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

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Abstract
Stress exposure triggers cognitive and behavioral impairments that influence decision-making processes. Decisions under a context of uncertainty require complex reward-prediction processes that are known to be mediated by the mesocorticolimbic dopamine (DA) system in brain areas sensitive to the deleterious effects of chronic stress, in particular the orbitofrontal cortex (OFC). Using a decision-making task, we show that chronic stress biases risk-based decision-making to safer behaviors. This decision-making pattern is associated with an increased activation of the lateral part of the OFC and with morphological changes in pyramidal neurons specifically recruited by this task. Additionally, stress exposure induces a hypodopaminergic status accompanied by increased mRNA levels of the dopamine receptor type 2 (Drd2) in the OFC; importantly, treatment with a D2/D3 agonist quinpirole reverts the shift to safer behaviors induced by stress on risky decision-making. These results suggest that the brain mechanisms related to risk-based decision-making are altered after chronic stress, but can be modulated by manipulation of dopaminergic transmission.

1. Introduction

Decision-making processes are complex and influenced by multiple factors, but can be described as a basic algorithm consisting of representation, valuation and action selection

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steps, in which computation of the value associated with each potential action is the determinant element (Rangel et al., 2008). Research in both animals and humans revealed that the attribution of value, factoring expectation (the balance between value and effort) and uncertainty (the probability of a given outcome), is carried by a network comprising the medial prefrontal cortex (mPFC) and the orbitofrontal cortex (OFC), as well as subcortical limbic regions, including the dorsal striatum and nucleus accumbens (Doya, 2008). In addition, manipulations of the dopamine (DA) system, one of the main neurotransmitter systems modulating mPFC/OFC activity, have also been shown to impair decision-making processes (Simon et al., 2009; St Onge et al., 2010; Zeeb et al., 2009), specifically those involving behavior under uncertain/risk contexts (St Onge and Floresco, 2009).

Chronic stress exposure triggers plastic changes in brain areas involved in valuation and decision-making, including the prefrontal cortex and the striatum (Cerqueira et al., 2007a). More importantly, we have also shown that these changes induced by prolonged stress correlate with altered decision-making processes, such as a shift from goal-directed to habit-based choices (Dias-Ferreira et al., 2009) and an impairment in pavlovian-to-instrumental transfer (Morgado et al., 2012). However, to the best of our knowledge, no study has addressed the impact of chronic stress on decisions involving risk/uncertainty in animals. Thus, in the present study we used a paradigm in which rats choose between certain (safe) and uncertain (risky) options, with similar overall expectations and predictability (Morgado et al., 2014) to assess the impact of chronic stress exposure on risk-taking behavior. Subsequently, we correlated behavioral performance with alterations in the neuronal structure and in the dopaminergic content of brain regions differentially activated between stressed and control animals. Finally, we tested whether treatment with a DA (D2/D3) agonist, quinpirole, was able to revert the stress-induced changes in risk-based decision-making.

2. Experimental procedures

2.1. Animals

The subjects were 126 male Wistar rats (Charles River Laboratories, Barcelona, Spain), aged 2 months and weighing 250–300 g at the start of the experiment. The animals were housed in pairs under standard laboratory conditions (lights on from 8:00 A.M. to 8.00 P.M.) and had access to food and water ad libitum, except during behavioral testing, when food was restricted (see below).

All experiments were conducted in accordance with local regulations (European Union Directive 2010/63/EU) and National Institutes of Health guidelines on animal care and experimentation and approved by Direção Geral Veterinária (DGV; the Portuguese National Institute of Veterinary).

2.2. Experimental workflow

The experimental work was divided in two separate experiments (Fig. 1). In the first (Chronic Unpredictable Stress (CUS) vs controls), 80 animals were trained daily in the neutral condition (see below) for 20 days, until individual performances stabilized. Animals were then assigned, matching for risk preference on the behavioral task (controls: 20.96 ± 0.83 choices; CUS: 20.57 ± 0.85 choices; $t=0.33$, $P=0.74$), to be either stressed or serve as controls (40 animals each). Stress was induced according to the protocol below for 28 days, during which controls were only gently handled three times a week. The day after this period of stress/handling, 20 animals of each group had blood collected in the morning for corticosterone determinations and were sacrificed in the afternoon; their brains were processed as follows: 10 CUS and 10 controls were used for Golgi reconstructions and the remainder 10 CUS and 10 controls were macrodissected in ice and used for RT-qPCR and HPLC analyses. Also the day after the last stress exposure/handling, the remaining 40 animals (20 controls and 20 CUS), started testing on the risk-based decision-making task (1 session per day). A subgroup of these (10 controls and 10 CUS) was sacrificed on the same day, 90 min after completing the session (under the neutral condition) and their brains used for c-fos staining. The other 10 controls and 10 CUS went through the entire paradigm, consisting of 8 days in the neutral condition, 8 days in the risk-favorable condition and 8 days in the safe-favorable condition, without interruption. Importantly, this sequence of conditions was similar for all animals, as previously described by Morgado et al. (2014). Behavioral analysis only took into account the last 5 days of each condition, when individual animal performances were more stable. Each of these 20 animals was then sacrificed 90 min after the last behavioral session and their brains processed for Golgi-c-fos.

In the second experiment (quinpirole treatment), 46 animals were first trained in the task during 20 days, as above, and subsequently assigned to one of four groups, matched according to performance on the behavioral task: controls receiving vehicle, controls receiving quinpirole, CUS receiving vehicle and CUS receiving quinpirole. Animals were then handled three times a week (control groups) or submitted to the CUS protocol for 28 days, after which they were all tested in 3 consecutive 8-day decision-making paradigms: neutral, risk-favorable and safe-favorable, as above (1 session/day). Fifteen minutes before each daily session, throughout the entire period of testing, animals of the quinpirole groups (controls and stressed) received *ip* quinpirole hydrochloride in 0.9% saline (0.15 mg/kg; Sigma-Aldrich) while the remainder received *ip* vehicle. Drug dosage was selected in accordance with previous reports showing behavioral effects on decision-making tasks (Boulougouris et al., 2009; Kurylo and Tanguay, 2003) and absence of impact on locomotory parameters (Morgado et al., 2014). Similarly to experiment 1, and to avoid interference of the acute effects of quinpirole on behavior (Morgado et al., 2014), the three first days of each paradigm were not considered for data analysis.

2.3. Chronic Unpredictable Stress (CUS)

Animals assigned to the stress group were daily exposed, for 60 min, to one of five different stressors: cold water (18 °C), vibration, restraint, overcrowding and exposure to a hot air stream, randomly distributed throughout 28 days. This chronic stress paradigm is considered to better mimic the variability of stressors encountered in daily life (Sousa et al., 1998). Controls were carefully handled during the same period.

2.4. Biometric parameters

To assess the efficacy of the stress protocol, serum corticosterone levels of both stressed and control animals were measured the day after the last stress exposure/handling, in the morning (up to 1 h after "lights on"). Blood was collected via tail venipuncture, centrifuged at 13,000 rpm for 10 min and serum removed and stored at −80 °C until use. Serum total corticosteroid levels were measured by radioimmunoassay using a commercial kit (R&D Systems, Minneapolis, MN, USA), according to manufacturer's instructions. The intra- and inter-assay coefficients of variation were less than 5% and 10%, respectively. The

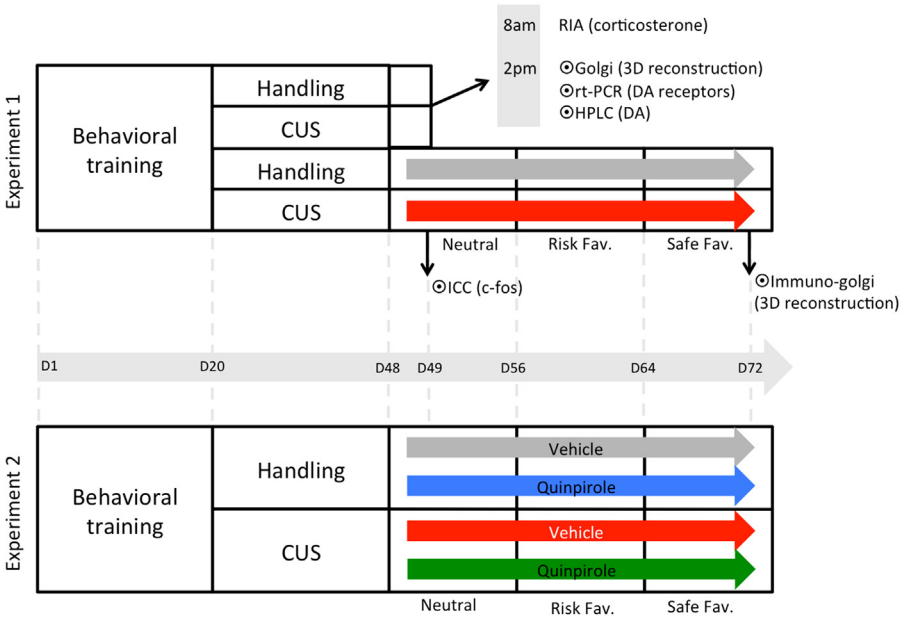

Figure 1 Experimental outline. Two parallel experiments were conducted. First, the effects of chronic stress on risk-based decision-making were assessed and a neurochemical, morphological and genetic analysis was performed. Secondly, a dopamine agonist quinpirole was used to revert the behavioral effects of chronic stress. CUS (Chronic Unpredictable Stress).

evolution of the weight of each animal was used as an additional measure of stress efficacy.

2.5. Risk-based decision-making paradigm

Behavioral training and testing was performed in 5-hole operant chambers (OCs; TSE Systems, Germany) within sound attenuating cubicles. Each chamber has five apertures mounted into a wall and a pellet dispenser in the opposite side to deliver rewards.

The decision-making paradigm was recently described (Morgado et al., 2014). Each daily session lasted for 30 min or 100 trials, whichever occurred first. In each trial, rats could choose between a "safe" hole (resulting in the delivery of "*s*" pellets with 100% probability) and 4 "uncertain/risk" holes (resulting in the delivery of "*r*" pellets with 25% probability), where the number of pellets rewarded upon each nose-poke in the safe ("*s*") or risky ("*r*") holes was manipulated to create three different conditions (neutral $s=1$ and $r=4$, risk favorable $s=1$ and $r=8$ and safe favorable $s=2$ and $r=4$); "uncertain/risk" holes, which were randomly allocated, in each trial, to 4 of the 5 apertures, were illuminated while the "safe" hole was not. After each choice, animals received reward at the pellet dispenser, the home cage light was switched off and a new trial started 5 s later. The total number of trials, the number of choices (nose-pokes) and omissions (no-nose-poke) and the number of pellets received in each trial, as well as the total time spent, were automatically registered by the software. Importantly, this design of risky and safe choices in the neutral condition evens the overall outcome of either option, allowing an analysis of risk-taking behaviors independently of reward value or delay. Of note, the sequence of paradigms was kept constant across all animals and consisted of an initial training period in the neutral condition, that lasted for 20 days and allowed animals to learn the task and reach a baseline performance level, followed (after the 28 days of stress/handling) by testing for 8 days in each of the three conditions: neutral, risk-favorable and safe-favorable (Morgado et al., 2014).

In order to facilitate motivation for task performance, a food deprivation regimen (15 g rat chow per day) was initiated 24 h before behavioral training/testing to maintain the subjects at approximately 90% of their free-feeding body weight (Dias-Ferreira et al., 2009; Morgado et al., 2014).

2.6. Dopamine quantification by High-Performance Liquid Chromatography (HPLC)

Following decapitation, brains were rapidly removed and discrete brain regions, specifically the OFC and anterior insula, dissected from the left or the right hemispheres (for HPLC and RT-qPCR in a pseudo-random, balanced distribution). Animals were anesthetized, decapitated, and heads were immediately snap-frozen in liquid nitrogen. Brain areas of interest were rapidly dissected on ice under a stereomicroscope, observing anatomical landmarks. Briefly, serial sections of the rat brain were collected using a stainless steel brain matrix (Stoelting Inc, USA; 1.0 mm) and laid down on an ice filled petri dish. The anterior insula and orbitofrontal cortex were recognized by comparing anatomical landmarks with a reference brain atlas (Paxinos and Watson, 2007). For the anterior insula (4.20 mm to 2.52 mm from bregma) a rectangular shaped area defined by the claustrum (medial border) and the cortical surface (lateral border) was defined. The rhinal fissure defined the lower limit, while the upper tip of the lateral lining of the claustrum defined the upper limit. For the OFC (5.16 mm to 4.20 mm from bregma) a triangular shaped area was defined by the ventral part of the cortex (base), the lateral part of the claustrum (medial border) and an imaginary line that reflected a similar angle to the base (lateral border). This is easily identifiable due to the higher density of cells that translates in differences of luminescence. The samples were collected to an Eppendorf and were snap-frozen (dry ice) and stored at −80 °C until use.

For HPLC, ice-cold samples were homogenized and deproteinized in 150 μL of 0.2 N perchloric acid solution containing 7.9 mM $Na_2S_2O_5$ and 1.3 mM Na_2EDTA. The homogenate was centrifuged at 20,000*g* for 45 min at 4 °C and the supernatant was stored at −80 °C. The analysis was performed by reverse-phase ion pair High-Performance Liquid Chromatography (HPLC) with an Electrochemical Detector (ED), as previously described with minor modifications (Kyratsas et al., 2013). Specifically, the mobile phase consisted of an acetonitrile phosphate buffer (15-17%), pH 3.0, containing 300 mg/L 5-octylosulfate sodium salt as the ion-pair reagent and

20 mg/L Na_2EDTA. Reference standards were prepared in 0.2 N perchloric acid solution containing 7.9 mM $Na_2S_2O_5$ and 1.3 mM Na_2EDTA. The working electrode was glassy carbon along with a reference electrode (Ag/AgCl) and the columns were Thermo Aquasil C18, 250 mm × 4.6 mm, 5 μm (Thermo Electron, Cheshire, UK). The voltage of the working electrode was set at +800 mV in the LC4C amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA) and the flow rate of the LC1150 HPLC pump (GBC Inc, Braeside, Australia) was set at 1.0 mL/min. Quantification of dopamine was done by comparison of the area under the curve with that of reference standards using HPLC software (Chromatography Station for Windows).

2.7. Gene expression measurements by real time quantitative PCR (RT-qPCR)

RT-qPCR analysis was used to measure the mRNA levels of the following genes: dopamine receptor D1A (Drd1a), dopamine receptor D2 (Drd2) and dopamine receptor 3 (Drd3). The reference gene for hypoxanthine guanine phosphoribosyl transferase (Hprt) (accession number from GenBank: NM_012583) was used as an internal standard for the normalization of the expression of selected transcripts, since we have first confirmed that its expression is not influenced by the experimental conditions. Expression level of Hprt in terms of CT in OFC in the control was 18.6 and in CUS was 19.3 ($t=-1.82$, $P=0.10$). Regarding the Insular cortex, the expression levels were in the control of 18.3 and in CUS 19.3 ($t=-1.87$, $P=0.09$).

All accession numbers and primer sequences are available on request. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed with iScript cDNA Synthesis Kit for RT-PCR (Bio-Rad, Laboratories, Hercules, CA, USA). Primers were designed using Primer3 software (Rozen and Skaletsky, 2000), on the basis of the respective GenBank sequences. All melting curves exhibited a single sharp peak at the expected temperature.

2.8. c-fos immunohistochemistry

Animals were sacrificed 90 min after the end of the behavioral task (Leite-Almeida et al., 2014; Morgado et al., 2014) with a sub-lethal injection with pentobarbital and transcardially perfused with phosphate buffer (PBS) followed by 4% paraformaldehyde. Brains were removed, post-fixed in PFA for 4 h, kept in 8% sucrose at 4 °C and cut in 50 μm coronal sections. c-fos immunohistochemistry was as described previously (Morgado et al., 2014) using anti-Fos primary antibody (1:2000; PC38 Anti-c-Fos (Ab-5), Calbiochem, Darmstadt, Germany) polyclonal swine anti-rabbit secondary antibody (1:200 in PBS-T; E0353, DAKO, Glostrup, Denmark), avidin–biotin complex (ABC, 1:200, Vector Laboratories, Burlingame, CA, USA) and 0.0125% diaminobezidine tetrahydrochloride (DAB; Sigma-Aldrich). All procedures were performed at room temperature.

The number of c-fos positive cells was counted within the boundaries of the medial prefrontal cortex (prelimbic cortex (PrL), infralimbic cortex (IL) and cingulate cortex (Cg1)), OFC (medial (MO), ventral (VO) and lateral (LO) parts), somatosensory cortex (SSC), motor cortex (MC), insula, dorsal striatum (dorsolateral striatum (DLS) and dorsomedial striatum (DMS)) and nucleus accumbens (shell (NAcS) and core (NAcC)) as previously described (Morgado et al., 2014).

2.9. Golgi staining

The day after the last stress exposure, animals from each group were transcardially perfused with 0.9% saline under deep pentobarbital anesthesia. Brains were removed and processed for 3D morphometric analysis of neurons; briefly, they 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 (see Cerqueira et al., 2007c for details).

2.10. Imuno-Golgi staining

To analyze neurons specifically recruited during the task, a method that combines c-fos immunohistochemistry with Golgi-impregnation was performed. Stressed and control rats, 90 min after the end of the behavioral task, were transcardially perfused with 0.9% saline under deep pentobarbital anesthesia and processed according to the protocol described by Pinto et al. (2012) using primary c-fos antibody (1:1000; Calbiochem), secondary anti-rabbit antibody Alexa Fluor 594 (1:500; invitrogen) and DAPI (1 μg/ml). Sections were mounted in superfrost slides using Vectashield mounting medium (Vector Laboratories).

2.11. Neuronal 3D-dendritic structure analysis

Pyramidal neurons of lateral part of OFC (lOFC) cortex and of insula (Zilles and Wree, 1995) were analyzed. The criteria used to select neurons for reconstruction were those described by Uylings et al. (1986). In order to minimize selection bias, slices containing the region of interest were randomly searched and the first 10 neurons fulfilling the above criteria (maximum of 3 neurons per slice) were selected. For each selected neuron, all branches of the dendritic tree were reconstructed at 600 × magnification using a motorized microscope (Axioplan 2, Carl Zeiss, Germany), attached to a camera (DXC-390, Sony Corporation, Tokyo, Japan) and Neurolucida software (MicroBrightField Bioscience) and analyzed using NeuroExplorer software (MicroBrightField Bioscience).

2.12. Statistical analysis

Data was analyzed using SPSS (version 19.0; IBM). Results are expressed as group means ± SE. Results of the first experiment (Control and stress groups) were compared using Student's *t* test. Results of the second experiment (controls and stressed animals treated or not with quinpirole) were analyzed with a two-way ANOVA (stress*treatment); individual group comparisons were then made post hoc using Tukey's honestly significant differences test. For all analyses, differences were considered to be significant only when $p<0.05$.

3. Results

3.1. Efficacy of the stress protocol

Our stress protocol was effective, as revealed by a decreased body weight gain (Experiment 1: controls: 100.28 ± 9.63 g; stress: 65.93 ± 19.13 g; $t=5.07$, $p<0.001$) and increased corticosterone levels (Experiment 1: controls: 59.49 ± 11.46 ng/mL; stress: 154.94 ± 23.11 ng/mL; $t=-3.70$, $p<0.001$) of stress animals compared with controls.

3.1.1. Experiment 1

3.1.1.1. Stress biases decisions to safe options. During training (done in the neutral condition, before stress exposure), animals increased the number of completed trials in each session, inversely decreasing total time spent to do so; totally completed trials were achieved by all animals on the 8th day of training (data not shown). On

average, animals performing the task chose the safe hole approximately 20% of the times and any of the four risk holes 80% of the times. This near-random pattern of choices, that was established relatively early and maintained during the entire protocol (as previously described in Morgado et al., 2014), suggests that at baseline animals have no preference for either option.

Compared to handling, exposure to CUS significantly increased preference for safe options in the neutral condition (controls: 21.05±0.25 choices; CUS: 26.48±0.84 choices $t=-6.206$, $P<0.05$, Figure 2, panel A). More importantly, this stress-triggered bias toward safe was also present even in the uncertain/risk favorable paradigm (controls: 17.83±0.51 choices; CUS: 21.75±1.02 choices; $t=-3.43$, $P<0.05$) and more evident in the safe-favorable condition (controls: 24.9±1.17; choices CUS: 31.83±1.04 choices; $t=-4.03$, $P<0.05$) (Figure 2, panel A).

3.1.1.2. Stress bias to safer behavior is paralleled by changes in the orbitofrontal cortex. This stress-induced altered pattern of choice was accompanied by a distinct task-triggered c-fos activation pattern. Indeed, previously chronically stressed animals displayed, after task performance, a significantly increased activation of the lOFC (controls: 635±38 cells/mm^2; CUS: 831±60 cells/mm^2; $t=-2.32$, $P<0.05$) and the insula (controls: 205±19 cells/mm^2; CUS: 282±15 cells/mm^2; $t=-2.50$, $P<0.05$), but not any other brain region, when compared to controls (vOFC - controls: 873±110 cells/mm^2; CUS: 981±95 cells/mm^2; $t=-0.74$, P=0.48. mOFC - controls: 608±68 cells/mm^2; CUS: 694±54 cells/mm^2; $t=-0.10$, $P=0.34$. PrL - controls: 543±33 cells/mm^2; CUS: 510±69 cells/mm^2; $t=0.31$, $P=0.76$. IL - controls: 544±18 cells/mm^2; CUS: 459±41 cells/mm^2; $t=1.88$, $P=0.13$. Cg1 - controls: 557±7 cells/mm^2; CUS: 398±7 cells/mm^2; $t=-1.58$, $P=0.15$. NAcC - controls: 481±5 cells/mm^2; CUS: 529±8 cells/mm^2; $t=-0.44$, $P=0.67$. NAcS - controls: 465±38 cells/mm^2; CUS: 454±53 cells/mm^2; $t=0.17$, $P=0.87$. DLS - controls: 412±42 cells/mm^2; CUS: 429±41 cells/mm^2; $t=-0.31$, $P=0.77$. DMS - controls: 427±65 cells/mm^2; CUS: 452±86 cells/mm^2; $t=-0.24$, $P=0.82$. SSC - controls: 241±32 cells/mm^2; CUS: 207±33 cells/mm^2; $t=0.73$, $P=0.48$. MC - controls: 273±28 cells/mm^2; CUS: 213±23 cells/mm^2; $t=1.60$, $P=0.15$.) (Figure 2, panel B). Of note, as c-fos induction is relatively fast and transient and animals were not being stressed/handled for at least 24 h at time of sacrifice, differences in activation pattern most probably reflect a different response to the task rather than a direct stress effect (Coggeshall, 2005).

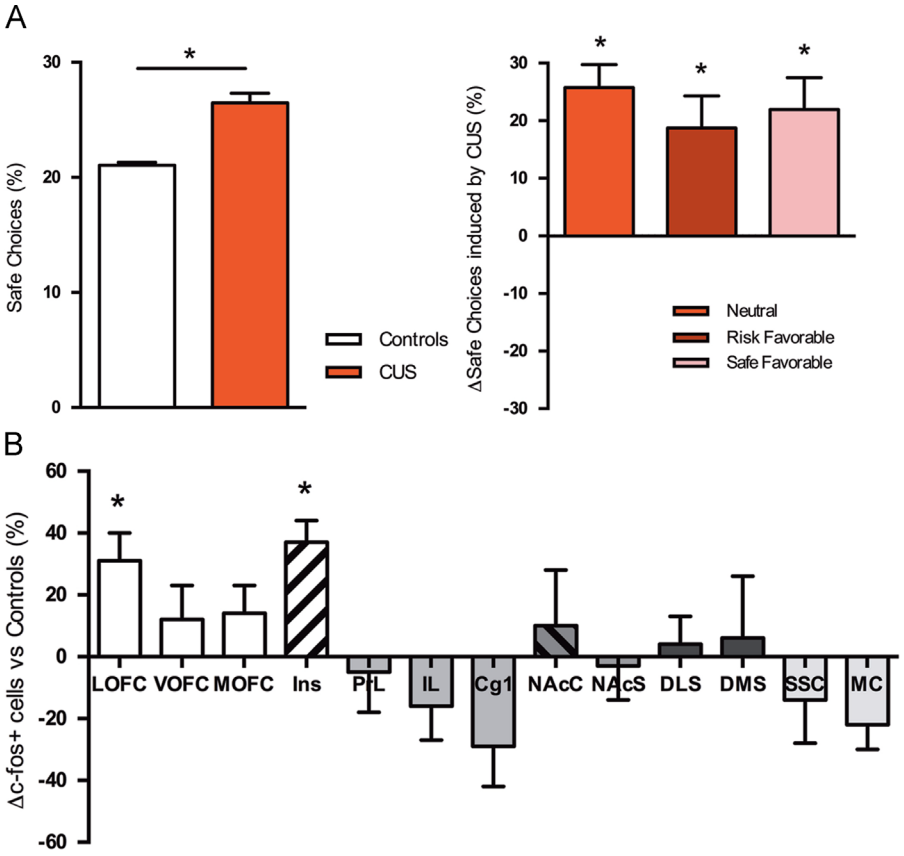

Figure 2 Effects of chronic stress on risk-taking behavior. (A) Chronic stress significantly decreased risk choices among the three different protocols tested. *$p<0.05$ vs non-stressed controls. (B) Increased (in %) in the density of c-fos positive cells in orbitofrontal cortex (OFC) and insular cortex (Ins) in chronically stressed animals performing the task when compared with non-stressed controls. No significant differences were found in other brain areas examined. L/V/MOFC (lateral/ventral/medial OFC), PrL (prelimbic cortex), IL (infralimbic cortex), Cg1 (cingulate cortex), NaccC/S (Nacc Core/Shell), DLS/DMS (dorsomedial/lateral striatum), SSC (somatosensory cortex), MC (motor cortex). *$p<0,05$ vs non-stressed controls.

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

Given the previous result, we also quantified the mRNA levels of the different DA receptors in the OFC and anterior insula. Immediately after stress, expression levels of the mRNAs encoding the *Drd1* ($t=-0.04$, $P=0.97$) and *Drd3* (-0.49, $P=0.64$) receptors did not differ between controls and stressed animals. However, there was a significant up-regulation of *Drd2* mRNA in this brain region ($t=-3.42$, $P<0.05$) (Figure 3, panel B). No changes on expression levels of the mRNAs encoding the *Drd1* ($t=1.39$, $P=0.21$), *Drd2* ($t=-1.70$, $P=0.14$) and *Drd3* ($t=-0.80$, $P=0.46$) were found among anterior insula (Figure 3, panel B).

Finally, we performed a three-dimensional morphometric analysis of pyramidal neurons from the lOFC and insula cortices, immediately after stress. When compared with controls, chronically stressed animals displayed a significant increase in the length of apical dendrites of OFC neurons ($t=-2.96$, $P<0.05$), while basal dendrites of the OFC and apical and basal dendrites of the insula were not affected (Figure 3, panels C and D). To assess whether these changes were present in the neurons specifically activated by the behavioral task, we performed the immuno-Golgi staining in brains collected 90 min after task completion (24 days after the last stressor); this approach confirmed our hypothesis, by revealing a similar decrease in the apical dendrites of lOFC c-fos positive cells ($t=-2.60$, $P<0.05$) (Figure 3, panel D).

3.1.2. Experiment 2

3.1.2.1. D2/D3 agonist quinpirole reverts stress effects on behavior. Given our observations of a decreased dopamine content and overexpression of drd2 mRNA in the OFC after chronic stress, but also previous studies showing the effect of D2/D3 agonist quinpirole in decision making (Morgado et al., 2014; St Onge et al., 2010; St Onge and Floresco, 2009; Zeeb et al., 2009), we decided to test its effect on reward prediction and our risk-based decision-making behavior. Confirming our previous results, chronically stressed

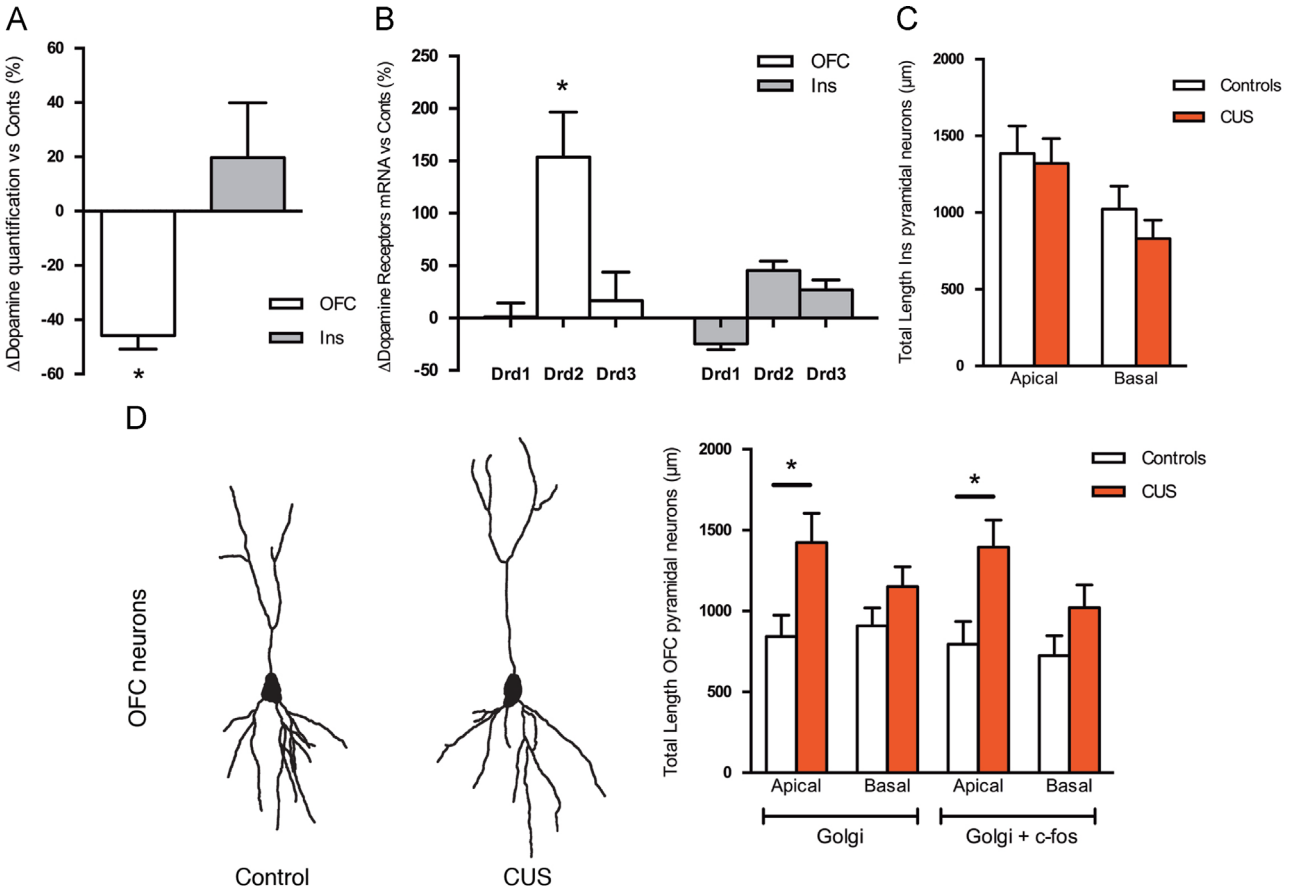

Figure 3 Neurochemical and neural effects of chronic stress. (A) The effects of chronic stress on HPLC DA levels in orbitofrontal cortex (OFC) and insula as compared with non-stressed controls. *$p<0.05$ vs non-stressed controls. (B) The effects of Chronic Unpredictable Stress on dopamine D1, D2 and D3 receptors mRNA levels (in %) in orbitofrontal cortex (OFC) and insular cortex (Ins) when compared with non-stress controls. *$p<0.05$ vs non-stressed controls. (C) Morphometric analysis of apical and basal dendrites in the insular pyramidal neurons in animals submitted to chronic stress when compared with non-stressed controls. (D) Morphometric analysis of apical and basal dendrites in the OFC pyramidal neurons in animals submitted to chronic stress when compared with non-stressed controls. Neurons specifically recruited by the task were assessed with imunoGolgi staining (Golgi+c-fos). *$p<0.05$ vs non-stressed controls.

animals displayed an increased preference for safe choices in all three different paradigms. Interestingly, acute administration of quinpirole before behavioral testing (during which animals were not being stressed) completely reverted that bias making their pattern of choices undistinguishable from that of untreated controls (neutral: effect of stress - $F_{(1,42)}=11.06$, $p<0.01$, effect of quinpirole - $F_{(1,42)}=4.02$, $p=0.51$, interaction - $F_{(1,42)}=8.36$, $p<0.01$; risk favorable: effect of stress - $F_{(1,42)}=6.59$, $p<0.05$, effect of quinpirole - $F_{(1,42)}=3.28$, $p=0.77$, interaction - $F_{(1,42)}=4.13$, $p<0.05$; safe favorable: effect of stress - $F_{(1,42)}=9.04$, $p<0.01$, effect of quinpirole - $F_{(1,42)}=1.64$, $p=0.27$, interaction - $F_{(1,42)}=4.11$, $p<0.05$), (Figure 4). Of notice, treatment with quinpirole had no effect on the choices of non-stressed animals in any of the paradigms (Figure 4).

4. Discussion

Stress has a strong impact on brain function and could lead either to beneficial or detrimental effects (McEwen, 1998). Chronic stress impairs, amongst others, behavioral flexibility (Cerqueira et al., 2007a), pavlovian to instrumental transfer (Morgado et al., 2012) and decision-making (Dias-Ferreira et al., 2009). Surprisingly, however, no study has to our knowledge explored the impact of chronic stress on the willingness to take risks, as we have done in the present work (Morgado et al., 2015). A recent paper by Pabst et al. (2013) has shown that, in humans, acute stress exposure before the task increases the preference for risky options, which can be correlated with an increase in salivary cortisol reflecting the activation of the hypothalamus-pituitary-adrenal (HPA) axis. Interestingly, these data are in line with results by Koot et al. (2013) revealing that acute corticosterone administration, which partially mimics HPA axis activation, promotes the choice of unfavorable conditions. However, both studies seem to be in contradiction with the present findings that chronic stress increases the preference for safe options. These contrasting and opposing effects of acute versus chronic stress have been described in other behavioral domains (more importantly in cognition, where acute stress enhances while chronic stress impairs memory, see Lupien et al., 2009 for a review) and might represent a fundamental aspect of the two-facets of the stress response. Indeed, while acute stress can be considered adaptive (Diamond et al., 1992), chronic or prolonged stress becomes maladaptive, in line with its negative impact in several dimensions of brain function (Sousa and Almeida, 2012), and, as shown herein, also risk preference. In line with our data, Kandasamy et al. (2014) recently described that chronic elevation of cortisol levels induced by administration of hydrocortisone promotes a risk aversive behavior among healthy volunteers.

Despite these considerations, our observations suggest that animals submitted to chronic stress change their valuating systems, overrating losses and, subsequently, avoiding 'risk' options that imply the possibility of not receiving any reward. Importantly, the present study also shows, using the expression of the c-fos protein, that this behavioral effect is associated with an over-activation of the lateral part of OFC and insular cortex. Intriguingly, but significantly, these are exactly the same two regions that mediate the effect of acute corticosterone administration on a rodent Iowa gambling task described above (Koot et al., 2013), which strongly suggests these areas to be key to the impact of stress and glucocorticoids on tasks involving uncertainty/risk. The OFC is critically involved on assigning and updating reward values, encoding a wide range of other variables indispensable for decision-making, including expected outcomes (Schoenbaum et al., 1998), effort associated to each option (Kennerley et al., 2009; Roesch and Olson, 2005), confidence in the decision (Kepecs et al., 2008) and the probability of win (Kennerley et al., 2009). Interestingly, rodent lesion studies have highlighted that the OFC encodes specific information about the outcome rather than its general affective value (Burke et al., 2008). Additionally, we had previously shown that chronic stress biased behavior from goal-directed to habit based choices, which was mediated by a shift from an atrophied medial prefrontal loop to a hypertrophied orbitofrontal network (Dias-Ferreira et al., 2009). In accordance to this finding, we have also identified a deleterious impact of chronic stress in pavlovian to instrumental transfer, a function highly dependent on the integrity of the OFC (Morgado et al., 2012). Furthermore, the insular cortex was also over-activated during the task in stressed animals. This brain region is involved in representations of bodily internal states and needs (Naqvi and Bechara, 2009) and in risk-aversion signaling (Clarke et al., 2008; Preuschoff et al., 2008). Insula lesion studies have shown an increase in risky non-advantageous choices (Clarke et al., 2008), which is in line with our observation of insular over-activation associated to a risk-aversion pattern of choice.

Together with hyperactivation of these regions, we found a stress-induced significant reduction of DA in OFC, but not in the insular cortex, accompanied with overexpression of Drd2 mRNA; this suggests that DA reduction and subsequent upregulation of Drd2 are implicated in the processes leading to the observed behavioral changes. This is in line with previous studies revealing a role for stress-induced hypodopaminergic status (in the PFC) in the genesis of working memory

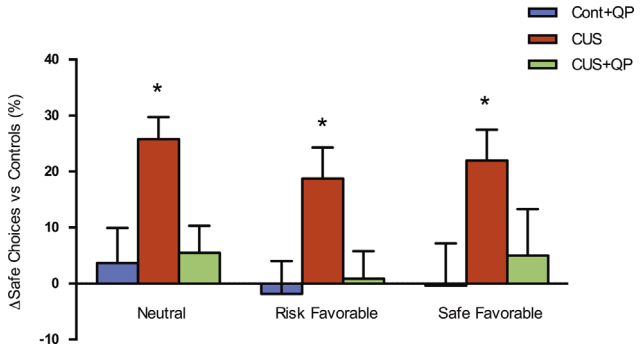

Figure 4 Quinpirole effects on decision-making. Chronic stress exposure, as compared with non-stressed controls, significantly increases the frequency of safe choices (in %) in all testing conditions, an effect that is reverted by D2/D3 agonist quinpirole. Neutral: risky and safe choices associated with the same reward magnitude in the long run (4 pellets at 25% vs 1 pellet at 100%); risk favorable: risky choices associated with higher reward magnitude in the long run (8 pellets at 25% vs 1 pellet at 100%); safe favorable: safe choices associated with higher reward magnitude in the long run (4 pellets at 25% vs 2 pellets at 100%). CUS (Chronic Unpredictable Stress); QP (quinpirole). *$p<0.05$ vs non-stressed controls.

(Mizoguchi et al., 2000) and decision-making deficits (Gruber et al., 2010; Tseng and O'Donnell, 2004); of notice, the latter were ascribed to a lack of inhibitory actions of D2 receptors on NMDA-induced responses (Tseng and O'Donnell, 2004). Irrespective of the underlying mechanism, our observation that the stress-induced bias on risk-based decision-making can be pharmacologically reverted by a D2/D3 agonist, quinpirole, clearly confirms the involvement of the dopaminergic system in this process. These observations are in accordance with the hypothesis that stress induced hypodopaminergic state could mediate its behavioral effects on risk-based decision-making through hyperactivation of OFC. Indeed, two recent studies pointed out the role of dopaminergic system in stress resilience (Zurawek et al., 2013) and social aversion induced by chronic stress (Barik et al., 2013). Moreover, the dopaminergic system has also been implied in the behavioral alterations induced by acute stress on decision-making paradigms (Shafiei et al., 2012). Interestingly, no changes in insular DA levels were found, which points to a possible involvement of other neurotransmitters such as GABA and glutamate in these processes (de Kloet et al., 2005).

Previous studies have reported effects of dopaminergic agents on decision-making behaviors, associating dopaminergic agonists with increased rates of risk choices (Morgado et al., 2014; Riba et al., 2008; St Onge et al., 2010; St Onge and Floresco, 2009). As chronic stressed animals were risk-aversive, it could be argued that quinpirole effects observed in our study could be explained by an unspecific increasing of risk-prone behavior induced by dopaminergic activation. However, if this were true, one would expect that non-stressed quinpirole-treated animals would also increase their frequency of risk choices, which was not verified herein. This absence of quinpirole effects on control animals contrasts with our prior observations (Morgado et al., 2014) and points out the discrepancy between acute (previous study) and chronic (present work) effects of dopaminergic agents on decision-making. Indeed, the fact that quinpirole was administered daily but only the 5 last days of each paradigm were analyzed allows the dopaminergic system do adapt to increasing D2/D3 stimulation by downregulating receptor expression, for example. Additionally, contradictory data on the literature reported impaired performance on gambling tasks induced by dopaminergic agonists (Zeeb et al., 2009) and related lower dopaminergic levels with higher risk choices in Iowa Gambling Task (IGT) (Sevy et al., 2006) supporting the idea that effects of dopaminergic drugs on decision-making cannot be explained by an oversimplistic view and could be dependent on basal levels of DA, available dopaminergic receptors, specific features of decision-making tasks and duration of treatment.

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

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Author contributions

PM, NS and JJC designed the experiments. PM, BR, JJC performed the behavioral and pharmacological tests. PM, BR, HLA, JMP participated in the stereological reconstructions and c-fos analyses, PM, FM and AJR did the RT-qPCR determinations and CD and NK the DA HPLC measurements. PM, NS and JJC performed the statistical analyses, draw the figures and wrote the first version of the manuscript, which was then revised by all authors.

Conflict of interest

The authors declare no competing financial interests.

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