioral effects of corticosteroids and stimulated research on the actions of these hormones on brain circuits. Further studies during the 1970s and 1980s revealed that excessive or prolonged exposure to corticosteroids induces pyramidal cell loss and dendritic atrophy in the hippocampus (Sapolsky et al., 1985; Sapolsky and Pulsinelli, 1985), and may accelerate ageing (DeKosky et al., 1984; Landfield et al., 1978;

Sapolsky et al., 1987; Sapolsky et al., 1985, 1986a). These results were extended to the stress response, of which high glucocorticoid release became a hallmark (Sapolsky et al., 1986b). These deleterious effects of stress upon brain circuits, which until ten years ago referred almost exclusively to the hippocampus, were summarized in Sapolsky's commentary in Science entitled "Why stress is bad for your brain" (Sapolsky, 1996). However, as emphasized by Selye, the stress response is primarily beneficial for the organism as it promotes adaptation, and only becomes harmful if its intensity or duration exceeds a certain individual threshold (i.e., when maladaptation prevails) (Selye, 1936, 1976).

The impact of stress on brain circuits and consequent behavioral changes are highly dependent on the duration and type of stressor. While a prolonged exposure to stressors and/or sustained release of glucocorticoids usually triggers behavioral deficits, the acute release of stress hormones has a positive impact on certain behaviors (de Kloet et al., 2005; Joels et al., 2006; McEwen, 2007; Sapolsky, 2004; Sousa et al., 2008). Indeed, stress hormones, including glucocorticoids, released during learning (as a response to a novel challenge), are necessary for establishment of enduring memories. Several studies, using different behavioral paradigms, have revealed that the acute release of corticosterone that occurs in association with training (at similar levels to those found after exposure to stress) strengthens spatial memory in a time- and context-dependent manner (Lupien and McEwen, 1997; Sandi et al., 1997; Sandi and Rose, 1994). Furthermore, corticosterone administered after training facilitates extinction of passive and active avoidance responses, thereby promoting the elimination of behaviors that may be no longer relevant (Bohus and de Kloet, 1981; Joels et al., 2006).

On the other hand, it has been shown that a chronic exposure to stressors, mainly through the release of glucocorticoids, affects the function of several regions of the limbic system, which is associated with a differential structural modulation of these brain networks (McEwen, 2007;

Sapolsky, 1996; Sapolsky, 2004; Sousa et al., 2008). Interestingly, some of these structural changes are reflected on the volume of these regions/sub-regions, which does not seem to correlate with changes in neuronal numbers [as originally reported (Sapolsky et al., 1985)], but rather with dendritic reorganization of the projection neurons of these regions (Cerqueira et al., 2007; Cerqueira et al., 2005; Radley et al., 2004; Sousa et al., 1998; Sousa et al., 2008; Sousa et al., 2000; Sousa et al., 1999; Watanabe et al., 1992; Wellman, 2001). Indeed, the chronic stress-induced dendritic atrophy of pyramidal neurons in the hippocampus (Sousa et al., 2000; Watanabe et al., 1992) and mPFC (Radley et al., 2004), and hypertrophy of pyramidal neurons in the lateral orbital frontal cortex (Liston et al., 2006) and of pyramidal and stellate neurons in the basolateral amygdala (Vyas et al., 2002) is accompanied with deficits in spatial reference (Luine et al., 1994) and working memory (Mizoguchi et al., 2000), behavioral flexibility (Cerqueira et al., 2007), and fear conditioning (Conrad et al., 1999). Importantly, these behaviors not only rely on the functional integrity of these regions but also on their dynamic interaction (Salzman and Fusi, 2010; Wang and Morris, 2010), and chronic stress has been shown to interfere with hippocampus-mPFC (Cerqueira et al., 2007) and amygdala-mPFC (Lee et al., 2011) functional interactions, which have been proposed to decline through modulation of NMDA glutamate receptor expression in pyramidal neurons of mPFC (Cerqueira, 2006; Lee and Goto, 2011). Taken together, these studies emphasize the view that chronic stress should be seen as a disruptor of brain circuits rather than simply a cause of dysfunction in an isolated brain region. Furthermore, a systems level perspective beyond the traditional limbic system could lead to a better understanding of complex traits of stress-related behavior, as depression, addiction or compulsivity (Cleck and Blendy, 2008; de Kloet et al., 2005; Koob, 2008; Pittenger and Duman, 2008).

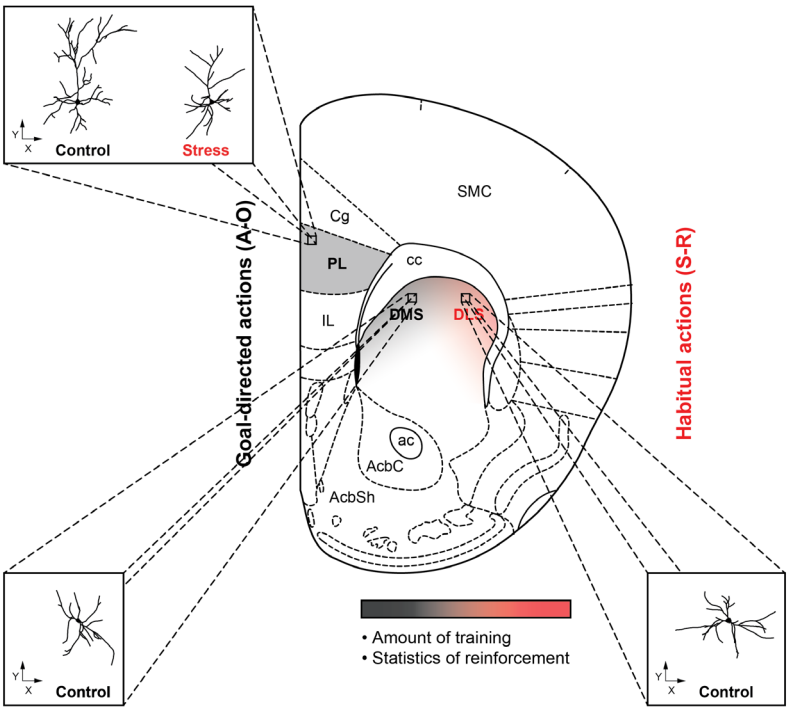

Figure 1.3. The impact of chronic stress on cortico-basal ganglia circuits will be investigated beyond the traditional limbic systems.
The previously reported chronic stress effects on the dendritic structure of pyramidal neurons in the mPFC raise interesting hypotheses regarding an extension of these effects on the structure and function of cortico-basal ganglia circuits (e.g., MSNs in the associative and sensorimotor striatum), with possible adaptive and/or maladaptive influences on action performance.
The diagram illustrating a coronal section of the mouse brain was adapted from (Paxinos and Franklin, 2001). Abbreviations are as in Figure 1.1.

AIMS: THE ROLE OF STRESS IN CORTICO-BASAL GANGLIA LOOP PROCESSING AND INSTRUMENTAL CONDITIONING.

A possible role of stress in the way actions are performed, either based on its antecedents (stimulus) or its consequences (outcome), would be potentially interesting given its adaptive and/or maladaptive effects. This becomes a clearer hypothesis considering the impact of chronic stress on brain circuits, specifically on mPFC that takes part of the cortico-basal ganglia circuits mediating these different modes of performing the same action (Figure 1.3). Therefore, in the next chapters we aim at:

• Evaluate the impact of a previous chronic exposure to an unpredictable environment, capable of eliciting a stress response, on the fine balance between goal-directed and habitual action strategies.

• Investigate the impact of chronic stress on the structure and function of corticostriatal circuits underlying these different action strategies.

We hope to have arrived safely to the second crossroad. Now let's see what happens next![3]

[3]Reference to the metaphor used in the first paragraph of this chapter.

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2

CHRONIC STRESS CAUSES FRONTOSTRIATAL REORGANIZATION AND AFFECTS DECISION-MAKING

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CONTENTS

SUMMARY

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.

INTRODUCTION

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 (Balleine et al., 2007; Yin and Knowlton, 2006), 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 (Dickinson, 1985). Habitual responses no longer need the evaluation of their consequences and can be elicited by particular situations or stimuli (Balleine et al., 2007; Yin and Knowlton, 2006), 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 (Balleine et al., 2007), 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 (McEwen, 2007; Sapolsky, 2004). Stress-induced deficits in spatial reference and working-memory (Mizoguchi et al., 2000) and behavioral flexibility (Cerqueira et al., 2007a) are associated with synaptic and/or dendritic reorganization in both the hippocampus (Sousa et al., 2000) and the medial prefrontal cortex (mPFC) (Radley et al., 2004). 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 (Balleine and Dickinson, 1998) and the dorsomedial striatum (DMS) (Yin et al., 2005) have been implicated in the acquisition and execution of goal-directed actions, whereas sensorimotor circuits, namely, the dorsolateral striatum (DLS) (Yin et al., 2006), are necessary for habit formation, we examined the effects of chronic stress on these brain areas.

RESULTS

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 (see Experimental Procedures) that combines different stressors in an unpredictable manner to avoid the resilient effect of behavioral control over stressors (Amat et al., 2005). Twenty-one days of stress exposure decreased body-weight gain (Figure 2.1A), reduced the thymus/body-weight ratio (Figure 2.1B), and resulted in persistently raised serum corticosterone levels (Figure 2.1C), 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.

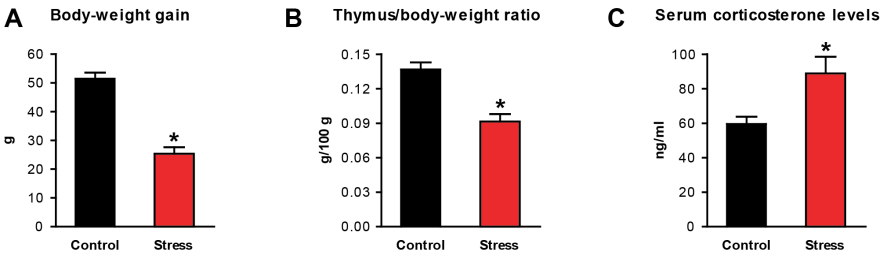

Figure 2.1. Biometric parameters used as an index of stress treatment efficacy.
(A) Body-weight gain of rats during stress exposure or handling (t_{38} = 8.348, $p < 0.001$).
(B) Thymus/body-weight ratio from animals sacrificed after the treatment period (t_{8} = 5.088, p = 0.001).
(C) Corticosterone levels measured in blood serum collected at least 8 h after the last stress exposure (t_{26} = 2.804, p = 0.009).
Error bars denote SEM. *$p < 0.05$.

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 (Adams and Dickinson, 1981; Yin and Knowlton, 2006). Rats (n = 8 per group) were trained to press a lever for a particular outcome (pellets or sucrose, counterbalanced) under a random ratio schedule which was previously shown to bias for goal-directed behavior (Adams and Dickinson, 1981; Dickinson, 1985; Hilario et al., 2007). 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 (Figure 2.2A). To evaluate if 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 (Figure 2.2B). 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 – see Experimental Procedures; 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 (Adams and Dickinson, 1981; Dickinson, 1985; Hilario et al., 2007), the actions of control animals became highly sensitive to sensory specific satiety (Figure 2.2C; 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 (Figure 2.2C; 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 (Katz, 1982), but rather because stressed animals rapidly shift to a habitual strategy as training progresses. The amount of reinforcer consumed during the ad

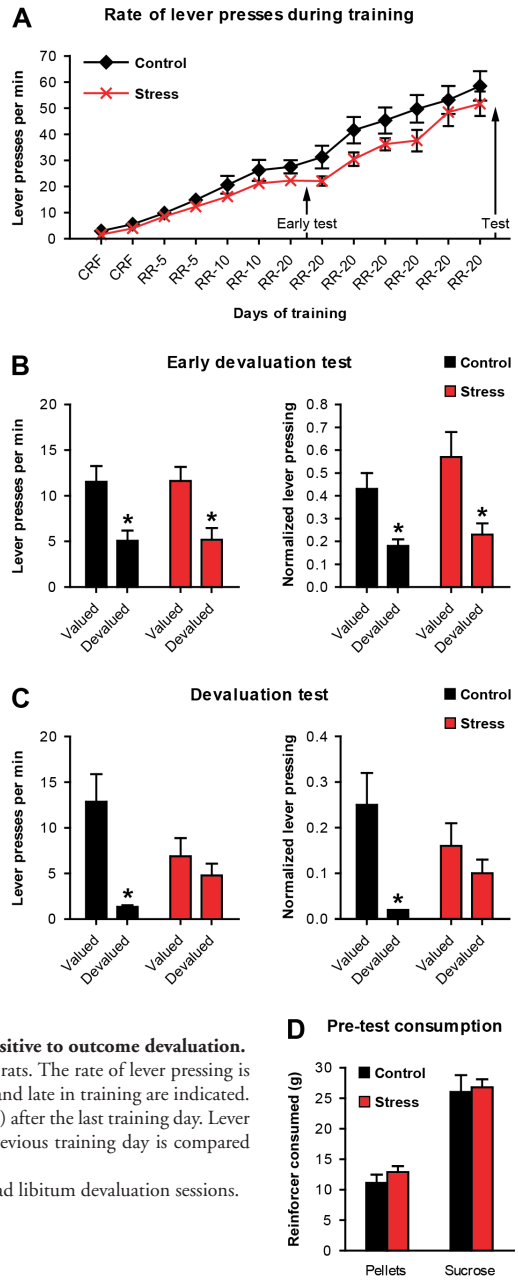

Figure 2.2. Chronic stress biases behavioral responding to become insensitive to outcome devaluation.
(A) Acquisition of the lever-pressing task in control and chronically stressed rats. The rate of lever pressing is depicted for each daily session. Reversible devaluation tests performed early and late 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. *$p < 0.05$.

libitum devaluation sessions was similar in stressed and control animals (Figure 2.2D; 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 (Hammond, 1980; Yin and Knowlton, 2006). 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 (Figure 2.3A). 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 represented as mean ± 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; see Experimental Procedures).

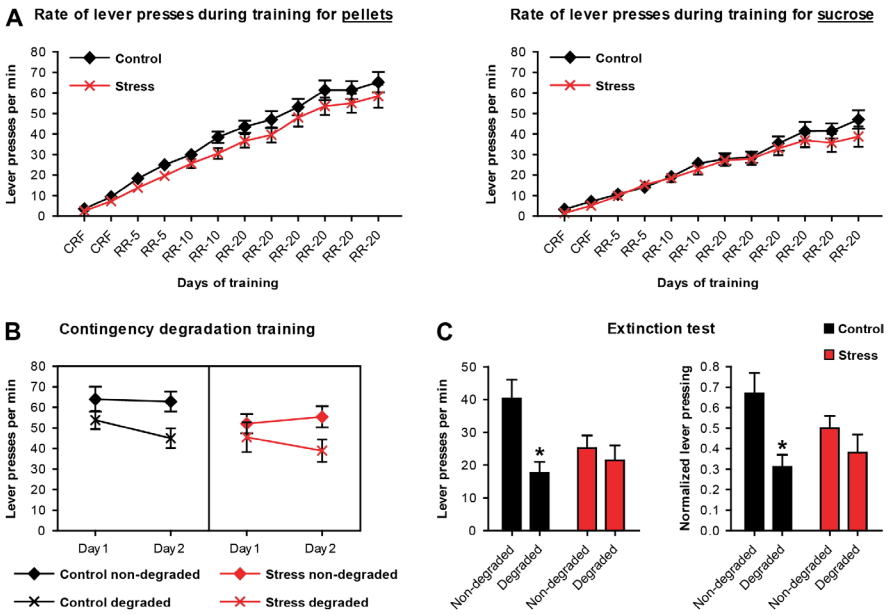

Figure 2.3. Chronic stress predisposes choices to be insensitive to changes in action-outcome contingency.
(A) Acquisition of the lever-pressing task in control and chronically stressed rats. The rate of lever pressing is depicted for each daily session for pellets and for sucrose.
(B) Performance for 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.

After 2 days of forced-choice degradation training in which both groups changed their behavior (Figure 2.3B; 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 (Yin et al., 2005)] (Figure 2.3C). Control animals significantly reduced their responses on the degraded lever compared to 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.

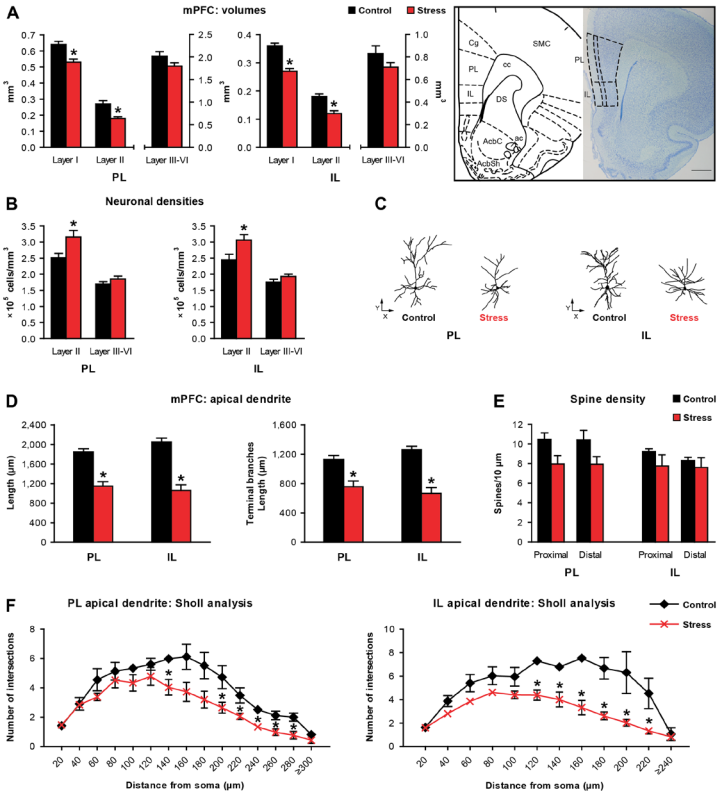

Figure 2.4. Chronic stress results in selective atrophy within the external layers of both PL and IL mPFC sub-regions.
Several structural measurements of control and chronically stressed rats are compared. (A and B) Stereological estimations of (A) volumes and (B) neuronal densities. (A, right) Outlining between regions and layers is represented; diagram was adapted from (Paxinos and Watson, 1998) 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.

These data indicate that previous exposure to chronic stress biases decision-making and predisposes 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 (Balleine and Dickinson, 1998; Yin et al., 2005) or sensorimotor (Hi-

lario et al., 2007; Yin et al., 2006) corticostriatal circuits (Jedynak et al., 2007; Killcross and Coutureau, 2003; Nelson and Killcross, 2006). 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 (Balleine and Dickinson, 1998; Killcross and Coutureau, 2003). Volumetric estimations showed a selective atrophy of external cortical layers in both mPFC sub-regions of stressed animals (Figure 2.4A; 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 (Figure 2.4B; 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 (Figures 2.4C and 2.4D). These effects suggest a loss of neu-

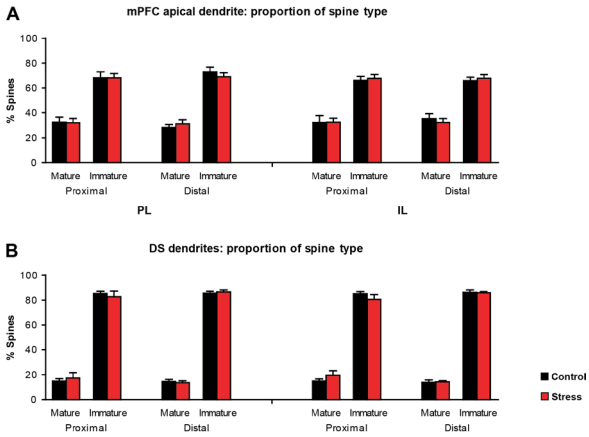

Figure 2.5. Morphological classification of dendritic spines in Golgi-impregnated neurons in the mPFC and DS.
Percentage of spine type of control and chronically stressed rats is compared. To simplify reading: mushroom, wide and ramified spines were classified as mature; and, thin spines as immature (Harris et al., 1992; Kasai et al., 2003; Takumi et al., 1999).
(A) Pyramidal neurons in layer II/III of the PL and IL cortices (PL: proximal mature, t_8 = 0.092, p = 0.929; immature, t_8 = −0.017, p = 0.987; distal mature, t_8 = −0.740, p = 0.481; immature, t_8 = 0.806, p = 0.444; IL: proximal mature t_8 = −0.026, p = 0.980; immature, t_8 = −0.355, p = 0.732; distal mature, t_8 = 0.567, p = 0.587; immature, t_8 = −0.460, p = 0.658).
(B) MSNs in the DMS and DLS (DMS: proximal mature, t_8 = −0.498, p = 0.632; immature, t_8 = 0.498, p = 0.632; distal mature, t_8 = 0.478, p = 0.646; immature, t_8 = −0.478, p = 0.646; DLS: proximal mature, t_8 = −1.074, p = 0.314; immature, t_8 = 1.074, p = 0.314; distal mature, t_8 = −0.177, p = 0.864; immature, t_8 = 0.177, p = 0.864).
Error bars denote SEM.

ronal connectivity that does not seem to result from spine loss (Figure 2.4E; 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 (Figure 2.5A), but rather to an impoverished arborization confined to distal portions (Figure 2.4F; 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 (Figure 2.6).

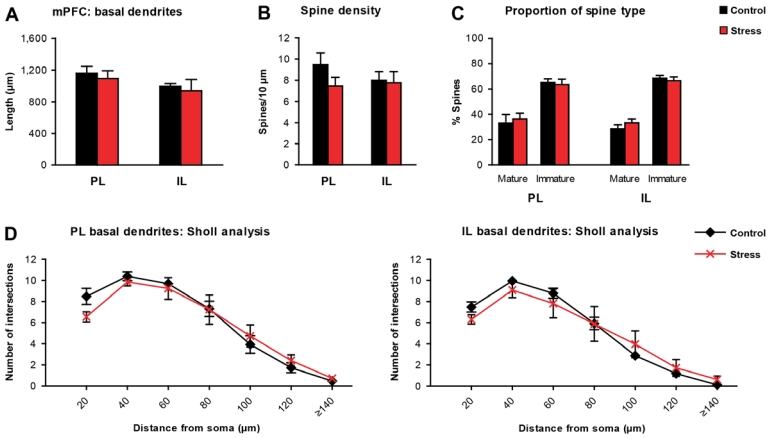

Figure 2.6. Morphometric analysis in 3D of basal dendrites in Golgi-stained pyramidal neurons of superficial layers (II/III) of PL and IL mPFC subregions.

Several measurements of control and chronically stressed rats are compared. (A) Length (PL: $t_8 = 0.508$, $p = 0.625$; IL: $t_8 = 0.371$, $p = 0.720$), (B) spine density (PL: $t_8 = 1.458$, $p = 0.183$; IL: $t_8 = 0.166$, $p = 0.872$), (C) percentage of spine type (for morphological classification, see Figure 2.5; PL: mature, $t_8 = -0.414$, $p = 0.690$; immature, $t_8 = 0.328$, $p = 0.751$; IL: mature, $t_8 = -1.081$, $p = 0.311$; immature, $t_8 = 0.513$, $p = 0.622$), and (D) differential rearrangement (PL: stress effect, $F_{1,8} = 0.074$, $p = 0.792$; IL: stress effect, $F_{1,8} = 0.023$, $p = 0.883$) of basal dendrites. Error bars denote SEM.

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 (Liston et al., 2006) and has been implicated in decision-making (Kepecs et al., 2008), 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 (Figure 2.7). In addition, no differences were found in the motor and somatosensory cortices (Figure 2.8).

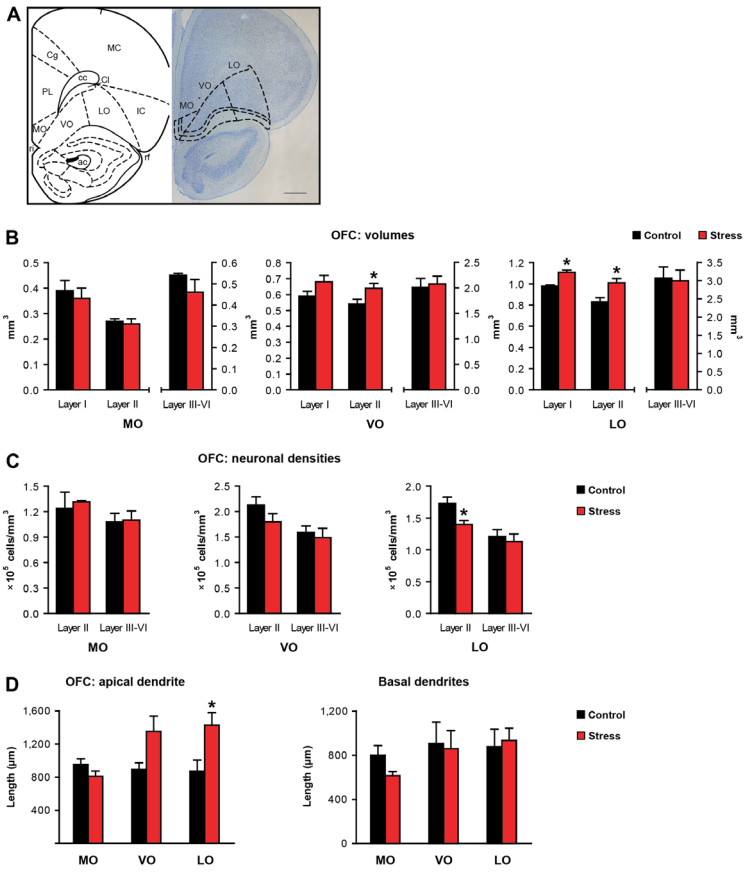

Figure 2.7. Chronic stress has a differential impact on OFC subregions.
Several structural measurements of control and chronically stressed rats are compared. (A to C) Stereological estimations of (B) volumes (MO: layer I, $t_8 = 0.620$, $p = 0.552$; layer II, $t_8 = 0.364$, $p = 0.725$; layer III-VI, $t_4 = 1.440$, $p = 0.219$; ventral orbital, VO: layer I, $t_8 = -1.776$, $p = 0.114$; layer II, $t_8 = -2.368$, $p = 0.045$; layer III-VI, $t_8 = -0.304$, $p = 0.769$; LO: layer I, $t_8 = -4.628$, $p = 0.002$; layer II, $t_8 = -2.931$, $p = 0.019$; layer III-VI, $t_8 = 0.189$, $p = 0.855$) and (C) neuronal densities (MO: layer II, $t_4 = -0.410$, $p = 0.703$; layer III-VI, $t_8 = -0.194$, $p = 0.851$; VO: layer II, $t_8 = 1.516$, $p = 0.168$; layer III-VI, $t_8 = 0.419$, $p = 0.686$; LO: layer II, $t_8 = 2.847$, $p = 0.022$; layer III-VI, $t_8 = 0.522$, $p = 0.616$). (A) Outlining between regions and layers is represented; diagram was adapted from (Paxinos and Watson, 1998) and corresponding brain slice stained with Giemsa (3.70 mm from bregma). Cg, cingulate cortex; MC, motor cortex; cc, corpus callosum; Cl, claustrum; IC, insular cortex; ri, rhinal incisura; rf, rhinal fissure; ac, anterior commissure. Scale bar, 800 µm.
(D) Morphometric analysis in 3D of Golgi-stained pyramidal neurons of superficial layers (II/III): length of apical and basal dendrites (apical dendrite: MO, $t_8 = 1.497$, $p = 0.173$; VO, $t_8 = -2.280$, $p = 0.052$; LO, $t_8 = -2.718$, $p = 0.026$; basal dendrites: MO, $t_8 = 1.933$, $p = 0.089$; VO, $t_8 = 0.189$, $p = 0.854$; LO, $t_8 = -0.286$, $p = 0.782$).
Error bars denote SEM. *$p < 0.05$.

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 (Voorn et al., 2004) and has been implicated in goal-directed actions (Yin et al., 2005), and the DLS or sensorimotor striatum, which is critical for habit formation (Yin et al., 2006) and receives input from the sensorimotor cortices (Voorn et al., 2004) and, more laterally, from the LO cortex (Schilman et al., 2008). 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

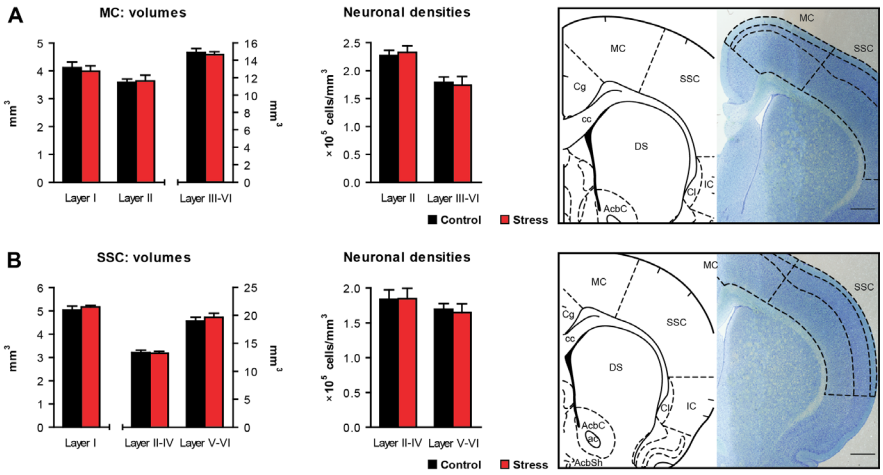

Figure 2.8. Chronic stress has no overall effect in motor and somatosensory cortices.
Stereological estimations of volumes and neuronal densities of control and chronically stressed rats are compared.
(A) Motor cortex (MC; volumes: layer I, $t_8 = 0.464$, $p = 0.655$; layer II, $t_8 = -0.210$, $p = 0.839$; layer III-VI, $t_8 = 0.453$, $p = 0.663$; neuronal densities: layer II, $t_8 = -0.359$, $p = 0.729$; layer III-VI, $t_8 = 0.248$, $p = 0.810$).
(B) Somatosensory cortex (SSC; volumes: layer I, $t_8 = -0.711$, $p = 0.498$; layer II-IV, $t_8 = 0.188$, $p = 0.855$; layer V-VI, $t_8 = -0.629$, $p = 0.547$; neuronal densities: layer II-IV, $t_8 = -0.052$, $p = 0.960$; layer V-VI, $t_8 = 0.339$, $p = 0.743$).
(A and B, right) Outlining comprising the entire motor and somatosensory cortices, and between distinguishable layers is represented; diagram was adapted from (Paxinos and Watson, 1998) and corresponding brain slice stained with Giemsa (1.00 mm from bregma). Cg, cingulate cortex; cc, corpus callosum; DS, dorsal striatum; Cl, claustrum; IC, insular cortex; AcbC, core, and AcbSh, shell, of nucleus accumbens; ac, anterior commissure. Scale bar, 800 μm. Error bars denote SEM.

habitual behavior (Figure 2.9A; see Experimental Procedures) (Yin et al., 2006; Yin et al., 2005) 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 ($t_8 = -2.343$, $p = 0.047$) (Figure 2.9A); 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 (Figures 2.9B, 2.9C, and 2.9E). We found a significant increase in dendritic arbors of DLS neurons (Figure 2.9C, length: $t_8 = -2.527$, $p = 0.035$; terminal branches

length: $t_8 = -2.563$, $p = 0.033$; Figure 2.9E, $F_{1,8} = 5.016$, $p = 0.055$) and a non-significant trend toward a reduction in the dendrites in DMS neurons (Figure 2.9C, length: $t_8 = 1.682$, $p = 0.131$; terminal branches length: $t_8 = 1.550$, $p = 0.160$; Figure 2.9E, $F_{1,8} = 2.820$, $p = 0.132$) of stressed animals. No significant effects of stress were observed in spine density (Figure 2.9D; 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 (Figure 2.5B). 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.

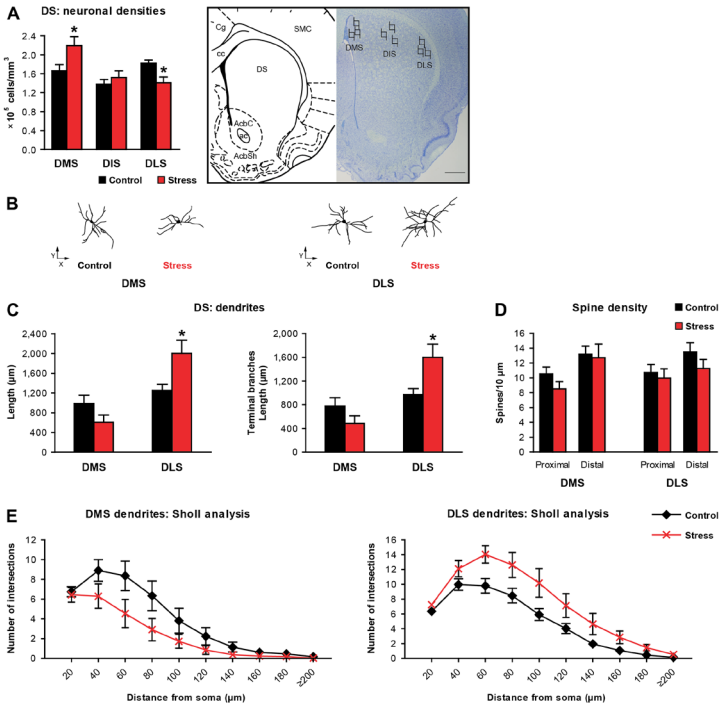

Figure 2.9. Chronic stress induces opposing modulating effects in DMS and DLS networks.
Several structural measurements of control and chronically stressed rats are compared. (A) (Left) Stereological estimation of neuronal densities. (Right) Sampling of the DMS, DIS, and DLS regions is illustrated; diagram was adapted from (Paxinos and Watson, 1998) and corresponding brain slice stained with Giemsa (1.00 mm from bregma). Abbreviations are as in Figure 2.4. 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.

DISCUSSION

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 (Balleine and Dickinson, 1998) and the DMS (Yin et al., 2005) can bias behavior to be more habitual, whereas inactivation of the DLS (Yin et al., 2006) 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 (Yin and Knowlton, 2006). 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 (Balleine and Dickinson, 1998), DMS (Yin et al., 2005), and DLS (Yin et al., 2006), 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 (Schilman et al., 2008), presents a clear hypertrophy. [The MO which projects to more medial striatal areas (Schilman et al., 2008) 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 (Killcross and Coutureau, 2003). 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 (Corbit et al., 2001; Yin et al., 2008). 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 (Sousa et al., 2000) and amygdala (Vyas et al., 2002) 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 (Dickinson, 1985). 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 (McEwen, 2007; Sapolsky, 2004). 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 (Cleck and Blendy, 2008; Koob, 2008), but certainly has a broader impact spanning activities from everyday life decisions to economics.

EXPERIMENTAL PROCEDURES

Animals

All procedures were carried out in accordance with European Union Directive 86/609/EEC and NIH guidelines on animal care and experimentation, and approved by the Portuguese DGV and NIAAA ACUC. Fifty-six male Wistar rats, aged 3 months and weighing 400-500 g, were housed 2 per cage and used as experimental subjects. From these animals, 8 controls and 8 stressed rats were tested for outcome devaluation, 15 controls and 15 stressed rats were assigned for contingency degradation; for biometric and structural analysis, 5 stressed animals and 5 controls were not submitted to behavioral training/testing but sacrificed after stress exposure or handling, respectively.

Five male Long-Evans rats, between 6 and 12 months old and weighing on average 200 g more than experimental subjects were used as residents on the social defeat stress procedure. In order to increase their territorial status, Long-Evans rats were individually housed, when cages were changed part of the old bedding was mixed with the new bedding, and were pair housed with strain and age-matched sterilized females the day before each encounter.

All animals were kept under standard laboratory conditions: 12 h light/dark cycle, at 22ºC, relative humidity of 55%, and ad libitum access to food and water, except for experimental subjects during behavioral assessment (see behavioral procedures).

Chronic unpredictable stress

Animals assigned to the stress group were exposed once a day to one of three stressors: social defeat, forced swimming and restraint. Stressors were randomly distributed throughout a 21 day period and arbitrarily scheduled within three different times of the day. Controls were handled daily during the same period and at the same schedules. This type of chronic stress paradigm, mixing different stressors (endowed with physical and psychological components) that are presented in an unpredictable schedule, has previously been shown to result on persistently elevated plasma levels of corticosterone (Sousa et al., 1998) and is thought to better mimic the variability of stressors encountered in daily life (Joels et al., 2004; Sousa et al., 1998).

Social defeat was based on the resident-intruder paradigm (Rygula et al., 2006). In brief, 15 min after the female had been removed from the resident's cage, the experimental male (intruder) was placed inside. The animals were allowed to interact for a maximum of 10 min but usually experimental subjects took no more than 3-5 min to be defeated by the residents, as indicated by the overall behavior and submissive posture (escape, freezing, defensive upright, vocalization). Immediately afterwards, the intruder was physically separated from the resident by an acrylic divider with holes within the resident cage for further 60 min. To avoid individual differences in intensity of defeat, each day the intruders were confronted with another resident, randomized to maximize the time between repeated encounters. In forced swimming, animals were placed inside a 20-cm-diameter cylinder half-filled with 24 ± 1ºC water during 10 min. Regarding restraint stress, rats were immobilized inside sized-fit PVC tubes for 30 min.

Biometric parameters

As an index of stress treatment efficacy, body weights were recorded on a weekly basis throughout the study and corticosterone levels were

measured in blood serum, collected via tail venipuncture at least 8 h after the last stress exposure (4 h before "lights off") and before introduction to food deprivation, by radioimmunoassay. From the animals sacrificed after stress exposure or handling, post-mortem thymus weights were also assessed.

Behavioral procedures

Following stress exposure, behavior was assessed using two different instrumental tasks. Behavioral training and testing took place in operant chambers (30.5 cm L × 24.1 cm W × 21.0 cm H) 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. Twelve hours after the last stress exposure and 18 h before training started, animals were placed in a food deprivation schedule, having access to food during 1 h per day after the training 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 for and the devaluation test were based on previous work (Hilario et al., 2007). During training, one reinforcer was delivered in the operant chamber contingent upon lever pressing, and the other reinforcer was presented freely in the home cage and used as a control for the devaluation test. The reinforcer and lever used were counterbalanced across groups. Following one day for a magazine training session (30 min, on average 30 reinforcers delivered on a random time 60 s schedule), animals were trained (one session per day during 30 min or until 30 reinforcements) in increasing difficulty schedules of reinforcement: 2 days of continuous

reinforcement (CRF), 2 days of random ratio-5 (RR-5), 2 days of RR-10 and finally 7 days of RR-20 (on average one reinforcer every 20 lever presses). Using a reversible devaluation paradigm, animals were tested at two different phases of training: after the first day of RR-20 (early devaluation), and again after the last training day. The devaluation test commenced 24 h after the previous training day, and lasted 2 days. On each day rats were given ad libitum exposure to one of the reinforcers for 1 h in a separate cage. Rats were allowed to consume either the reinforcer earned by lever pressing (devalued condition), or the one they received for free in their home cage (valued condition), so devaluation was achieved by sensory specific satiety. The amount of reinforcer consumed during the ad libitum session was recorded, to check if all subjects were consuming at least 5 g of each reinforcement and to test for free reinforcer consumption between groups. Immediately after, rats were given a 5 min test in extinction with the training lever extended. The order of the valued and devalued condition tests (day 1 or day 2) was counterbalanced across groups.

Procedures for contingency degradation were conducted similarly to what has been described in previous work (Yin et al., 2005). Each animal was trained to press left and right levers for pellets and sucrose respectively, with these contingencies counterbalanced across groups. Animals had 2 sessions per day, one for each lever/reinforcer, with at least 1 h break between sessions and the order of the sessions alternated each day. Similarly to training for outcome devaluation, following one day for magazine training sessions (30 min for each reinforcer, on average 30 reinforcers delivered on a random time 60 s schedule) animals were trained (during 30 min or until 30 reinforcements for each session) in increasing difficulty schedules of reinforcement: 2 days of CRF, 2 days of RR-5, 2 days of RR-10 and finally 6 days of RR-20. Correct acquisition of the response-outcome associations was evaluated after the first day of RR-20 (early in training) using a devaluation test. Devaluation was achieved by the same procedure described above but

followed, immediately after each feeding session, by a 5 min choice extinction test on the two levers. After 6 days of RR-20, animals were trained in degradation for 2 days in which, for each animal, one instrumental outcome continued to be obtained in a RR-20 schedule, while the other instrumental outcome was delivered noncontiguously such that its probability of delivery in each second of the training session was equally likely if the animals responded appropriately or not (random time schedule adjusted to the average reinforcement rate of the last day of acquisition training). For half of the rats, the response-pellet contingency was degraded, and for the other half the response-sucrose contingency was degraded. As for the acquisition training, 2 sessions were given each day, one for each lever (during 30 min or until 30 reinforcements), with a break between sessions and the order of the sessions alternated. After degradation training, the rats received a 5 min choice extinction test on the two levers as the primary test of the effects of contingency degradation training.

Histological procedures

In order to examine the mechanisms through which previous exposure to chronic stress predisposes animals to readily shift from goal-directed actions to habits, structural analyses were performed after chronic stress exposure but before behavioral training. The day after the last stress exposure, 5 animals from each group were transcardially perfused with 0.9% saline under deep pentobarbital anesthesia. Brains were removed and split into two hemispheres by a midsagittal section. Right hemispheres were processed for stereology [see (Cerqueira et al., 2005) for details]; briefly, hemispheres were postfixed in 4% paraformaldehyde for 4 weeks, subsequently embedded in glycolmethacrylate and 30 μm coronal sections were collected and stained with Giemsa [the shrinkage factor, calculated according to (Madeira et al., 1990), was 1.07 for both controls and stressed rats]. Left hemispheres were processed for 3D morphometric analysis of neurons [see (Cerqueira et al., 2007b) for

details]; briefly, hemispheres were immersed in Golgi-Cox solution for 14 days, then transferred to a 30% sucrose solution (minimum 3 days) before 200 µm coronal sections were collected and developed.

Structural analysis

To minimize bias, each brain was coded to keep the experimenter blind to the treatment.

Volumes and neuronal densities were estimated for: PL and IL subregions of the mPFC; MO, VO and LO subregions of the OFC; the entire MC (comprising areas M1 and M2); the entire SSC (comprising areas S1 and S2). Neuronal densities were estimated for: DMS, DLS and DIS (intermediate area between DMS and DLS).

Region boundaries of the mPFC, OFC, entire MC and SSC were outlined according to (Paxinos and Watson, 1998), based on clear cytoarchitectural differences (Cerqueira et al., 2005; Palomero-Gallagher and Zilles, 2004; Zilles and Wree, 1995). Each mPFC subregion and the entire MC were further divided in three easily distinguishable layers: layer I, layer II, and layer III-VI [see (Cerqueira et al., 2005) for details]; the same criteria was applied to OFC subregions. Since all SSC subregions were included as a whole, the division considered the layers that are commonly distinguishable to all subregions: layer I, layer II-IV, and layer V-VI (Palomero-Gallagher and Zilles, 2004; Zilles and Wree, 1995).

In the striatum, neuronal densities and 3D morphometric analysis were performed within conservative limits targeting the dorsomedial and dorsolateral areas shown previously to have distinct behavioral (Yin et al., 2006; Yin et al., 2005) and physiological (Partridge et al., 2000) roles, and cortical connectivity (Voorn et al., 2004) (as illustrated in Figure 2.9A). Although analyses of volume in these striatal subregions were consistent with neuronal densities measurements (data not shown),

we reasoned that the absence of clear intrastriatal landmarks to delineate each striatal subregion could render these analyses harder to reproduce and bias our results, and therefore we opted for using the more accurate measure of neuronal density in estimating changes in the dorsal striatum.

Volumes and neuronal densities estimations were performed using StereoInvestigator software and a camera attached to a motorized microscope. Cavalieri's principle was used to assess the volume of each region [see (Cerqueira et al., 2005) for details]. Briefly, every 4th (for PL, IL, MO, VO and LO), 24th (for MC and SSC) and 12th (for DMS, DLS and DIS) section was used and its cross-sectional area was estimated by point counting at a final magnification of ×112. For this we randomly superimposed onto each area a test point grid in which the interpoint distance, at tissue level, was: 75 µm for IL and MO layers I/II; 100 µm for IL and MO layer III-VI, and PL, VO and LO layers I/II; 150 µm for PL, VO and LO layer III-VI; 250 µm for MC layers I/II, SSC layer I, and DMS, DLS and DIS; and 350 µm for MC layer III-VI and SSC layers II-IV/V-VI. The volume of the region of interest was calculated from the number of points that fell within its boundaries and the distance between the systematically sampled sections.

Average neuronal density was estimated by using an adaptation of the optical fractionator method [see (Cerqueira et al., 2005) for details]. Briefly the following sampling scheme was used: (a) as for volume estimation, every 4th, 24th and 12th section, depending on the region, were evaluated; (b) the boundaries of the mPFC, OFC, MC and SSC layers/regions were defined as above; in contrast, the limits of the DMS and DLS followed a "restricted" criterion and included only the most dorsomedial and dorsolateral areas of the DS (as illustrated in Figure 2.9A) – this strategy was used to ensure that the estimation of the neuronal density was confined to the DMS and DLS, respectively; (c) a grid of virtual 3D boxes (30 × 30 × 15 µm) was superimposed on each section and the sampling was calculated to estimate density in 5

boxes per area; and (d) neurons were counted whenever their nucleus (identified by size, shape and the presence of prominent nucleoli) came into focus within the counting box.

Dendritic arborization and spine numbers and shape were analyzed for pyramidal neurons in layer II/III of the PL and IL cortices, and for MSNs of the DMS and DLS; dendritic arborization was also analyzed for pyramidal neurons in layer II/III of the MO, VO and LO cortices. The criteria used to select pyramidal neurons for reconstruction of both basal and apical trees were those described in (Cerqueira et al., 2007b), and were partially adapted to MSNs: (a) location as dorsal-medial and -lateral as possible for DMS and DLS, respectively (sampling following the same approach as for neuronal densities; for illustration, see Figure 2.9A); (b) full impregnation of the neurons along the entire length of the dendritic tree; (c) dendrites without significant truncation of branches; and (d) relative isolation from neighboring impregnated neurons, astrocytes or blood vessels. In order to minimize selection bias, slides containing the region of interest were randomly searched and the first 10 neurons fulfilling the criteria (maximum of 3 neurons per section) were selected. For each selected neuron, all branches of the dendritic tree were reconstructed at 600× (oil) magnification using a motorized microscope with a camera attached and Neurolucida software. A 3D analysis of the reconstructed neurons was performed using NeuroExplorer software. Several aspects of dendritic morphology were examined: total dendritic length; number of basal dendrites; number of dendritic branches; terminal branches length; and arrangement of dendritic material by a 3D version of Sholl analysis of intersections [see (Cerqueira et al., 2007b) for details]. Dendritic spine density was determined in branches that were either parallel or at acute angles to the coronal surface of the section. Segments were randomly selected in basal branches and proximal and distal apical branches of pyramidal neurons [see (Cerqueira et al., 2007b) for details], and in proximal and distal parts of the dendritic tree of MSNs [see (Li et al., 2003) for details].

To assess changes in spine morphology, spines in the selected segments were classified into mature (mushroom, wide and ramified spines) and immature (thin spines) (Harris et al., 1992; Kasai et al., 2003; Takumi et al., 1999) and the proportion of spines in each category was calculated for each neuron. All measurements for individual neurons from each animal were averaged.

Statistics

Statistical analyses were done using SPSS software. Parametric tests were applied since all clusters of data were normally distributed, as indicated either by Kolmogorov-Smirnov's or Shapiro-Wilk's tests (contingency degradation extinction data had to be square root transformed to assume a normal distribution). Repeated measures ANOVA were used to evaluate acquisition of lever presses, contingency degradation training and Sholl analysis, followed by post hoc analyses when appropriate. As per the experimental design, during the devaluation tests and contingency degradation extinction test, planned comparisons using a paired t-test were made between valued and devalued or degraded and non-degraded conditions for each group, with the null hypothesis being that there is no statistical difference between conditions and the alternative hypothesis that the two conditions are different (Hilario et al., 2007). Pre-test consumption during devaluation, all other structural data and biometric parameters were analyzed using unpaired t-tests. Statistical significance was accepted for $p < 0.05$. Results are represented as means ± SEM (although not indicative of the variability of the difference in paired tests).

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3

CHRONIC STRESS FUNCTIONALLY BIASES FRONTOSTRIATAL CIRCUITS TOWARD THE EXECUTION OF HABITS

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CONTENTS

SUMMARY

The ability to shift between different action strategies is necessary for adaptation to different environments. We previously showed that chronic unpredictable stress promotes a bias toward the execution of habits versus goal-directed actions. We also found a stress-induced divergent structural reorganization of the corticostriatal circuits mediating these different action strategies, suggesting a relative dominance of the sensorimotor striatum over associative frontostriatal circuits. Here, by following the simultaneous activity of neuronal ensembles in these circuits, we show that habitual action performance after chronic stress developed concomitantly with a decline in functional frontostriatal interactions, and a shift in the pattern of action-related activity in dorsal striatum, with the associative striatum becoming less engaged than sensorimotor striatum as training progressed. Chronic stress did not affect the baseline or dynamic range of firing rate, suggesting that the changes in frontostriatal activity were specific to action performance, corresponding to a shift in action mode.

INTRODUCTION

In everyday life actions can be learned and performed using different strategies. For instance, when we turn on the light for the first time entering our new living room, two concurrent associations occur: one, encoding the contingency between getting the light turned on (outcome) and the previous execution of the action over the light switch; another, reinforcing the link between going through the door (stimulus) and executing the action over the switch. The subsequent times entering the living room we could perform the same action either using a goal-directed (action-outcome) or habitual (stimulus-response) strategy, although physically the two movements could be very similar. During the following days/weeks these action strategies would compete for the performance of the action. However, it is easy to conceive that if no major changes occur between performing the action and getting the

outcome, it becomes simpler and more efficient to create a new habit and follow the rule of flipping on the light switch as we go through the door. Nevertheless, this imbalance toward the use of a habitual action strategy should be flexible enough to allow for online adjustments when action-consequences change; for instance, if a movement detector is now triggering the light as we enter the living room, it would be no longer appropriate to perform an action over the switch. Therefore, a successful adaptation to unpredictable environments depends on the ability to use appropriate action strategies according to the circumstances (Adams, 1982; Adams and Dickinson, 1981a; Balleine et al., 2009; Dickinson, 1985).

Successful adaptation to unpredictable environments also relies on a proper stress response (McEwen, 2007; Sapolsky, 2004). Indeed, a previous exposure to stressful events was shown to affect the mode in which actions are performed in the near future (Dias-Ferreira et al., 2009; Graybeal et al., 2011; Schwabe and Wolf, 2009; Soares et al., 2012). We previously showed that chronic unpredictable stress promotes a bias toward the execution of habits versus goal-directed actions, and that this predisposition was associated with opposing structural changes in the brain circuits mediating these different action strategies (Dias-Ferreira et al., 2009). Here, we address how such a priming effect of stress impacts on the neuronal dynamics in the brain circuits mediating these different action strategies as animals learn to perform them.

Distinct corticostriatal circuits have been implicated in the control of goal-directed and habitual action strategies. Lesions of associative corticostriatal circuits involving the prelimbic (PL) subregion of medial prefrontal cortex (mPFC) (Balleine and Dickinson, 1998) and the dorsomedial striatum (DMS) (Yin et al., 2005b) biased action performance that otherwise would be goal-directed to become habitual, whereas inactivation of the sensorimotor striatum – dorsolateral striatum (DLS) – (Yin et al., 2006) shifted a habitual action performance into a goal-

directed one, suggesting that these distinct corticostriatal circuits seem not only to support but also to compete for performing the same action (Balleine et al., 2009; Daw et al., 2005). Our previous results revealed a divergent structural reorganization of these frontostriatal circuits after chronic unpredictable stress, with dendritic atrophy of pyramidal neurons in the mPFC and medium spiny neurons (MSNs) in the DMS, and hypertrophy of MSNs in the DLS (Dias-Ferreira et al., 2009). This relative advantage of the sensorimotor network after chronic stress raised the hypothesis that the proposed competition between these corticostriatal circuits would already be biased when new actions had to be learned and performed in the near future. Here, we recorded the simultaneous activity of neuronal ensembles in these frontostriatal circuits in controls and chronically stressed mice during the learning of novel actions and the development of different action strategies. We uncovered that habitual action performance in stressed mice emerges concomitantly with a shift in the pattern of action-related activity in dorsal striatum, with DMS becoming less engaged than DLS, and with a decline in functional frontostriatal interactions.

RESULTS

Chronic stress favors a shift toward habitual performance of de novo learned actions

We first investigated if previous exposure to chronic unpredictable stress in mice affected the way de novo learned actions would be performed, as we have previously shown in rats. In two independent experiments (aimed at testing different features of action-outcome behavior), one group of mice (Figure 3.1; n = 16 per group) was trained to press a lever for a particular outcome (pellets or sucrose, counterbalanced), and another group of mice (Figure 3.2; n = 33 per group) was trained in two different action-outcome pairs – e.g., pressing the left lever would lead to pellets, and pressing the right lever would lead to sucrose – with one

training session per day for each pair (counterbalanced). Training started with 4 days of continuous reinforcement (D1-4.CRF), and progressed from day-to-day on increasing random ratio (RR) schedules of reinforcement to RR-20 (on average 1 reinforcer every 20 lever presses) that was maintained from day 3 (D3-RR) onward (see Experimental Procedures). In both instrumental tasks (Figure 3.1A and 3.2A; see Figure 3.2A for corresponding statistics), control and stressed mice increased lever pressing across training days ($F_{2.253,67.587}$ = 84.012, p < 0.001), and there was no interaction with ($F_{2.253,67.587}$ = 0.623, p = 0.558) or main effect of stress exposure ($F_{1,30}$ = 0.003, p = 0.960). Although, at this level of analysis, action performance seemed indistinguishable between stressed and control mice, we questioned whether both were performing their actions on the basis of their consequences, by testing for two different features of action-outcome behavior.

In the first instrumental task (Figure 3.1), we examined whether stressed and control mice were performing their actions based on the expected value of predicted outcomes (Adams and Dickinson, 1981b; Yin and Knowlton, 2006). In accordance with previous studies (Dickin-

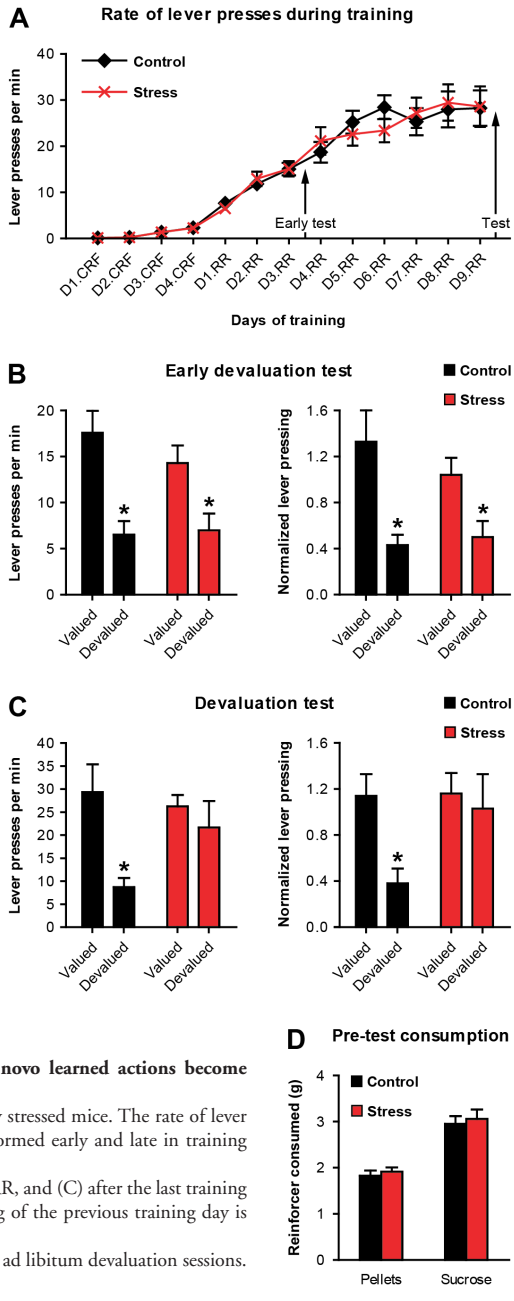


Figure 3.1. Chronic stress favors a shift to a habitual strategy as de novo learned actions become insensitive to outcome devaluation.

(A) Lever press performance throughout training for control and chronically stressed mice. The rate of lever pressing is depicted for each daily session. Reversible devaluation tests performed early and late in training are indicated.

(B and C) Devaluation test performed (B) after the first day of RR-20 – D3.RR, and (C) 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.

(D) Amount of reinforcer consumed by control and stressed mice during the ad libitum devaluation sessions. Error bars denote SEM. *p < 0.05.

son, 1985; Hilario et al., 2007), control mice trained under random ratio schedules of reinforcement biased action performance toward goal-directed strategies. In two reversible devaluation tests performed early in training (after the first day of RR-20 – D3.RR; Figure 3.1B) and after the last training day (Figure 3.1C), control mice significantly reduced their actions over the lever 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 – see Experimental Procedures; Figure 3.1B, early devaluation: lever presses per min, $t_{15} = 4.142$, $p = 0.001$; normalized lever pressing, $t_{15} = 4.126$, $p = 0.001$; Figure 3.1C, devaluation test: lever presses per min, $t_{15} = 4.222$, $p = 0.001$; normalized lever pressing, $t_{15} = 4.424$, $p < 0.001$). On the other hand, actions performed by stressed mice that early in training also showed to be sensitive to sensory-specific satiety (Figure 3.1B; lever presses per min, $t_{15} = 3.006$, $p = 0.009$; normalized lever pressing, $t_{15} = 3.011$, $p = 0.009$), with increased training became insensitive to the expected value of the outcome, as indicated by the lack of a devaluation effect (Figure 3.1C; lever presses per min, $t_{15} = 1.268$, $p = 0.224$; normalized lever pressing, $t_{15} = 1.122$, $p = 0.280$). The early devaluation test demonstrates that this insensitivity did not arise from an inability of the stressed mice to learn the relation between the action and the outcome, or from stress effects on food valuation or hedonics (Katz, 1982); also, the amount of reinforcer consumed during the ad libitum devaluation session was similar between control and stressed mice (Figure 3.1D; pellets: $t_{30} = -0.618$, $p = 0.541$; sucrose: $t_{30} = -0.404$, $p = 0.689$). This shift toward a habitual strategy was further confirmed following lever press training in the second instrumental task (Figure 3.2), as stressed mice failed to adjust their action choices (Figure 3.2C) after the contingency between one of the actions and the respective outcome had been degraded (Figure 3.2B).

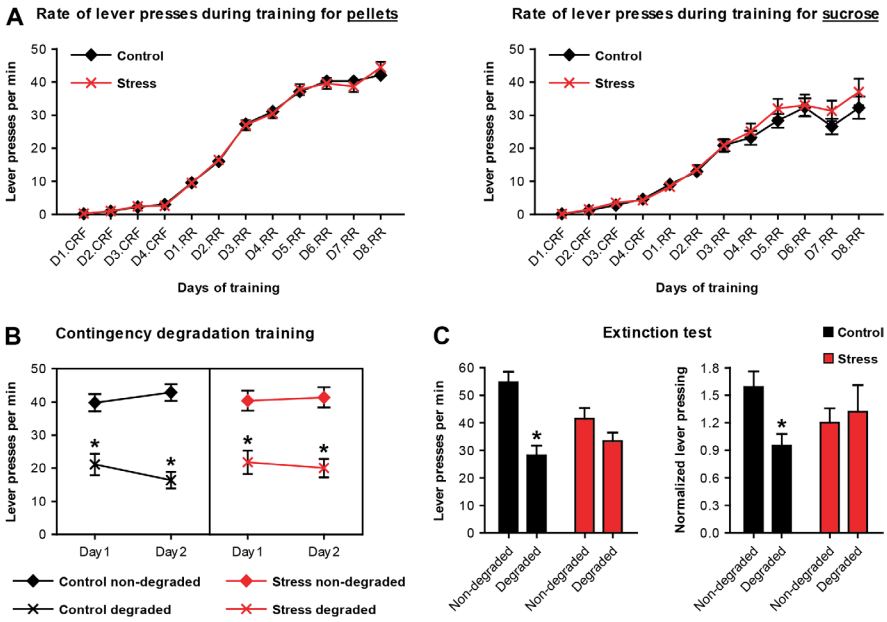


Figure 3.2. Chronic stress predisposes de novo action performance to become insensitive to changes in action-outcome contingency.

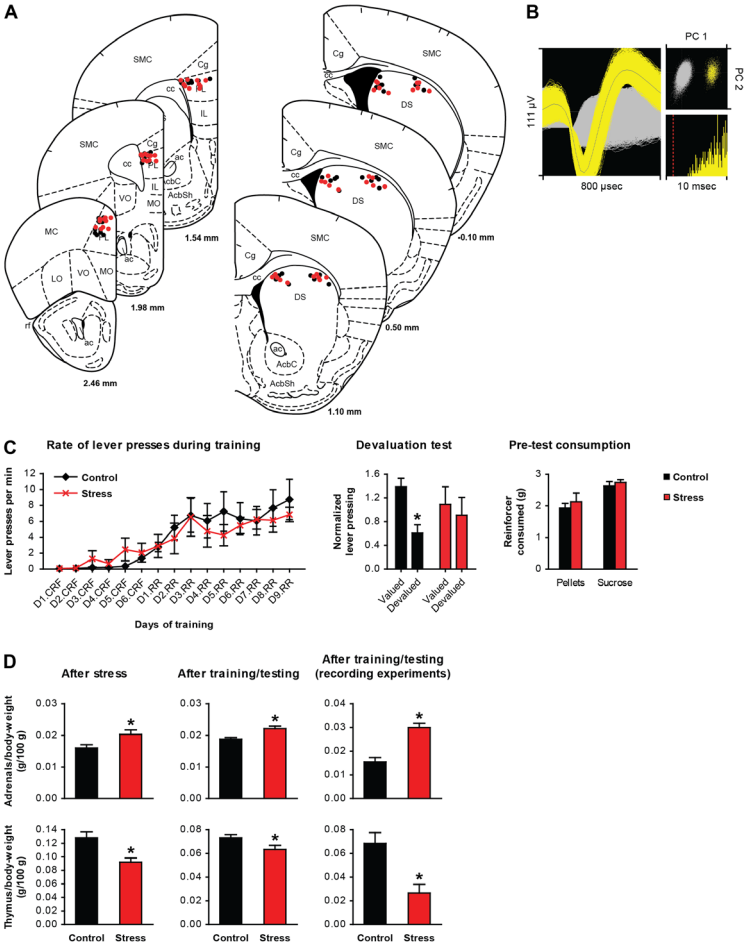
(A) Lever press performance throughout training for control and chronically stressed mice. The rate of lever pressing is depicted for each daily session for pellets and for sucrose. At this level of analysis, action performance seemed indistinguishable between control and stressed mice (pellets: training effect, $F_{4.633,296.506} = 675.825$, $p < 0.001$; training × stress interaction, $F_{4.633,296.506} = 0.520$, $p = 0.747$; stress effect, $F_{1,64} < 0.001$, $p = 0.988$; sucrose: training effect, $F_{2.105,134.708} = 159.063$, $p < 0.001$; training × stress interaction, $F_{2.105,134.708} = 0.877$, $p = 0.423$; stress effect, $F_{1,64} = 0.479$, $p = 0.491$).

(B and C) Therefore, we investigated whether the actions performed by stressed and control mice would still rely on the contingency between getting the outcome and the previous execution of the action (Hammond, 1980; Yin and Knowlton, 2006). (B) After the last training day – D8.RR, 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) for each animal (see Experimental Procedures). After 2 days of forced-choice degradation training in which both groups changed their lever pressing rate (degradation effect: control, $F_{1,32} = 43.790$, $p < 0.001$; stress, $F_{1,32} = 43.877$, $p < 0.001$; training × degradation interaction: control, $F_{1,32} = 4.694$, $p = 0.038$; stress, $F_{1,32} = 0.347$, $p = 0.560$), (C) mice were given a critical choice test between the degraded and non-degraded lever, in extinction [to avoid the confounding effects of consumption and reinforcement (Yin et al., 2005)]. Lever pressing in absolute number and normalized to the lever pressing of the last training day – D8.RR, is compared between levers for each group (lever presses per min: control, $t_{32} = 3.903$, $p < 0.001$; stress, $t_{32} = 1.323$, $p = 0.195$; normalized lever pressing: control, $t_{32} = 2.668$, $p = 0.012$; stress, $t_{32} = -0.318$, $p = 0.753$). These data indicates that stressed animals failed to choose the action that was necessary to obtain the outcome, which did not seem to arise from an inability to learn the action-outcome relations as shown by their clear preference toward the valued lever in a devaluation test performed early in training (after the first day of RR-20 – D3.RR; lever presses per min: control valued, 14.476 ± 1.767; devalued, 7.752 ± 0.691; $t_{32} = 3.774$, $p = 0.001$; stress valued, 16.206 ± 2.003; devalued, 6.052 ± 0.756; $t_{32} = 5.169$, $p < 0.001$; normalized lever pressing: control valued, 0.617 ± 0.074; devalued, 0.382 ± 0.051; $t_{32} = 2.964$, $p = 0.006$; stress valued, 0.667 ± 0.067; devalued, 0.266 ± 0.033; $t_{32} = 6.092$, $p < 0.001$; results are means ± SEM), but rather because stressed animals were already using habitual action strategies when one of the action-outcome contingencies was changed.

Error bars denote SEM. *$p < 0.05$.

A shift in the pattern of action-related neural activity emerges in the dorsal striatum with habit formation after chronic stress

We recorded the simultaneous activity of neuronal ensembles in the PL subregion of mPFC, DMS and DLS during the first instrumental task (Figures 3.3A and 3.3B, and Table 3.1; see Experimental Procedures) as distinct action strategies emerged in stressed (n = 5) and control mice (n = 5). Stressed mice implanted with multi-electrode arrays and recorded through out lever press training also became insensitive to outcome devaluation (Figure 3.3C), and showed significant biometric markers of the long-term impact of stress (Figure 3.3D) in contrast to controls.



In the three simultaneously recorded areas, in both stressed and control mice, neurons showed either a phasic increase or decrease in firing rate related to lever press (Figure 3.4A; see Experimental Procedures). When considering each of the recorded areas individually along training (Figure 3.4B), the mPFC presented no stress effect or change in the proportion of these lever press-related neurons as training progressed (training × stress interaction: $F_{2,16} = 0.190$, $p = 0.828$; stress effect: $F_{1,8} = 0.246$, $p = 0.633$; training effect: $F_{2,16} = 0.126$, $p = 0.883$). In the dorsal striatum, while no significant effects of stress and training were found in the DLS (training × stress interaction: $F_{2,16} = 0.573$, $p = 0.575$; stress effect: $F_{1,8} = 2.148$, $p = 0.181$; training effect: $F_{2,16} = 0.363$, $p = 0.701$), the proportion of neurons showing lever press-related activity in the DMS of control and stressed animals progressed in an opposite trend (training × stress interaction: $F_{2,16} = 2.804$, $p = 0.090$; or just considering D1.RR and D9.RR days of training, $F_{1,8} = 5.223$, $p = 0.052$; stress effect: $F_{1,8} = 0.083$, $p = 0.780$; training effect: $F_{2,16} = 0.255$, $p = 0.778$).

Figure 3.3. Multi-site multi-electrode recordings during the emergence of distinct lever press strategies in control and chronically stressed mice.

(A) Depiction of the verified placement of each row of electrodes for the 2 × 8 multi-electrode arrays bilaterally implanted with the eight electrode rows along the anteroposterior axis. One array targeted (right hemisphere) the PL subregion of mPFC and a second array (left hemisphere) the dorsal striatum, with one row targeting the DMS and the other the DLS. Each row is illustrated by three dots distributed along the anteroposterior axis represented by three diagrams (distance from Bregma is indicated). Diagrams were adapted from (Paxinos and Franklin, 2001). Black dots, control; red dots, stress. MC, motor cortex; LO, lateral orbital cortex; VO, ventral orbital cortex; MO, medial orbital cortex; rf, rhinal fissure; ac, anterior commissure; SMC, sensorimotor cortices; Cg, cingulate cortex; cc, corpus callosum; IL, infralimbic cortex; AcbC, core, and AcbSh, shell, of nucleus accumbens; DS, dorsal striatum.

(B) Example of an isolated single unit in the mPFC. (Left) Depiction of the extracellularly recorded waveforms of the unit (yellow) and noise (gray), (bottom right) interspike-interval histogram, and (top right) projection of the clusters correspondent to the unit (yellow) and the noise (gray) based on analysis of the first two principal components of the waveforms recorded.

(C) (Left) Lever press performance throughout training for control and chronically stressed mice during recordings. The rate of lever pressing is depicted for each daily session (training effect: $F_{3.136,28.227} = 10.869$, $p < 0.001$; training × stress interaction: $F_{3.136,28.227} = 0.585$, $p = 0.637$; stress effect: $F_{1,9} = 0.072$, $p = 0.795$). (Center) Devaluation test performed after the last training day. Normalized lever pressing is compared between the valued and the devalued condition for each group (control: $t_5 = 2.857$, $p = 0.036$; stress: $t_4 = 0.309$, $p = 0.773$). (Right) Amount of reinforcer consumed by control and stressed mice during the ad libitum devaluation sessions (pellets: $t_9 = -0.670$, $p = 0.520$; sucrose: $t_9 = -0.585$, $p = 0.573$).

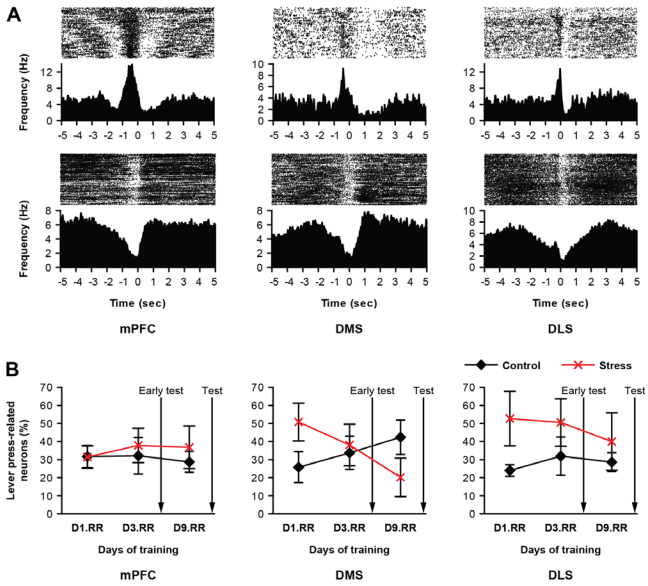
(D) Biometric parameters used as an index of the long-term impact of stress. (Top) Post-mortem adrenals and (bottom) thymus weight are compared between control and chronically stressed mice sacrificed after stress exposure (adrenals weight: $t_{36} = -2.430$, $p = 0.020$; thymus weight: $t_{26} = 3.463$, $p = 0.002$), and behavioral training/testing (adrenals weight: $t_{92} = -3.553$, $p = 0.001$; thymus weight: $t_{79.294} = 2.264$, $p = 0.026$), as well as after recording experiments (adrenals weight: $t_9 = -5.923$, $p < 0.001$; thymus weight: $t_9 = 3.477$, $p = 0.007$).

Error bars denote SEM. *$p < 0.05$.

Table 3.1. Number of units recorded in each brain area across training days.

Group	D1.RR			D3.RR			D9.RR		
	mPFC	DMS	DLS	mPFC	DMS	DLS	mPFC	DMS	DLS
Control	71	44	54	71	42	51	50	43	55
Stress	113	42	59	114	48	57	115	40	48

A detailed analysis of the proportion of positively and negatively modulated neurons showing lever press-related activity (Figure 3.5A) revealed this divergent engagement of the DMS, since the proportion of positive modulated neurons decreased significantly as goal-directed lever pressing became habitual in stressed animals. Furthermore, when considering the three simultaneously recorded areas for each day of training analyzed (Figure 3.4C), a clear effect of stress in the pattern of lever press-related activity emerged only in the last day of training, revealing that the shift toward habitual lever pressing in stressed animals is accompanied with a smaller engagement of the DMS in relation to the DLS (brain region × stress interaction: D1.RR, $F_{2,16} = 1.724$, $p = 0.210$; D3.RR, $F_{2,16} = 0.516$, $p = 0.606$; D9.RR, $F_{2,16} = 3.772$, $p = 0.045$, post hoc, stress DMS – DLS, $p < 0.05$).



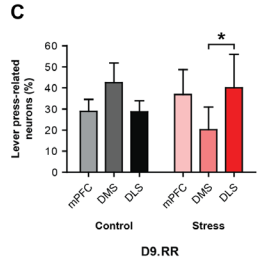


Figure 3.4. As training progresses a divergent pattern of lever press-related activity emerges in the dorsal striatum of stressed and control animals.
(A) Examples of neurons showing (top) positive and (bottom) negative modulation of firing rate in relation to lever press for the PL subregion of mPFC, DMS and DLS. The spiking activity for the same neuron is represented in a raster plot where each dot indicates a spike, and a peri-event time histogram (PETH) with time zero as the time of lever press.
(B and C) Proportion of neurons displaying lever press-related activity in the PL subregion of mPFC, DMS and DLS of control and chronically stressed mice. The percentage of lever press-related neurons is depicted (B) for each recorded area through out lever press training (devaluation tests corresponding to Figure 3.1 are indicated), and (C) for the last day of training across the three simultaneously recorded areas.
Error bars denote SEM. *$p < 0.05$.

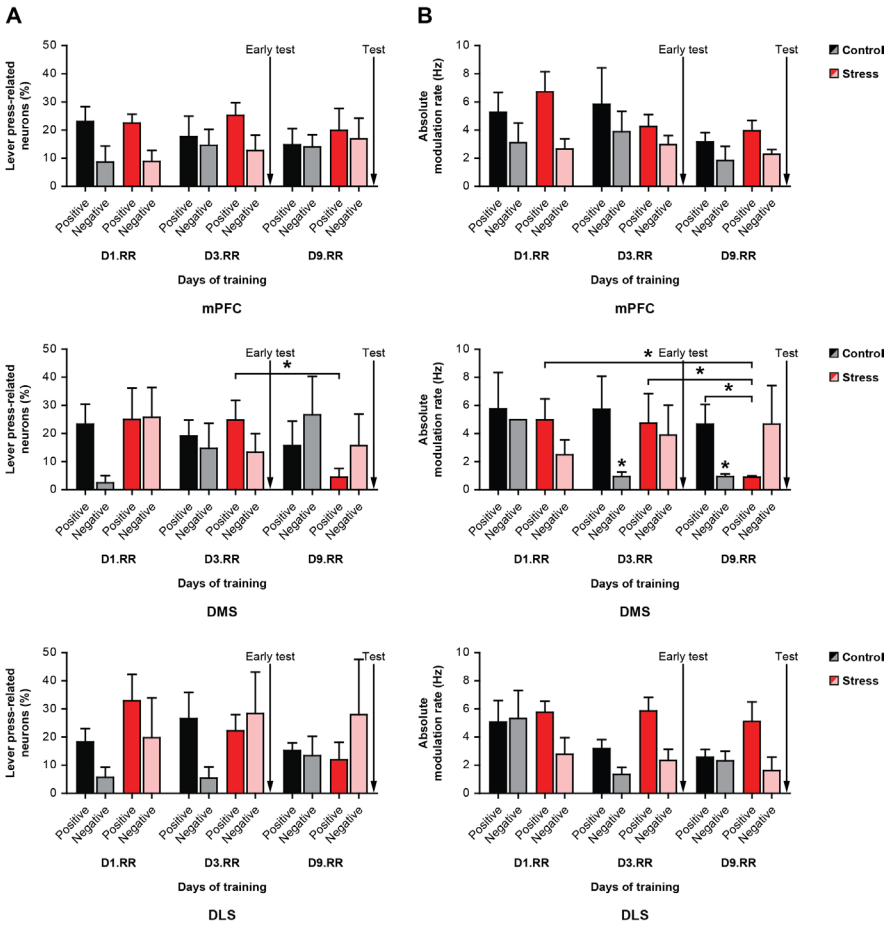


Figure 3.5. Analysis separating neurons showing a phasic increase or decrease in firing rate related to lever press.

(A) The percentage of positive and negative modulated neurons showing lever press-related activity in the PL subregion of mPFC, DMS and DLS of control and chronically stressed mice is depicted through out lever press training (devaluation tests corresponding to Figure 3.1 are indicated). Corrected multiple comparisons are performed between positive and negative modulation for each day of training and group (all, $p > 0.05$), and between control and stressed mice for each day of training and type of modulation (all, $p > 0.05$). Main effects of training for each group and type of modulation are also investigated (DMS: control negative, $\chi^2_2 = 7.429$, $p = 0.024$; stress positive, $\chi^2_2 = 6.421$, $p = 0.040$, post hoc D3.RR – D9.RR, $p < 0.05$; all remaining, $p > 0.05$).

(B) The absolute modulation rate of neurons showing positive and negative changes related to lever press in the PL subregion of mPFC, DMS and DLS of control and chronically stressed mice is depicted through out lever press training (devaluation tests corresponding to Figure 3.1 are indicated). Corrected multiple comparisons are performed between positive and negative modulation for each day of training and group (DMS: D3.RR control and D9.RR control, $p < 0.05$; all remaining, $p > 0.05$), and between control and stressed mice for each day of training and type of modulation (DMS: D9.RR positive, $p < 0.05$; all remaining, $p > 0.05$). Main effects of training for each group and type of modulation are also investigated (DMS: stress positive, $H_2 = 8.300$, $p = 0.016$, post hoc D1.RR – D9.RR and D3.RR – D9.RR, $p < 0.05$; all remaining, $p > 0.05$).

Error bars denote SEM. *$p < 0.05$.

We next examined the magnitude of the modulation around the lever press in neurons that showed lever press-related activity (Figure 3.4A; see Experimental Procedures). A balanced average of this firing rate modulation was calculated across neurons showing positive and negative changes in relation to lever press to investigate the net modulation (Figure 3.6). When considering each of the recorded areas individually along training (Figure 3.6A), the mPFC presented no stress effect or significant change in the net modulation as training progressed (training × stress interaction: $F_{2,202} = 0.043$, $p = 0.958$; stress effect: $F_{1,202} = 0.276$, $p = 0.600$; training effect: $F_{2,202} = 2.276$, $p = 0.105$). In the dorsal striatum, while no significant effects of stress and training were found in the DLS (training × stress interaction: $F_{2,105} = 0.618$, $p = 0.541$; stress effect: $F_{1,105} = 2.079$, $p = 0.152$; training effect: $F_{2,105} = 1.102$, $p = 0.336$), the DMS of stressed animals became significantly inhibited when compared to controls in the last day of training (training × stress interaction: $F_{2,84} = 0.311$, $p = 0.734$; stress effect: $F_{1,84} = 6.943$, $p = 0.010$, post hoc D9.RR, $p < 0.05$; training effect: $F_{2,84} = 1.973$, $p = 0.145$). The analysis separating the absolute modulation rate of neurons showing positive and negative changes in relation to lever press (Figure 3.5B) dissected some of the possible contributions to this overall inhibition of DMS activity. In stressed animals the positive modulation of lever press-related neurons decreased significantly in the last day of training, also becoming significantly lower than the positive modulation in controls. Fur-

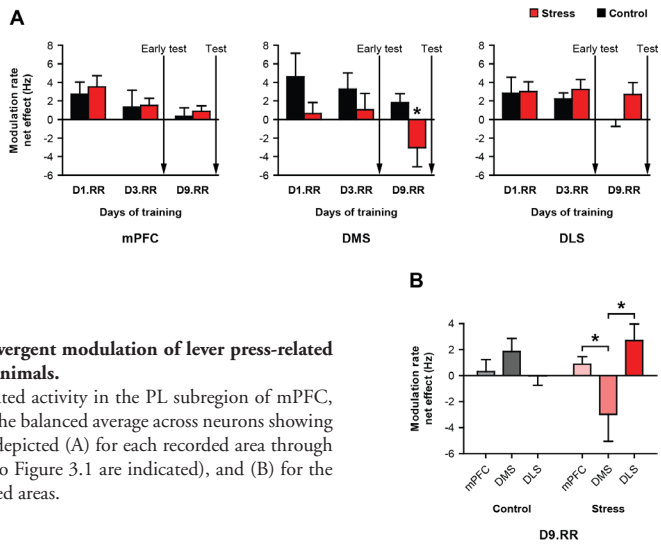


Figure 3.6. Distinct action strategies emerge with a divergent modulation of lever press-related activity in the dorsal striatum of stressed and control animals.
Net effect of the firing rate modulation of lever press-related activity in the PL subregion of mPFC, DMS and DLS of control and chronically stressed mice. The balanced average across neurons showing positive and negative changes in relation to lever press is depicted (A) for each recorded area through out lever press training (devaluation tests corresponding to Figure 3.1 are indicated), and (B) for the last day of training across the three simultaneously recorded areas.
Error bars denote SEM. *p < 0.05.

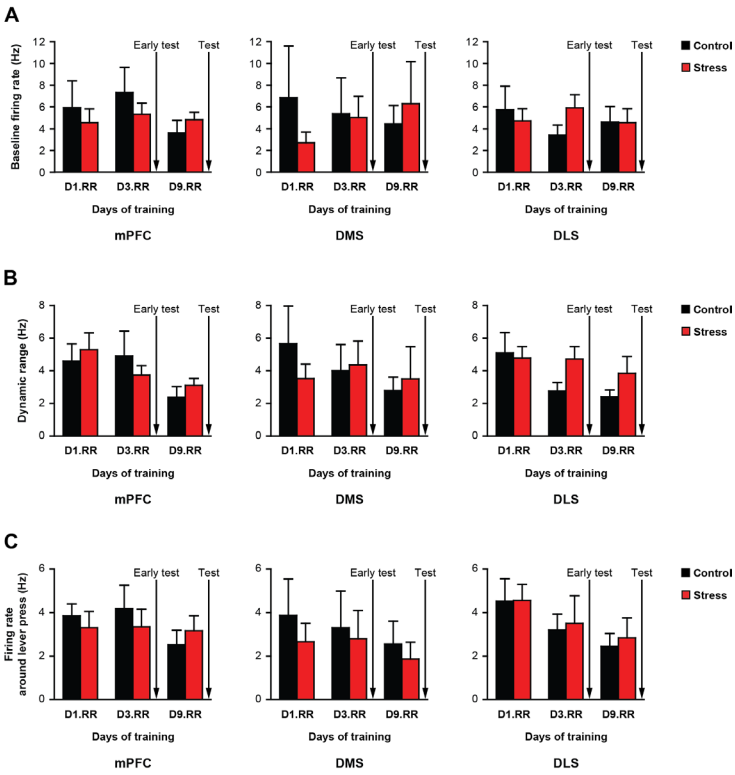


Figure 3.7. Chronic stress does not affect the baseline firing rate, the dynamic range of firing rate, or the firing rate average around lever press.

(A) The firing rate average of baseline activity across lever press-related neurons in the PL subregion of mPFC, DMS and DLS of control and chronically stressed mice is depicted through out lever press training (devaluation tests corresponding to Figure 3.1 are indicated). Baseline activity was considered from 5 to 2 sec before lever press (Figure 3.4A; see Experimental Procedures). Planned comparisons are performed between control and stressed mice for each day of training (mPFC: D1.RR, U = 415.000, p = 0.743; D3.RR, U = 598.000, p = 0.569; D9.RR, U = 317.000, p = 0.236; DMS: D1.RR, U = 71.000, p = 0.151; D3.RR, U = 130.000, p = 0.913; D9.RR, U = 62.000, p = 0.343; DLS: D1.RR, U = 167.000, p = 0.815; D3.RR, U = 180.000, p = 0.448; D9.RR, U = 113.000, p = 0.812). General main effects of training for each group are also investigated (mPFC: control, H_2 = 0.314, p = 0.855; stress H_2 = 2.273, p = 0.321; DMS: control, H_2 = 0.112, p = 0.946; stress H_2 = 2.571, p = 0.277; DLS: control, H_2 = 0.835, p = 0.659; stress H_2 = 0.637, p = 0.637).

(B) The dynamic range of the changes in firing rate related to lever press in the PL subregion of mPFC, DMS and DLS of control and chronically stressed mice is depicted through out lever press training (devaluation tests corresponding to Figure 3.1 are indicated). Planned comparisons are performed between control and stressed mice for each day of training (mPFC: D1.RR, U = 395.000, p = 0.532; D3.RR, U = 616.000, p = 0.710; D9.RR, U = 325.000, p = 0.285; DMS: D1.RR, U = 71.000, p = 0.151; D3.RR, U = 130.000, p = 0.913; D9.RR, U = 56.000, p = 0.206; DLS: D1.RR, U = 167.000, p = 0.815; D3.RR, U = 145.000, p = 0.094; D9.RR, U = 95.000, p = 0.356). General main effects of training for each group are also investigated (mPFC: control, H_2 = 2.626, p = 0.269; stress H_2 = 4.038, p = 0.133; DMS: control, H_2 = 4.608, p = 0.100; stress H_2 = 1.941, p = 0.379; DLS: control, H_2 = 5.940, p = 0.051; stress H_2 = 1.334, p = 0.513).

(C) The firing rate average around lever press across neuronal ensembles in the PL subregion of mPFC, DMS and DLS of control and chronically stressed mice is depicted through out lever press training (devaluation tests corresponding to Figure 3.1 are indicated). Firing rate around lever press was considered from -2 to 2 sec around the event. The effects of stress as training progressed are investigated (mPFC: training × stress interaction, $F_{2,16}$ = 1.932, p = 0.177; stress effect, $F_{1,8}$ = 0.059, p = 0.814; training effect, $F_{2,16}$ = 2.993, p = 0.079; DMS: training × stress interaction, $F_{1.252,10.017}$ = 0.116, p = 0.795; stress effect, $F_{1,8}$ = 0.262, p = 0.623; training effect, $F_{1.252,10.017}$ = 1.079, p = 0.342; DLS: training × stress interaction, $F_{2,16}$ = 0.029, p = 0.971; stress effect, $F_{1,8}$ = 0.068, p = 0.801; training effect, $F_{2,16}$ = 3.149, p = 0.070).

Error bars denote SEM.

thermore, when considering simultaneously the three recorded areas for each day of training analyzed (Figure 3.6B), an effect of stress in the net modulation pattern emerged only in the last day of training, revealing that habitual lever pressing in stressed animals emerged with a significant inhibition of DMS when compared to a positively modulated DLS and mPFC (brain region × stress interaction: D1.RR, $F_{2,125}$ = 1.289, p

= 0.279; D3.RR, $F_{2,144}$ = 0.635, p = 0.531; D9.RR, $F_{2,122}$ = 5.454, p = 0.005, post hoc stress mPFC – DMS and DMS – DLS, $p < 0.05$). These asymmetric changes in the modulation pattern of lever press-related activity strikingly resemble and extend the alterations observed in the proportion of lever press-related neurons after chronic stress. Taken together, these findings indicate that as training progressed, a shift in the pattern of lever press-related activity emerged in dorsal striatal circuits, with DLS being more engaged and DMS becoming progressively less engaged in stressed animals compared to controls. Remarkably, chronic stress effects on neuronal activity were not observed during baseline firing rate (Figure 3.7A) or the dynamic range of firing rate (Figure 3.7B), suggesting that the observed shift in neuronal activity emerged during lever press training leading to a shift in action mode.

Functional frontostriatal interactions decline with habit formation after chronic stress

We also investigated whether the emergence of habitual action performance in stressed mice was associated with changes in the functional interaction between these frontostriatal circuits. The dorsal striatum receives a massive, but topographically organized, input from pyramidal neurons in the PL subregion of mPFC; this projection is broader toward the DMS (Voorn et al., 2004). Importantly, medium spiny neurons in the dorsal striatum are also interconnected by local axon collaterals (Wilson and Groves, 1980). Therefore, we quantified the interaction between mPFC and DMS, mPFC and DLS, and DMS and DLS neurons, by calculating the coherence between spiking activity in these neuronal ensembles as training progressed (see Experimental Procedures). The analysis of the coherograms plotting the spike-spike coherence around lever press revealed a decrease in the coherence between mPFC and DMS (Figure 3.8A), and mPFC and DLS (Figure 3.8B) neurons in stressed animals as training progressed, which did not seem to be the case for controls. Indeed, the analysis across animals considering

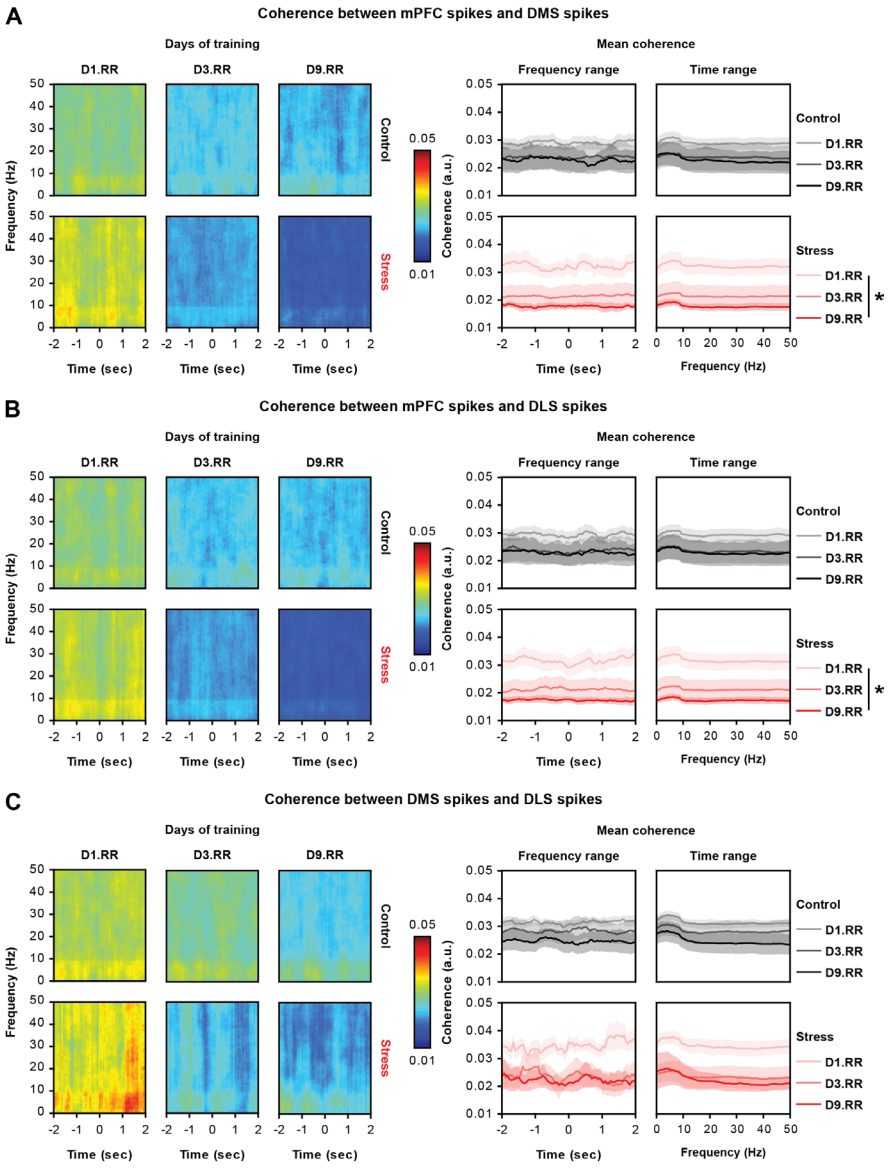


Figure 3.8. Spike-spike coherence between the mPFC and the dorsal striatum decreases with habit formation after chronic stress.

Spike-spike coherence between (A) mPFC and DMS, (B) mPFC and DLS, and (C) DMS and DLS neurons of control and chronically stressed mice through out lever press training. (Left) Coherograms showing coherence around lever press (time zero) and across frequency bands, and (right) coherence averaged in the frequency range and in the time range are depicted for each day of training analyzed for both (top) controls and (bottom) stressed mice.

(Right) Shaded regions denote SEM. *$p < 0.05$.

the mean coherence in the frequency range between mPFC and DMS, and mPFC and DLS neurons showed no changes in controls as training progressed (training effect: mPFC-DMS, $F_{2,8}$ = 1.382, p = 0.305; mPFC-DLS, $F_{2,8}$ = 0.747, p = 0.504), whereas in stressed animals a significant decrease emerged with habitual action strategies (training effect: mPFC-DMS, $F_{2,8}$ = 4.995, p = 0.039, post hoc D1.RR – D3.RR, p < 0.05; mPFC-DLS, $F_{2,8}$ = 5.166, p = 0.036, post hoc D1.RR – D3.RR, p < 0.05). These results were further confirmed in the analysis across animals considering the mean coherence in the time range (control: training effect, mPFC-DMS, $F_{2,8}$ = 0.610, p = 0.567; mPFC-DLS, $F_{2,8}$ = 0.751, p = 0.502; stress: training effect, mPFC-DMS, $F_{2,8}$ = 4.995, p = 0.039, post hoc D1.RR – D3.RR, p < 0.05; mPFC-DLS, $F_{2,8}$ = 5.165, p = 0.036, post hoc D1.RR – D3.RR, p < 0.05), which also revealed that this changes were consistent across frequency bands (control: training × frequency interaction, mPFC-DMS, $F_{1.697,6.790}$ = 1.246, p = 0.337; mPFC-DLS, $F_{1.722,6.888}$ = 0.425, p = 0.642; stress: training × frequency interaction, mPFC-DMS, $F_{2.097,8.387}$ = 0.545, p = 0.607; mPFC-DLS, $F_{1.821,7.285}$ = 1.831, p = 0.226). Interestingly, the coherence between DMS and DLS neurons (Figure 3.8C) showed a non-significant trend throughout training for both stressed animals (training effect: mean coherence in the frequency range, $F_{2,8}$ = 4.108, p = 0.059; mean coherence in the time range, $F_{2,8}$ = 4.120, p = 0.059) and controls (training effect: mean coherence in the frequency range, $F_{2,8}$ = 1.072, p = 0.387; mean coherence in the time range, $F_{2,8}$ = 1.079, p = 0.385). It is noteworthy that this decrease in coherence between mPFC and dorsal striatum neurons of stressed animals did not stem from overall changes in firing rate (Figures 3.7A and 3.7B), particularly around lever press (Figure 3.7C), or from a stress effect on the rate of lever pressing (Figure 3.3C) throughout training, as previous studies related coherence with these factors (Koralek et al., 2012; Lepage et al., 2011), thus suggesting that the observed decrease in functional frontostriatal interactions emerged with habitual action performance in chronically stressed animals.

DISCUSSION

The present results reveal that the performance of de novo learned actions after chronic unpredictable stress is accompanied by a progressive decline in functional frontostriatal interactions, and by a shift in the pattern of action-related activity in dorsal striatum, with DLS being more engaged and DMS becoming progressively less engaged as action performance became habitual. Chronic stress did not affect frontostriatal activity early in training, and did not affect baseline firing rate or the dynamic range of firing rate, suggesting that the observed shift in neuronal activity emerged concomitantly and was specific to the shift in action strategy observed in stressed animals.

According to our previous findings (Dias-Ferreira et al., 2009), de novo action learning and performance after a chronic exposure to stress would occur under rewired frontostriatal circuits. We now show that regardless of the relative advantage of DLS in the beginning of training, action-related activity in the DLS only took advantage over DMS as action performance became habitual in stressed animals. This observation could actually reflect a bias that was present in the network since the beginning of action performance, and that would lead to an imbalance in the proposed competition between these frontostriatal circuits for the control of action mode (Balleine et al., 2009; Daw et al., 2005). This competition can stem from intrastriatal competition between the associative and sensorimotor circuits, with the DMS directly gating the access of DLS to the control of action performance (Thorn et al., 2010), or from a relative advantage between different parallel circuits based on the preferential selection of different inputs arriving to the dorsal striatum. Each one or both of these mechanisms, that likely act in concordance in control animals, may be impacted by chronic stress exposure and underlie the bias toward habitual action performance. The stress effects over the structure of the PL subregion of mPFC (Dias-Ferreira et al., 2009; Radley et al., 2004) suggests a relative disadvantage of this input to dorsal striatum, particularly to DMS. By recording the simulta-

neous activity in these frontostriatal circuits, we found no chronic stress effects on the engagement of the PL cortex in action performance, but rather a progressive decline in functional frontostriatal interactions with habit formation in stressed animals. Together, these findings suggest that although chronic stress does not affect action-related activity in the PL subregion of mPFC, this cortical output would be differentially selected by dorsal striatum, probably by a mechanism of corticostriatal plasticity (Calabresi et al., 1992; Gerdeman and Lovinger, 2001; Hilario et al., 2007; Reynolds et al., 2001; Shen et al., 2008; Yin and Knowlton, 2006; Yin et al., 2005a; Yu et al., 2009). In this sense, as stressed animals become habitual, other glutamatergic inputs to the striatum, namely those arriving to the DLS, could become more relevant for the control of action performance[1]. For example, other cortical areas that send a direct input to the dorsal striatum could assume this control, for instance the sensorimotor cortices, particularly primary motor and somatosensory cortices, which target mostly the DLS (Voorn et al., 2004). However, the role of these cortical areas in the control of different action strategies is not clear and should be further explored. On the other hand, the infralimbic cortex, although not sending a direct input to the dorsal striatum (Voorn et al., 2004), could exert its control over habitual action performance via amygdalar circuits (Killcross and Coutureau, 2003; Vertes, 2004), particularly the central nucleus, which was also implicated in habit formation (Lingawi and Balleine, 2012). Finally, another subcortical circuit concerning a direct thalamic input to dorsal striatum should also be considered in the shift toward habitual action performance (Balleine et al., 2009).

[1]See Chapter 4 for further discussion of the potential mechanisms mediating the functional shift in frontostriatal activity and action strategies observed in stressed animals.

A previous chronic exposure to an unpredictable environment can elicit a physiological response – stress – that affects the mode in which actions will be performed in the near future. This bias toward the use of habitual action strategies could be interpreted as a preparatory response toward a context of uncertainty, where we cannot manipulate the probability of obtaining an outcome, and the use of a strategy in which actions would

be controlled by simple rules, a particular stimulus or state, can be highly advantageous. Nevertheless, this would only be the case if, even under some degree of uncertainty, the average outcome value or contingencies would not undergo major changes (Balleine et al., 2009; Derusso et al., 2010; Dickinson, 1985). In this sense, in a scenario where actions need to be adjusted to major changes in the policy or in our current needs, the developed bias toward the execution of habits versus goal-directed actions after exposure to chronic unpredictable stress might be highly detrimental. The herein revealed functional bias sheds light on how habitual action performance develops and is implemented in frontostriatal circuits of chronically stressed subjects, opening new avenues toward the understanding of the development of stress-related deviant behavior, as addiction and compulsivity (Cleck and Blendy, 2008; Ersche et al., 2012; Koob, 2008), or the maintenance of old habits affecting activities spanning from our everyday life to economics.

EXPERIMENTAL PROCEDURES

Animals

All procedures were carried out in accordance with European Union Directive 86/609/EEC and National Institutes of Health guidelines on animal care and experimentation, and approved by the Portuguese Direcção Geral de Veterinária and National Institute on Alcohol Abuse and Alcoholism Animal Care and Use Committee. One hundred and forty-eight male C57BL/6J mice, aged 3 months and weighing on average 27 g, were housed 4 per cage and used as experimental subjects. From these animals, 16 control and 16 stressed mice were tested for outcome devaluation, 33 control and 33 stressed mice were assigned for contingency degradation, and 7 control and 5 stressed mice were included in the recording experiments (individually housed after the surgery); for biometric analysis after stress, a total of 19 stressed animals and 19 controls, distributed between the above experiments, were not

submitted to behavioral training/testing but sacrificed after stress exposure or handling, respectively.

Twelve male C57BL/6J mice, between 6 and 12 months old and weighing on average 7 g more than experimental subjects were used as residents on the social defeat stress procedure. In order to increase their territorial status, resident mice were individually housed, when cages were changed part of the old bedding was mixed with the new bedding, and were pair housed with strain and age-matched sterilized females the day before each encounter.

Chronic unpredictable stress

Similarly to previous work (Dias-Ferreira et al., 2009), animals assigned to the stress group were exposed once a day to one of three stressors: social defeat, forced swimming and restraint. Stressors were randomly distributed throughout a 21-day period and arbitrarily scheduled within three different times of the day. Controls were handled daily during the same period and at the same schedules. This type of chronic stress paradigm, mixing different stressors (endowed with physical and psychological components) that are presented in an unpredictable manner to reduce the chances of adaptation, has previously been shown to result in hallmark signs of chronic hypercorticalism (Cullinan and Wolfe, 2000; Sousa et al., 1998), and is thought to better mimic the variability of stressors encountered in daily life (Joels et al., 2004).

Social defeat was based on the resident-intruder paradigm (Berton et al., 2006; Rygula et al., 2006). In brief, 15 min after the female had been removed from the resident's cage, the experimental male (intruder) was placed inside. The animals were allowed to interact for a maximum of 10 min but usually experimental subjects took no more than 2-4 min to be defeated by the residents, as indicated by the overall behavior and submissive posture (escape, freezing, defensive upright, vocalization). Immediately afterwards, the intruder was physically separated from the

resident by an acrylic enclosure with holes (10 cm L × 10 cm W × 10 cm H) within the resident cage for further 30 min. To avoid individual differences in intensity of defeat, each day the intruders were confronted with another resident, randomized to maximize the time between repeated encounters. In forced swimming, animals were placed inside a 20-cm-diameter cylinder half-filled with 24 ± 1ºC water during 5 min. Regarding restraint stress, mice were immobilized inside sized-fit PVC tubes for 15 min.

As a biometric index of the long-term impact of stress (Cullinan and Wolfe, 2000), post-mortem adrenals and thymus weight were assessed from the animals sacrificed after stress exposure or handling, and behavioral training/testing, as well as after recording experiments. Only perfectly excised adrenals and thymi were included in the analyses.

Behavioral procedures

After stress exposure, and following the same strategy as in previous work (Dias-Ferreira et al., 2009), behavior was assessed using two different instrumental tasks. Behavioral training and testing took place in operant chambers (21.6 cm L × 17.8 cm W × 12.7 cm H) housed within sound attenuating cubicles (Med-Associates). 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 20 mg regular "chow" pellets (formula F0071, Bio-Serv), or a liquid dipper or syringe pump that delivered 20 µl of 20% sucrose solution. A computer equipped with MED-PC IV software (Med-Associates) controlled the equipment and recorded lever presses and head entries. Twelve hours after the last stress exposure and 18 h before training started, animals were placed in a food deprivation schedule, having access to food during 1 h per day after the training session, allowing them to maintain a body weight above 85% of their baseline weight.

Water was removed on average 3 h before each daily session.

Training for and the devaluation test were based on previous work (Dias-Ferreira et al., 2009; Hilario et al., 2007). During training, one reinforcer was delivered in the operant chamber contingent upon lever pressing, and the other reinforcer was presented freely in the home cage and used as a control for the devaluation test. The reinforcer and lever used were counterbalanced across groups. Following 1 day for a magazine training session (30 min, on average 30 reinforcers delivered on a random time 60 s schedule), animals were trained (1 session per day during 30 min or until 30 reinforcements) in increasing difficulty schedules of reinforcement: 4 days of continuous reinforcement (D1-4.CRF), 1 day of random ratio-5 (D1.RR), 1 day of RR-10 (D2.RR) and finally 7 days of RR-20 (on average 1 reinforcer every 20 lever presses; D3-9.RR). Using a reversible devaluation paradigm, animals were tested at two different phases of training: after the first day of RR-20 – D3.RR (early devaluation), and again after the last training day – D9.RR. The devaluation test commenced 24 h after the previous training day, and lasted 2 days. On each day mice were given ad libitum exposure to one of the reinforcers for 1 h in a separate cage. Mice were allowed to consume either the reinforcer earned by lever pressing (devalued condition), or the one they received for free in their home cage (valued condition), so devaluation was achieved by sensory specific satiety. The amount of reinforcer consumed during the ad libitum session was recorded, to check if all subjects were consuming at least 0.5 g of each reinforcement and to test for free reinforcer consumption between groups. Immediately after, mice were given a 5 min test in extinction with the training lever extended. The order of the valued and devalued condition tests (day 1 or day 2) was counterbalanced across groups.

Procedures for contingency degradation were conducted similarly to what was previously described (Dias-Ferreira et al., 2009; Yin et al., 2005b). Each animal was trained to press a left and right lever for pel-

lets and sucrose respectively, with these contingencies counterbalanced across groups. Animals had 2 sessions per day, one for each lever/reinforcer, with at least 1 h break between sessions and the order of the sessions alternated each day. Similarly to training for outcome devaluation, following 1 day for magazine training sessions (30 min for each reinforcer, on average 30 reinforcers delivered on a random time 60 s schedule) animals were trained (during 30 min or until 30 reinforcements for each session) in increasing difficulty schedules of reinforcement: 4 days of CRF (D1-4.CRF), 1 day of RR-5 (D1.RR), 1 day of RR-10 (D2.RR) and finally 6 days of RR-20 (D3-8.RR). Correct acquisition of the response-outcome associations was evaluated after the first day of RR-20 – D3.RR (early in training) using a devaluation test. Devaluation was achieved by the same procedure described above but followed, immediately after each feeding session, by a 5 min choice extinction test on the two levers. After 6 days of RR-20, animals were trained in degradation for 2 days in which, for each animal, one instrumental outcome continued to be obtained in a RR-20 schedule, while the other instrumental outcome was delivered noncontiguously such that its probability of delivery in each second of the training session was equally likely if the animals responded appropriately or not (random time schedule adjusted to the average reinforcement rate of the last day of acquisition training – D8.RR). For half of the mice, the response-pellet contingency was degraded, and for the other half the response-sucrose contingency was degraded. As for the acquisition training, 2 sessions were given each day, one for each lever (during 30 min or until 30 reinforcements), with a break between sessions and the order of the sessions alternated. After degradation training, the mice received a 5 min choice extinction test on the two levers as the primary test of the effects of contingency degradation training.

In vivo extracellular recordings during behavior

In order to record the simultaneous activity of neuronal ensembles in

mPFC, DMS and DLS during behavior, the day after the last stress exposure, each mouse was implanted bilaterally with two multi-electrode arrays (Costa et al., 2004). The main electrode design used in this study consisted of an array of 2 × 8 platinum-coated tungsten electrodes (35 or 50 μm diameter; CD Neural Technologies), that was placed with the eight electrode rows along the anteroposterior axis, and gently lowered through craniotomies made in accordance to the array size, while simultaneously monitoring neural activity. For mPFC, the eight electrodes on each row were separated by 150 or 200 μm, the two rows distanced 200 or 250 μm, respectively, and the array was cut at a 45-50 degree angle to better fit the anteroposterior anatomy of the PL subregion of mPFC (more ventral toward the posterior part); craniotomies were centered at 2.0 mm anterior and 0.3 mm lateral to Bregma, and the most posterior microwire electrodes of the array were lowered ≈ 1.9-2.1 mm from the surface of the brain. For dorsal striatum, the eight electrodes on each row were separated by 200 μm, and the two rows distanced 1000 or 1250 μm, so that one row targeted the DMS and the other the DLS (Yin et al., 2009); craniotomies were centered at 0.5 mm anterior and 2.0 mm lateral to Bregma, and the microwire electrodes lowered ≈ 2.3-2.4 mm from the surface of the brain. Final placement of the electrodes was monitored online during the surgery based on neural activity, and then confirmed histologically at the end of the experiment (Figure 3.3A) after perfusion with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS, and brain postfixation in 4% paraformaldehyde in PBS for 24 h, vibratome sectioning (40-μm coronal slices), and cresyl violet staining. From the seven implanted controls, one was excluded from the study because the implant dropped, and another was excluded from the neural-recordings analysis since, after histological confirmation, one of the arrays was slightly misplaced.

Behavior and recordings started after 14 days of post-surgery recovery. Behavioral procedures were exactly the same as described above for the animals undergoing behavioral training/testing for outcome devalua-

tion, but now considering only one devaluation test – after the last training day –, and two minor changes in the lever press training procedure: each session took 60 min or until the same 30 reinforcements; and training progressed through the same schedules of reinforcement, but with 6 days of CRF (D1-6.CRF). These minor changes aimed at preventing potential differences in reinforcement history and action-outcome association that could emerge due to the mechanics of the recording wires.

Neural activity was recorded using the MAP system (Plexon). The activity was initially sorted using an online sorting algorithm (Plexon). Only cells with a clearly identified waveform and a relatively high signal-to-noise ratio were used (Costa et al., 2004). Behavioral timestamps and neural activity were synchronized and recorded together by sending TTL pulses from a Med-Associates interface board to the MAP recording system through an A/D board (Texas Instruments). At the end of the recording, cells were resorted using an offline sorting algorithm (Plexon) to further confirm the quality of the recorded cells and to label single- and multi-units accordingly (Costa et al., 2004). Single units displayed a clear refractory period in the interspike-interval histogram, with no spikes during the refractory period (larger than 1.3 ms; Figure 3.3B). Because initial separate analyses of single- and multi-unit data did not retrieve different results, the two data sets were combined. In agreement with previous studies (Costa et al., 2004; Costa et al., 2006; Jin and Costa, 2010), most of the units recorded in the cortex were identified as putative pyramidal neurons (≈ 80%), and the vast majority of the units recorded in the dorsal striatum were putative MSNs (≈ 95%).

Data Analysis

Analyses of neural activity were performed in Matlab (MathWorks) with custom-written programs, and the remaining statistical analyses were done in SPSS (IBM).

Lever press-related neurons and firing rate modulation throughout a session

Assessment of firing rate changes related to lever press onset was based on previous work (Jin and Costa, 2010). A peri-event time histogram (PETH), referenced to lever press, was constructed by averaging the number of spikes in 20-ms bins, shifted by 1 ms, and averaged across trials – lever presses during a session (Figure 3.4A). Distributions of the PETH from 5000 to 2000 ms before lever press were considered baseline activity. Firing rate around lever press was then compared to baseline activity by determining which 20-ms bins, slid in 1 ms steps during an epoch spanning from 2000 ms before and after the event, met the criteria for lever press-related activity. As for previous studies (Jin and Costa, 2010; Paton et al., 2006), the thresholds for a significant increase and decrease in firing rate were asymmetrically set around the baseline distribution: a significant increase in firing rate was defined if at least 20 consecutive overlapping bins had a firing rate larger than a threshold of 99% above baseline activity limited by 19 degrees of freedom (given the 20 consecutive bins necessary to reject the null hypothesis when compared to the baseline distribution); and a significant decrease in firing rate was defined if at least 20 consecutive overlapping bins had a firing rate smaller than a threshold of 99% below baseline activity considering infinite degrees of freedom, given that a minimum of 200 consecutive overlapping bins with no spikes was also considered a significant inhibition. The onset of press-related firing rate modulation was defined as the beginning of the first of the 20 or 200 consecutive significant bins, and the time window until the end of the consecutive significant bins was defined as the modulation period. The modulation peak/trough was defined as the maximal/minimal mean value of 20 consecutive bins within the modulation period. The modulation rate was then calculated from the difference between the modulation peak/trough and the average baseline firing rate.

Spike-spike coherence

The temporal relation between spikes for each pair of simultaneously recorded neurons from two different regions (mPFC-DMS, mPFC-DLS and DMS-DLS) was estimated by a coherence measure, as described previously (Koralek et al., 2012). The individual spectra (S_{xx} and S_{yy}) and cross-spectrum (S_{xy}) estimates of the simultaneous recorded spike trains were obtained using a multitaper method (Jarvis and Mitra, 2001) implemented with the Chronux cohgrampb function (http://chronux.org/), using the following parameters: 5 tapers; 500-ms window size; 50-ms time step. The coherence (C_{xy}) output was defined as:

$$C_{xy} = \frac{S_{xy}}{\sqrt{S_{xy} S_{yy}}}$$

Spike-spike coherence estimates were calculated relative to lever press, and coherence magnitude was averaged across trials, and across neuronal pairs for each animal. The obtained values were on scale with previous results for spike-spike coherence (Koralek et al., 2012), which has been shown to retrieve values that are generally lower than those for spike-field or field-field coherence (Zeitler et al., 2006).

Other statistical procedures

Statistics were performed on the values for each animal except for lever press-related firing rate modulation and associated analyses (Figures 3.3, 3.5B, 3.7A and 3.7B), because of the training-dependent occurrence of low numbers of simultaneously recorded lever press-related neurons per animal. In this case, the statistics were performed on the values for the lever press-related neurons recorded from all animals of the same experimental group.

Parametric tests were applied to data sets with a distribution not significantly different from a normal distribution, as indicated either by Kolmogorov-Smirnov's or Shapiro-Wilk's tests (including data sets that assumed a normal distribution after transformation, which was the case

of both devaluation tests for the animals only undergoing behavioral testing). General main effects were investigated using a two-way mixed ANOVA for lever press training, overall percentage of lever press-related neurons and firing rate around lever press, a two-way repeated measures ANOVA for within-subject planned analyses of contingency degradation training and spike-spike coherence, and a two-way independent ANOVA for modulation rate net effect. When appropriate, these were followed by independent t-tests and Fisher's protected least-significant difference (PLSD) tests, as post hoc analyses of general main effects of group and brain region, respectively. As per the experimental design, during the devaluation tests and contingency degradation extinction test, planned comparisons using a dependent t-test were made between valued and devalued or degraded and non-degraded conditions for each group, with the null hypothesis being that there is no statistical difference between conditions and the alternative hypothesis that the two conditions are different. Pre-test consumption during devaluation and biometric parameters were analyzed using independent t-tests.

The remaining data sets (Figures 3.5, 3.7A and 3.7B) did not fulfill the above-mentioned parametric assumptions. Statistics for percentage of lever press-related neurons that were positively or negatively modulated (Figure 3.5A) were performed on the values for each animal using non-parametric tests. Planned comparisons between type of modulation for each day of training and group, and between groups for each day of training and type of modulation were analyzed using Holm-Bonferroni-corrected Wilcoxon signed-rank tests and Mann-Whitney tests, respectively. General main effects of training for each group and type of modulation were investigated using Friedman's ANOVA followed, when appropriate, by Wilcoxon signed-rank tests as post hoc analyses.

Statistics for absolute modulation rate (Figure 3.5B), and baseline and dynamic range of firing rate (Figures 3.7A and 3.7B) were performed on the values for lever press-related neurons using non-parametric tests.

In the case of absolute modulation rate, planned comparisons between type of modulation for each day of training and group, and between groups for each day of training and type of modulation were analyzed using Holm-Bonferroni-corrected Mann-Whitney tests. In the case of baseline and dynamic range of firing rate, planned comparisons between group for each day of training were analyzed using Mann-Whitney tests. General main effects of training for each group and type of modulation, or for each group (according to the above cases) were investigated using Friedman's ANOVA followed, when appropriate, by Mann-Whitney tests as post hoc analyses.

Statistical significance was accepted for $p < 0.05$. Results are represented as mean ± SEM (although not indicative of the variability of the difference between dependent samples).

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4

DISCUSSION

CONTENTS

In this dissertation we point out a new dimension through which stress can trigger maladaptive effects. We show that a previous exposure to an unpredictable environment that elicited a physiological response – stress – is sufficient to change the mode in which de novo learned actions would be performed in the near future. Chronic unpredictable stress biased action strategies to become habitual, as actions were no longer performed based on their consequences (action-outcome association) but rather based on simple rules, and driven by particular stimuli or states (stimulus-response association; see Figure 4.1 for a possible model illustrating this behavioral bias).

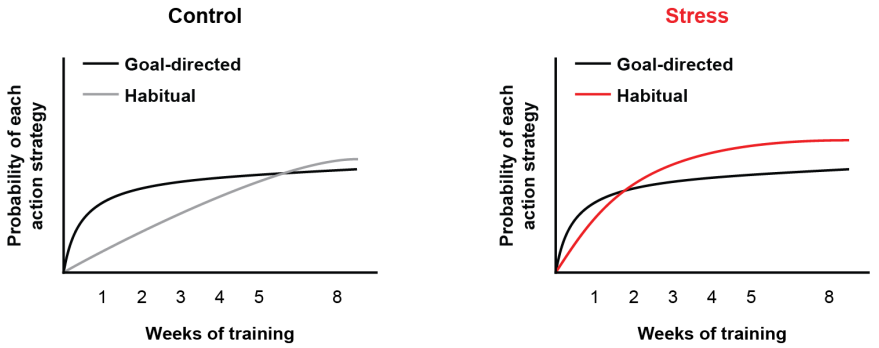

Figure 4.1. Possible model for the chronic stress induced bias toward the execution of habits versus goal-directed actions.
Actions can be driven by association with their antecedents (stimuli or states) or with their consequences (outcomes). The relative weight of each of these associations at the time of action performance will bias the probability of executing the action using a habitual or a goal-directed strategy, respectively (Balleine et al., 2009). In this thesis we show that a previous exposure to an unpredictable environment promotes an earlier shift in the balance between these different modes of performing the same action.

When investigating the corticostriatal circuits known to mediate these different action strategies, we found a divergent structural reorganization after chronic unpredictable stress, with dendritic atrophy of pyramidal neurons in the medial prefrontal cortex (mPFC) and medium spiny neurons (MSNs) in the dorsomedial striatum (DMS), and conversely dendritic hypertrophy of MSNs in the dorsolateral striatum (DLS) (Figure 4.2). These data are consistent with previous studies showing that lesions of associative corticostriatal circuits involving the prelimbic (PL) subregion of mPFC (Balleine and Dickinson, 1998) and the DMS (Yin

et al., 2005b) biased action performance that would otherwise be goal-directed to become habitual; whereas inactivation of the sensorimotor striatum – DLS – (Yin et al., 2006) shifted performance from habitual responses toward goal-directed actions, which suggest that competing corticostriatal circuits underlie the ability of animals to switch between these two modes of performing the same action (Balleine et al., 2009; Daw et al., 2005; Hilario et al., 2012).

The relative structural advantage of the sensorimotor network after chronic unpredictable stress raised the hypothesis that the proposed competition between these corticostriatal circuits would already be biased when new actions had to be learned and performed (Figure 4.1). In order to further explore this possibility, we recorded the simultaneous activity of neuronal ensembles in these frontostriatal circuits throughout learning of novel actions. We reveal that habitual action performance in chronically stressed animals emerges concomitantly with a progressive decline in functional frontostriatal interactions, and a shift in the pattern of action-related activity in dorsal striatum, with DMS becoming progressively less engaged than DLS (Figure 4.3). Chronic stress effects on frontostriatal activity were not observed early in training, and were not generalized to changes in baseline firing rate or the dynamic range of firing rate, suggesting that the observed shift in neuronal activity emerged over the course of action performance leading to a shift in action mode (Figure 4.1).

FROM THE CIRCUITS…

The structural changes reported in Chapter 2 reflect the systems level impact of stress on brain circuits. It has been shown that chronic stress, mainly through the release of corticosteroids (McEwen, 2007; Sapolsky, 1996; Sousa et al., 2008), has a differential impact on several brain regions, with dendritic atrophy of pyramidal neurons in the hippocampus (Sousa et al., 2000; Watanabe et al., 1992) and mPFC

(Radley et al., 2004), and hypertrophy of pyramidal neurons in the lateral orbital frontal cortex (Liston et al., 2006) and of pyramidal and stellate neurons in the basolateral amygdala (Vyas et al., 2002). These circuits, besides regulating the hypothalamic–pituitary–adrenal (HPA) axis (Herman and Cullinan, 1997), play an important role in cognitive functions (Squire and Zola, 1996). Indeed, the stress-induced structural reorganization of these circuits is accompanied by deficits in spatial reference (Luine et al., 1994) and working memory (Mizoguchi et al., 2000), behavioral flexibility (Cerqueira et al., 2007), and fear conditioning (Conrad et al., 1999). In the present dissertation, the revealed stress-promoted bias toward the execution of habits (stimulus-response association) versus goal-directed actions (action-outcome association) is achieved by an integrative approach, with correlations at the structural and functional level that reflect the competitive nature of the circuits previously implicated in these different action strategies. We now have a clearer picture of the effects of chronic stress beyond the traditional limbic system (McEwen, 2007; Sapolsky, 2004), by extending this knowledge to the dorsal striatum, which constitutes the entry point of the basal ganglia – a set of nuclei involved in generating and selecting appropriate actions that lead to outcomes through learning (Balleine et al., 2009; Costa, 2011; Doya, 1999; Fee and Goldberg, 2011; Graybiel, 1995; Hikosaka, 1998; Wickens et al., 2003; Yin and Knowlton, 2006).

The dorsal striatum constitutes an optimal platform for selecting appropriate action strategies (Balleine et al., 2009; Costa, 2011; Yin and Knowlton, 2006). The competition between different action strategies could stem from a serial feedforward mechanism relying on the selection of cortical inputs to striatum (Costa, 2011; Wickens et al., 2003). The medial-lateral gradient of inputs impinging on the dorsal striatum from associative and sensorimotor cortices, but also from the thalamus and amygdala, (Voorn et al., 2004) can be modulated (strengthened or weakened) based on synaptic plasticity (Reynolds et al., 2001; Wickens et al., 2003; Yin and Knowlton, 2006). In addition to lesion studies

(Yin and Knowlton, 2004; Yin et al., 2005b), the blockade of NMDA (N-methyl-d-aspartate) glutamate receptors in the DMS promotes habit formation, which suggests that action-outcome learning, or even the online maintenance of this association over the course of action learning and performance depends on ongoing plasticity at glutamatergic synapses in the DMS (Yin et al., 2005a). From the excitatory, glutamatergic inputs received by DMS, the one from pyramidal neurons in the PL subregion of mPFC deserves special interest in the context of the stress-promoted bias toward habitual action performance, given the chronic stress effects on its structure (Cerqueira et al., 2007; Radley et al., 2004) and its role in action-outcome learning (Balleine and Dickinson, 1998; Ostlund and Balleine, 2005). Interestingly, when recording the simultaneous activity in these frontostriatal circuits we found a progressive decline in functional frontostriatal interactions with habit formation in stressed animals (Figure 4.3). Despite the dendritic atrophy of pyramidal neurons, chronic stress did not affect neuronal firing rate in PL cortex during action performance (nor baseline firing rate), which suggests that the functional consequences of these structural changes in PL cortex may be more apparent downstream. So one possible mechanism could be the differential selection of inputs in the dorsal striatum at the level of glutamatergic synapses, in which inputs from the PL cortex to DMS would be weakened through plasticity, while other striatal inputs[1] would be strengthened through a process of corticostriatal plasticity. This would be consistent with thinking of the PL cortex and DMS working in a feedforward serial manner, and hence relative atrophy of the PL cortex-DMS circuit, would give advantage to hypertrophic DLS circuits.

[1]See Discussion in Chapter 2 and 3 for other glutamatergic inputs to the dorsal striatum that could become more relevant for the control of action performance.

As previously introduced in Chapter 1, the medial-lateral functional gradient in the dorsal striatum does not only rely on a gradient of cortical inputs, but is also mirrored by a gradient of differential expression of synaptic plasticity (Gerdeman et al., 2003; Hilario and Costa, 2008; Partridge et al., 2000; Yin and Knowlton, 2006). Interestingly, some

of the molecular players that are responsible for this medial-lateral gradient in plasticity are affected by chronic stress (Cerqueira, 2006; Cunha et al., 2006; de Kloet et al., 2005; Gresch et al., 1994; Hill et al., 2008; Lee and Goto, 2011; Mizoguchi et al., 2000; Pittenger and Duman, 2008; Valenti et al., 2012). It is currently thought that long-term synaptic plasticity, either in the form of long-term potentiation (LTP) and long-term depression (LTD), is a physiological mechanism important for learning (Bliss and Lømo, 1973). At synapses between cortical pyramidal neurons and MSNs, LTP was found to occur more easily in the DMS, while LTD has been shown to be easier to induce in the DLS (Partridge et al., 2000). As previously mentioned and emphasized bellow, this functional gradient correlates well with the medial-lateral gradient of some of the molecules underlying both forms of synaptic plasticity (Gerdeman et al., 2003; Hilario and Costa, 2008; Yin and Knowlton, 2006). Importantly, chronic stress has been shown to interfere with hippocampus-mPFC (Cerqueira et al., 2007) and amygdala-mPFC (Lee et al., 2011) functional interactions, which have been proposed to decline through modulation of NMDA glutamate receptor expression in mPFC pyramidal neurons (Cerqueira, 2006; Lee and Goto, 2011).

LTP induction at glutamatergic inputs to MSNs requires the activation of both NMDA glutamate receptors and D1 dopamine receptors (Kerr and Wickens, 2001; Partridge et al., 2000; Shen et al., 2008), which are more prevalent in DMS than D2 dopamine receptors (Joyce et al., 1985; Savasta et al., 1986; Yin et al., 2009). Therefore, a potential modulation of NMDA receptors in frontostriatal synapses after chronic stress could underlie the decline in functional frontostriatal interactions and the bias toward habitual action performance. On the other hand, it was also shown that is possible to induce LTP at glutamatergic inputs to MSNs through activation of A_{2A} adenosine receptors in addition to NMDA glutamate receptors (Shen et al., 2008). Interestingly, A_{2A} adenosine receptors are robustly and selectively expressed by D2-

dopamine-receptor expressing MSNs (Fink et al., 1992; Schiffmann et al., 1991; Schwarzschild et al., 2006), and D2 dopamine receptors were shown to be more abundant in DLS than in DMS (Joyce et al., 1985; Yin et al., 2009). Also, the genetic deletion of A_{2A} adenosine receptors in the striatum selectively impairs habit formation (Yu et al., 2009). These data suggest that in stressed animals an overexpression of A_{2A} adenosine receptors in the dorsal striatum, similarly to what has been reported for the hippocampus (Cunha et al., 2006), could underlie the shift in the pattern of dorsal striatum activity and the bias toward habitual action performance.

Bidirectional synaptic plasticity at corticostriatal synapses can be triggered by two LTD mechanisms that seem to act on the same corticostriatal terminals (Mathur et al., 2011). Classically, LTD at corticostriatal synapses was characterized as being dopamine and endocannabinoid signaling dependent through action on pre-synaptic CB1 receptors (Gerdeman and Lovinger, 2001; Gerdeman et al., 2002; Kreitzer and Malenka, 2005), which are more expressed in DLS than DMS (Herkenham et al., 1991), hence explaining why endoccanabinoid-dependent LTD is easier to induce in the DLS (Gerdeman et al., 2003). As previously mentioned, this mechanism of corticostriatal plasticity most probably underlies the critical role of endocannabinoid signaling through CB1 receptors in habit formation (Hilario et al., 2007). Another mechanism, recently found, is dependent on serotonin (5-HT) activation of 5-HT_{1b} receptors (Mathur et al., 2011). Chronic stress has been shown to affect both serotonin (de Kloet et al., 2005; Pittenger and Duman, 2008) and endocannabinoid (Hill et al., 2008) signaling in several brain regions, so these LTD mechanisms in dorsal striatum could potentially be involved in stress induced re-organization of striatal circuits and bias toward habits.

Finally, dopamine is involved in both LTP and LTD forms of striatal plasticity (Gerfen and Surmeier, 2011; Shen et al., 2008). As mentioned

in Chapter 1, the dopamine source and clearance in dorsal striatum also present a medial-lateral gradient. Bidirectional manipulations of dopamine levels in DLS, through lesions of the SNc input or blockade of the dopamine transporter (DAT) with amphetamine, impaired or favored habit formation, respectively (Faure et al., 2005; Nelson and Killcross, 2006). It is also worth mentioning again that amphetamine sensitization induces divergent changes in spine density in MSNs, with an increase in DLS and a decrease in DMS MSNs (Jedynak et al., 2007). Interestingly, several studies report impairments in dopaminergic activity after chronic stress (Gresch et al., 1994; Mizoguchi et al., 2000; Valenti et al., 2012). However, the implication of dopamine [probably acting on corticostriatal circuits as a feedback signal through error prediction in reinforcement learning (Schultz et al., 1997)], or any other of the above-mentioned candidates, will certainly require more specific approaches, namely through monitoring and manipulating neurotransmitter activity, with spatial and temporal resolution over the course of habit formation after chronic stress, to shed light on a potential serial feedforward mechanism relying on the selection of striatal inputs through a process of synaptic plasticity.

Our findings reveal that after chronic stress exposure the inputs to dorsal striatum will interact with rewired MSNs, suggesting that de novo action performance after chronic stress would occur not only under unbalanced serial feedforward/corticostriatal interactions, with the information arriving to the striatum being processed predominantly by MSNs in the DLS, but also under unbalanced intrastriatal competing interactions. We found that chronic stress caused opposing structural changes in the dorsal striatal circuits mediating goal-directed and habitual action performance, with dendritic atrophy of MSNs in the DMS and hypertrophy of MSNs in the DLS (Figure 4.2). In Chapter 3 we report that regardless this relative advantage of DLS in the beginning of training, action-related activity in the DLS only took advantage over DMS as action performance became habitual in stressed animals. This

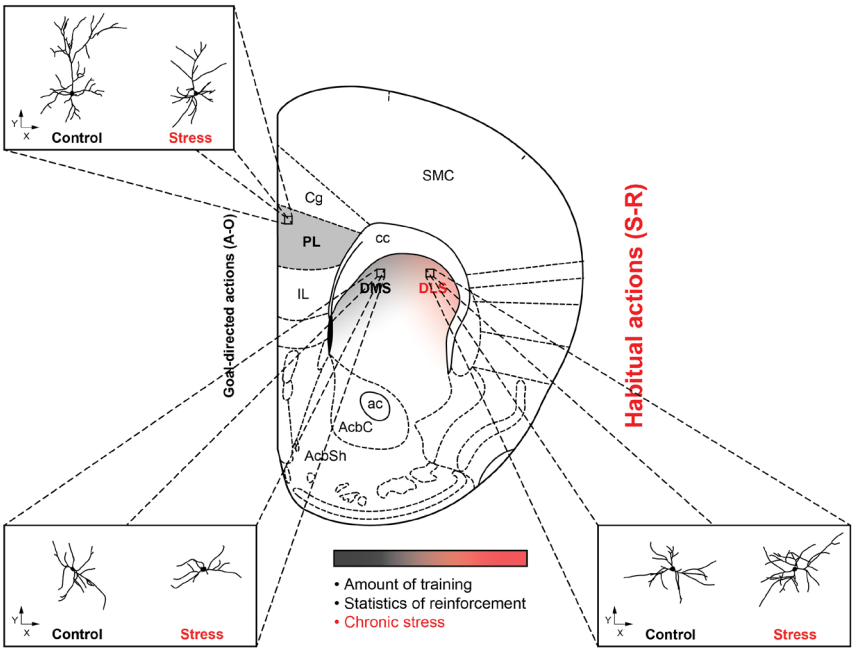

Figure 4.2. Chronic stress causes frontostriatal reorganization and promotes a bias toward the execution of habits versus goal-directed actions.
Depiction of the opposing structural changes caused by chronic stress in the associative and sensorimotor corticostriatal circuits underlying different action strategies, with dendritic atrophy of pyramidal neurons in the mPFC and MSNs in the DMS, and hypertrophy of MSNs in the DLS. The relative advantage of the sensorimotor network after chronic stress suggests an imbalance in the competition between these corticostriatal circuits for the control of action performance, leading to a bias toward the execution of actions based on their antecedents (stimulus-response – S-R) rather than taking into account their consequences (action-outcome – A-O). Adams and Dickinson noticed that the fine balance between these different action strategies relied on the amount of training and the statistics of reinforcement (Adams, 1982; Adams and Dickinson, 1981; Dickinson, 1985), here we reveal that this balance is also affected by a previous exposure to an unpredictable environment.
The diagram illustrating a coronal section of the mouse brain was adapted from (Paxinos and Franklin, 2001). Cg, cingulate cortex; SMC, sensorimotor cortices; IL, infralimbic cortex; cc, corpus callosum; AcbC, core, and AcbSh, shell, of nucleus accumbens; ac, anterior commissure.

observation could actually reflect a bias that was present in the network since the beginning of action performance (Figure 4.3), and that would lead to an imbalance in the proposed competition between these striatal circuits for the control of action mode (Balleine et al., 2009; Hilario et al., 2012) (Figure 4.1). In addition to the above discussed serial feedforward mechanism, suggesting that MSNs in the dorsal striatum become less driven by pyramidal neurons in the PL cortex with habit formation, this competition could stem from a intrastriatal competition mechanism

between MSNs in the DMS and MSNs in the DLS, with MSNs in the DMS gating the access of MSNs in the DLS to the control of action performance (Thorn et al., 2010). One possibility for this regulation or competition would be through direct feedback connectivity held by local axon collaterals (Wilson and Groves, 1980). Interestingly, the functional interactions between DMS and DLS did not decrease significantly with habit formation after chronic stress, suggesting that intrastriatal competition did not change throughout training. Therefore, the relative strength of parallel, albeit interactive, circuits that course between these dorsal striatal circuits, rather than intrastriatal competition, seem to be a better explanation for the functional bias observed in chronically stressed animals.

Dorsal striatum MSNs are inhibitory, GABA (γ-aminobutyric acid)-containing projection neurons (Kita and Kitai, 1988) that can be divided in two separate populations that project to the substancia nigra (striatonigral MSNs) and to the globus pallidus (striatopallidal MSNs) (Kawaguchi et al., 1990). The striatopallidal MSNs express D2 dopamine receptors (Gerfen et al., 1990), which are more common toward the DLS (Joyce et al., 1985; Yin et al., 2009), and the striatonigral MSNs express D1 dopamine receptors (Gerfen et al., 1990), which predominate over D2 dopamine receptors in the DMS (Joyce et al., 1985; Savasta et al., 1986; Yin et al., 2009). This level of functional organization based on the striatal output resembles the medial-lateral gradient discussed along this dissertation, and is specially interesting given that the impairment of LTP at the glutamatergic input to the striatopallidal pathway, through genetic deletion of A_{2A} adenosine receptors (Shen et al., 2008), impairs habit formation (Yu et al., 2009). Furthermore, regarding the lateral feedback inhibition held by axon collaterals, D2-expressing striatopallidal MSNs have more and stronger inhibitory projections to D1-expressing striatonigral MSNs than the inverse (Taverna et al., 2008; Tecuapetla et al., 2009). Therefore, a possible substrate for the bias in the competition between these different pathways in action control

after chronic stress could result from a potentiation of the glutamatergic transmission onto striatopallidal MSNs, also resulting in increased inhibition of striatonigral MSNs [a mechanism also proposed to underlie the functional reorganization in the striatum during the acquisition and consolidation of a skill (Yin et al., 2009)]. This is actually reflected on the shift in the pattern of action-related activity in dorsal striatum, with DLS being more engaged, and DMS becoming progressively less engaged, as action performance becomes habitual in chronically stressed animals. However, only by monitoring the activity in striatonigral and striatopallidal MSNs over the course of action performance, either by using in vivo extracellular recordings along with optogenetic based photoidentification methods (Lima et al., 2009) or by in vivo optical measurements using genetically encoded calcium indicators (Cui et al., 2012), the clarification of whether striatalpallidal MSNs can constitute the major striatal output enrolled in habitual action performance after chronic stress would be possible.

The structural and functional correlates of chronic stress induced bias toward habitual action performance presented in this dissertation (Figure 4.3) provide new insight into the effects of stress beyond the traditional limbic system (McEwen, 2007; Sapolsky, 2004), and call for a reappraisal of the impact of stress on brain circuits at the systems level, using approaches with better temporal and spatial resolution, such as opto and pharmacogenetics, that could lead to more precise behavioral correlates. Likewise, the underlying mechanisms of the revealed functional bias in frontostriatal circuits is most probably not limited to the herein proposed imbalance in serial feedforward/corticostriatal interactions and/or intrastriatal competing interactions. Many other alternatives spanning from chronic stress effects on a top-down arbitration over the access of associative and sensorimotor dorsal striatal circuits to the control of action performance (Isoda and Hikosaka, 2007), to chronic stress induced changes on the continuous internal state update by bottom-up circuits (Aponte et al., 2011) should

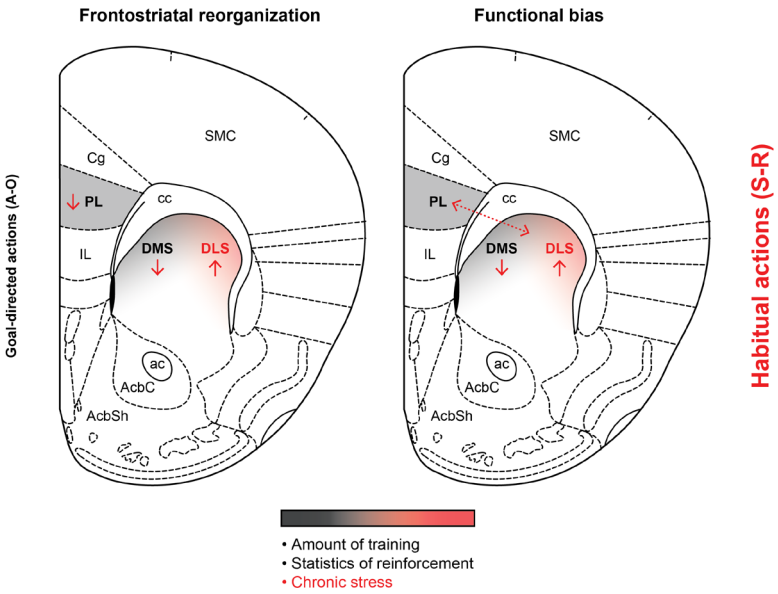

Figure 4.3. Structural and physiological correlates of the chronic stress induced bias toward habitual action performance.
In this dissertation we show that a previous chronic exposure to an unpredictable environment, capable of eliciting a physiological response – stress – important for adaptation, promotes a bias toward the execution of habits versus goal-directed actions. (Left) In Chapter 2, this predisposition is associated with a divergent structural reorganization of the corticostriatal circuits mediating these different action strategies, suggesting a relative advantage of the sensorimotor striatum over associative frontostriatal circuits after chronic stress. (Right) In Chapter 3, by following the simultaneous activity of neuronal ensembles in these circuits, we show that action performance after chronic stress progressed not only with a decline in functional frontostriatal interactions but also with a shift in the pattern of action-related activity in dorsal striatum, with the sensorimotor striatum being more engaged and the associative striatum becoming progressively less engaged as action performance becomes habitual.
The diagram illustrating a coronal section of the mouse brain was adapted from (Paxinos and Franklin, 2001). Abbreviations are as in Figure 4.2.

also be taken into consideration. For the time being, the present results provide further insight into how different action strategies are encoded, but especially how chronic unpredictable stress biases action strategies toward the performance of habits versus goal-directed actions, exposing some of the circuit changes underlying this adaptive and/or maladaptive response to a continuously changing environment.

… TO ADAPTIVE BEHAVIOR OR DISEASE

Selye defined stress as the physiological and adaptive response to any stimulus perceived as threatening or demanding (Selye, 1936). Normally, after exposure to a stressor, corticosteroids act in the brain to restore physiological and behavioral homeostasis (de Kloet et al., 2005; McEwen, 2007; Sapolsky, 2004). However, when the intensity, duration or unpredictability of stressors exceeds a certain individual-specific threshold, the recruitment of adequate adaptive mechanisms can fail (McEwen, 2007; Sapolsky, 1996; Selye, 1976). Chronic stress has been implicated in behavioral changes (de Kloet et al., 2005; McEwen, 2007; Sapolsky, 1996); in this dissertation we examined whether a previous exposure to a chronic unpredictable environment would affect adaptation to a new environment by altering the mode in which actions would be performed in the near future.

Actions can be driven by association with their antecedents (stimuli or states) or with their consequences (outcomes). The relative weight of these associations at the time of action performance will bias if the action is executed using a habitual or a goal-directed strategy, respectively (Balleine et al., 2009). Adams and Dickinson noticed that the fine balance between these different action strategies relied on the amount of training and the statistics of reinforcement (Adams, 1982; Adams and Dickinson, 1981; Dickinson, 1985; Dickinson et al., 1983), and more recently this balance was also shown to vary as a function of unpredictability (Derusso et al., 2010). Here, we show that a previous chronic exposure to an unpredictable environment, capable of eliciting a sustained stress response, promotes a bias toward habitual action performance (Figure 4.1).

We produce several lines of evidence indicating that a previous exposure to chronic unpredictable stress affects the ability of animals to perform actions based on their consequences. First, after training under schedules in which control animals were performing their actions based on the

expected value of predicted outcomes, stressed animals became insensitive to outcome devaluation by sensory specific satiety. Importantly, stressed animals were sensitive to outcome devaluation in a test performed early in training, demonstrating that this insensitivity did not arise from an inability of stressed animals to learn the relation between the action and the outcome, or from stress effects on food valuation or hedonics (Katz, 1982). Second, while the actions performed by control animals were still dependent on the contingency between getting the outcome and the previous execution of the action, stressed animals became insensitive to changes in action-outcome contingency, indicating that their actions were no longer performed because they were necessary to obtain the outcome. These two criteria are essential to characterize goal-directed instrumental performance (Dickinson, 1985; Dickinson and Balleine, 1993; Yin et al., 2008). It is important to emphasize that some Pavlovian responses can also be sensitive to outcome devaluation (Holland and Rescorla, 1975); therefore an impairment in goal-directed action performance should also be supported by manipulations of the action-outcome contingency allowing for the distinction from the stimulus-outcome contingency governing Pavlovian responses (Davis and Bitterman, 1971; Dickinson and Charnock, 1985; Rescorla, 1968; Yin et al., 2008). The results presented in this dissertation allow to infer that chronic unpredictable stress biased action strategies to become habitual, as actions were no longer performed based on their consequences (action-outcome association) but became controlled by simple rules, a particular stimulus or state (stimulus-response association).

The nature of the stimulus proposed to trigger habitual action performance is still a matter of debate. Given that the probability of performing an action is dependent on its consequences but also on its antecedents (e.g. discrete or more general/contextual cues), Pavlovian conditional responses to these antecedent stimuli can exert a strong influence on the selection and performance of instrumental actions (Estes, 1948; Holland, 2004). This effect can be tested using the

Pavlovian-to-instrumental transfer paradigm (PIT), in which animals are trained separately to associate a discrete cue with an outcome (Pavlovian training), and then to perform an instrumental action for the same outcome (instrumental training). Afterwards on probe trials, the impact of presenting the discrete cue on instrumental response is assessed. Two forms of PIT have been identified (Corbit and Balleine, 2005). The outcome-specific PIT is based on the choice between two actions mediated by the predictive status of a cue with respect to one specific outcome as opposed to the other. This form of PIT was recently shown to be impaired in chronically stressed animals, as instrumental responding related to a specific outcome was not specifically elicited by the correspondent cue (Morgado et al., 2012), which suggests that the herein revealed bias toward the use of a stimulus-response association after chronic stress is not mediated by this form of PIT. However, the effects of chronic stress on a general form of PIT, based on more generally arousing effects of a single outcome-related cue over the performance of a single instrumental action, remains to be tested. It is noteworthy that recent work has shown that more general/contextual cues seem to be sufficient to trigger habitual action performance (Gremel and Costa, 2012; Ostlund et al., 2010), suggesting a role for this general form of PIT in habit formation after chronic stress.

Uncertainty is not only a problem in decision-making, but is a prevalent quality in natural environments, and thus appropriate coping strategies must have been selected throughout evolution. Nature "equipped" us with the ability to generate and select novel actions on the basis of their consequences and on our needs at the time of a decision. The acquired behavioral plasticity or repertoire of actions that can be generated increases the probability of responding in an appropriate way to changing situations (Changeux and Dehaene, 1989; Costa, 2011). Fitness optimization in unpredictable environments is addressed in bet-hedging evolutionary theories (Seger and Brockmann, 1987). The bet-hedging diversifying trait preconizes that hedging our actions by

spreading the risk in a range of phenotypes can increase the chances of adaptation (Beaumont et al., 2009; Simons, 2011). So, how come did we develop a mechanism that reduces behavior variability by promoting automatization with habit formation after exposure to an unpredictable environment? Actually, an alternative trait to diversity bet-hedging, the conservative bet-hedging is often compared to an insurance policy, where the clients are willing to trade a small-expected monetary loss (i.e., by decreasing variability in action generation) in exchange of a stable financial situation (i.e., by ensuring an average reinforcement rate) (Simons, 2011). In this context, the bias toward habitual action performance could be interpreted as an adaptive response toward a context of uncertainty. When behavior is repeated regularly for extensive periods under uncertain situations where we cannot manipulate the probability of obtaining an outcome, but the average outcome value and contingencies are stable, general rules and habits can be advantageous (Balleine et al., 2009; Derusso et al., 2010; Dickinson and Charnock, 1985).

In this sense, the physiological response – stress – elicited by the exposure to an unpredictable environment could well be embedded with adaptive properties. However, we have to interact within a world of increasing complexity, where major changes in the policies, but also a continuous reshaping of our current needs demand a permanent readjustment of our everyday life decisions. In this sense, the chronic stress induced bias toward habitual action strategies can be maladaptive. It is important to mention that previous studies suggest that the herein reported chronic stress effects at the circuit and behavioral levels are reversible in young adults after a stress-free period (the so called vacations) (Bloss et al., 2010; Radley et al., 2005; Soares et al., 2012; Sousa et al., 2000), denoting the plastic potential of brain circuits. Understanding how stress shapes brain circuits, and how circuits generate actions, always keeping in mind the plastic potential of these circuits, could lead us to better clinical approaches for stress-related disorders. This is of special relevance given

the central role of habit formation in stress-related deviant behaviors, as addiction and compulsivity (Cleck and Blendy, 2008; Corbit et al., 2012; Ersche et al., 2012; Everitt and Robbins, 2005; Koob, 2008; Nelson and Killcross, 2006), or the maintenance of old habits affecting activities spanning from our everyday life to economics.

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Everything will be all right in the end. So if it's not all right, it is not yet the end.

Broadbent, G. (Producer), Parker, O. (Writer), & Madden, J. (Director) (2012). The Best Exotic Marigold Hotel [Motion picture]. United Kingdom: 20th Century Fox.