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ORIGINAL ARTICLE

The motivational drive to natural rewards is modulated by prenatal glucocorticoid exposure

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Exposure to elevated levels of glucocorticoids (GCs) during neurodevelopment has been identified as a triggering factor for the development of reward-associated disorders in adulthood. Disturbances in the neural networks responsible for the complex processes that assign value to rewards and associated stimuli are critical for disorders such as depression, obsessive–compulsive disorders, obesity and addiction. Essential in the understanding on how cues influence behavior is the Pavlovian–instrumental transfer (PIT), a phenomenon that refers to the capacity of a Pavlovian stimulus that predicts a reward to elicit instrumental responses for that same reward. Here, we demonstrate that *in utero* exposure to GCs (iuGC) impairs both general and selective versions of the PIT paradigm, suggestive of deficits in motivational drive. The iuGC animals presented impaired neuronal activation pattern upon PIT performance in cortical and limbic regions, as well as morphometric changes and reduced levels of dopamine in prefrontal and orbitofrontal cortices, key regions involved in the integration of Pavlovian and instrumental stimuli. Normalization of dopamine levels rescued this behavior, a process that relied on D2/D3, but not D1, dopamine receptor activation. In summary, iuGC exposure programs the mesocorticolimbic dopaminergic circuitry, leading to a reduction in the attribution of the incentive salience to cues, in a dopamine-D2/D3-dependent manner. Ultimately, these results are important to understand how GCs bias incentive processes, a fact that is particularly relevant for disorders where differential attribution of incentive salience is critical.

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INTRODUCTION

Early life stress or exposure to elevated levels of glucocorticoids (GCs) may increase the risk for the development of neuropsychiatric disorders, including those associated with reward deficits such as depression, obesity and addiction.[1–3] Although the neural circuits involved in their pathophysiology are complex, it is strongly believed that they are tightly linked to mesolimbic dopaminergic dysfunction.[4,5] Mesolimbic dopamine (DA) signaling has long been implicated in reward processing, but its precise contribution remains a topic of intense debate, in particular the role of accumbal DA.[6,7] Apart from the classical role in mediating the hedonic impact of a reward,[8] this circuit also seems crucial for reinforcement learning, being responsible for establishing stimulus–response associations (associative stamping) and, eventually, to enhance habit formation.[9] It is also hypothesized that changes in the activity of DA neurons encode a quantitative prediction error.[10] In addition, Berridge[6] has suggested that DA is responsible for the attribution of incentive salience to (otherwise neutral) cues that predict a reward, which triggers a motivational state of 'wanting' for both the cue and its associated reward. Regardless of the mechanism, mesolimbic DA seems to stamp in response–reward and stimulus–reward associations that are essential for the expression of motivated behaviors.[7]

Previous work from our lab has shown that prenatal exposure to GCs leads to morphological adaptations within the nucleus accumbens (NAc) and amygdala, together with a significant reduction in dopaminergic innervation arising from the ventral tegmental area (VTA).[11–13] As a result, these animals displayed persistent anhedonia but enhanced vulnerability for drug-seeking behavior in adulthood.[13,14] These symptoms may result from a complex differential attribution of incentive salience to natural versus non-natural rewards and their associated cues. In fact, individual differences in incentive salience attribution/'wanting' have been linked with propensity for addiction-like behaviors.[15] In this framework, we sought to further dissect if and how prenatal GC exposure alters the attribution of incentive salience to a cue predicting a natural reward (food). To do so, we used the Pavlovian–instrumental transfer (PIT) paradigm, which relies on the well-described phenomenon of a Pavlovian stimulus to invigorate an ongoing instrumental action.[16] The associative value of the cue and its motivational significance are crucial for proper transfer, a phenomenon that resembles cue-mediated increased drive for drugs seen in addicted individuals.[17,18] This test is often seen as a reflex of motivation for a specific reward, as it measures the ability of a cue to trigger the drive for a reward in the absence of both primary and secondary reinforcements.[16] In addition, we further evaluated the role of DA, and the impact of specific activation of either D1 or D2/D3 DA receptors in this type of behavior.

MATERIALS AND METHODS

Detailed explanation of all procedures is provided in the Supplementary Material.

Animals

All manipulations were conducted according to current regulations (European Union Directive 2010/63/EU). Pregnant Wistar Han rats were

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subcutaneously injected with the synthetic GC dexamethasone (DEX; *in utero* exposure to GC (iuGC) animals), at 1 mg kg^{-1} or with vehicle (CONT; control animals), on days 18 and 19 of gestation. Male offspring aged 3–4 months were used.

Behavioral procedures

Subjects and apparatus. Subjects were 6–10 iuGC and CONT experimentally naive rats, 3 months old at the start of the experiment. Rats had restricted access to water, with the bottles being removed from the home cages 90–60 min before the trainings and replaced 30–60 min after. The access to food was restricted to maintain the rats at ± 85% of their free feeding weight.

Two identical operant chambers (Med associates, St. Albans, VT, USA) housed in light- and sound-attenuating boxes, were used in the experiment. Each chamber contained a central, recessed magazine that provided access to 45-mg food pellets (Bio-Serve, Frenchtown, NJ, USA) or 100 µl of sucrose solution (20% wt/vol in water) delivered by a pellet dispenser and a liquid dipper, respectively, and two retractable levers that were located on each side of the magazine. Magazine entries were measured automatically by an infrared beam located at the mouth of the magazine. A 1-kHz tone and an amplified white noise, each with a sound of 80 dB, where available as discrete auditory cues. A 2.8 W, 100 mA house light positioned at the top-center of the wall opposite to the magazine provided illumination. A computer equipped with Med-PC software (Med Associates) controlled the equipment and recorded the data.

General PIT

The behavioral procedure was adapted from protocols previously described.[16,18,19]

Both groups of animals were given 10 sessions of Pavlovian training. Each training session included eight presentations of the conditioned stimulus (CS) - four presentations of the positive stimulus (CS+) and four presentations of the innocuous/negative stimulus (CS −). During each 2-min presentation of the CS+, the reward (food pellet) was delivered. The average intertrial interval (ITI) between CS presentations was 2 min. For half the rats in each group, the CS+ was the tone; and for the remaining rats, the CS+ was the amplified white noise. Magazine visits (MVs, number of times that the animal introduced the nose in the food magazine) were recorded during the CS period and during the ITI. Data are shown as the number of MVs performed during the CS+ period (8 min) and the number of MVs performed during the CS − period (8 min). Rats received one Pavlovian extinction session identical to the training, but under extinction (without reward).

For the instrumental training, animals were trained on random ratio (RR) schedule of reinforcement. During the training sessions, animals learned to press the lever (left and right levers counterbalanced) to receive the outcome (food pellet). Animals first received 2 days of continuous reinforcement (CRF) and were then shifted to an RR5 schedule (that is, each action delivered an outcome with a probability of 0.2). After 2 days of training, this was changed to an RR10 schedule (or a probability of 0.1) for two additional days. The training ended after 30 min of testing or after the animals earned 30 pellets. The number of times that the animals pressed the lever during the time of testing was registered. Data are presented as the number of lever presses per time of training. After training, animals were given a Pavlovian reminder, which was identical to the training.

Twenty-four hours later all rats were tested for PIT under extinction. The lever, which the animals learned to press was inserted into the chamber. In the first 8 min, the lever was available but no stimuli were presented; this period corresponding to a baseline performance interval (BPI). Each of the stimuli was then presented four times in a pseudorandom order. Each CS lasted for 2 min, separated by a 2-min ITI. The number of lever presses during both CS+ and CS − was assessed and plotted.

Selective PIT

The behavioral procedure used was adapted from protocols previously described.[20]

Pavlovian training comprised nine daily sessions in which each of two auditory CS (tone and white noise) was paired with a different outcome (pellet or sucrose solution). Each of the CS exposure that lasted for 2 min was presented four times per session using a pseudorandomized order, with an ITI of 2 min in average. Data were plotted as the number of MVs performed during both CS presentations (16 min in total) and the number of MVs performed during the ITI (pre-CS period).

Animals were then trained for the instrumental conditioning. Training was performed in two separate sessions per day (one session for each lever) and the order of training was alternated during days (average interval between the two sessions was 3 h). Each session finished after 30 rewards were delivered or 30 min had elapsed. In the first 2 days, lever pressing was in a CRF order. The probability of getting a reward decreased according to the following sequence: days 3–4, RR5; days 5–6, RR10. The number of lever presses per session was registered and plotted. After training, animals were given a Pavlovian reminder, which was identical to the training.

Twenty-four hours later, subjects were placed in the operant chamber to test for PIT transfer with both levers inserted. After a BPI that lasted for 8 min, four blocks of each auditory CS were presented randomly and lever presses were registered. During each stimulus presentation, lever presses were considered correct if it encoded the same reward as the audible sound. When encoding was different, the actions were considered incorrect. The number of lever presses performed during the test is plotted.

Operant behavior

Training and the devaluation test were described in previous work.[21] Briefly, animals were exposed to increasing difficulty schedules of reinforcement: 2 days of CRF, 2 days of RR5, 2 days of RR10 and finally 7 days of RR20. Animals were tested, using a reversible devaluation paradigm, at two different phases of training: after the first day of RR20 (early devaluation) and after the last training day (late devaluation). The devaluation test commenced 24 h after the previous training day and lasted 2 days. On each day rats were given *ad libitum* for 1 h, either the reinforcer earned by lever pressing (devalued condition) or the one received for free in the home cage (valued condition), so devaluation was achieved by sensory-specific satiety. Immediately after, rats were given a 5 min test in extinction.

Locomotor behavior

Locomotion was assessed using the open field test. Briefly, rats were injected with the drug and immediately after placed in the center of an arena (Med associates). The ambulation was monitored online over a period of 90 min. Total distance traveled was used as an indicator of locomotor activity.

Macrodissection

Rats were anesthetized, decapitated and the heads were immediately snap-frozen in liquid nitrogen. Brain areas of interest were rapidly dissected using Paxinos landmarks,[22] and stored at − 80 °C until use.

Western blot

Samples were treated as previously described.[13] Briefly, tissue was mechanically homogenized, centrifuged and the supernatant was quantified using the Bradford method. Fifty micrograms of the protein were run in SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were incubated with the primary antibodies: rabbit anti-DA receptor D1, rabbit anti-DA receptor D2, rabbit anti-DA receptor D3 and mouse anti-α-tubulin. The secondary antibodies were incubated at a 1:10 000 dilution (anti-rabbit and anti-mouse). Detection was performed using ECL kit and bands were quantified using ImageJ (http://rsbweb.nih.gov/ij/).

Immunohistochemistry

For c-fos activation analysis, all animals were submitted to the selective PIT protocol described above (nine sessions of Pavlovian training; six sessions of instrumental training; one session of PIT test). On the PIT test day half of the animals of each group (five CONT test animals and five iuGC test animals) performed the PIT test, whereas the other half did not perform the test (five CONT animals and four iuGC animals).

Rats that performed the test were sacrificed 110 min after initiation of PIT testing and the rats that did not perform the test were killed on the same day. Both groups were anesthetized with pentobarbital and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were processed and sectioned coronally on a vibratome at a thickness of 50 µm.

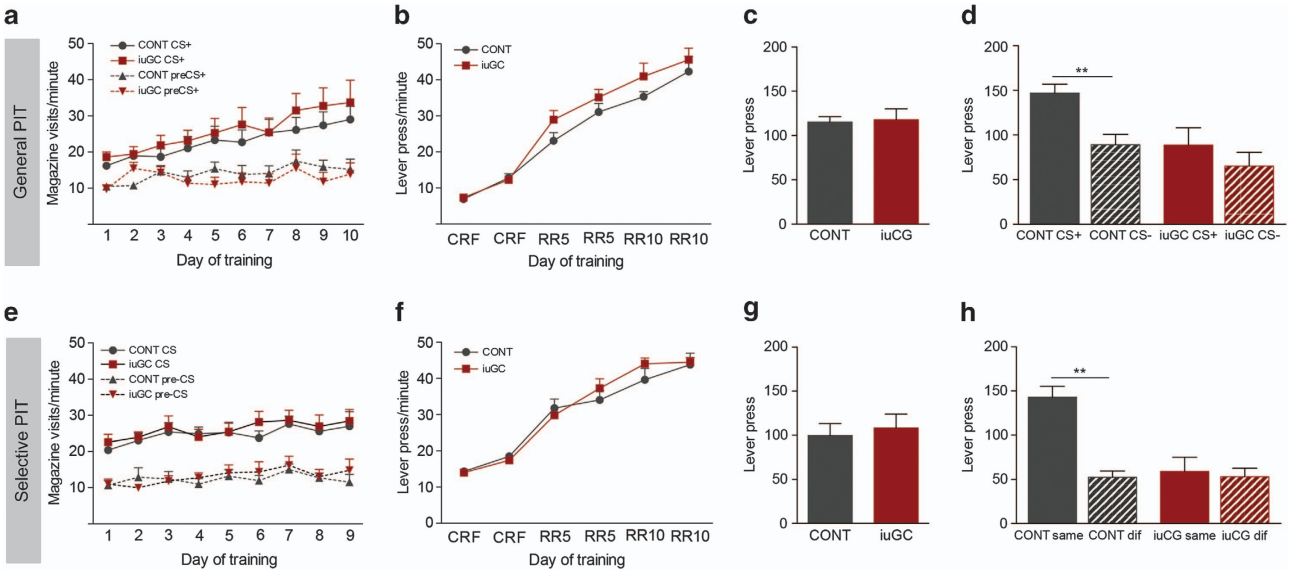


Figure 1. iuGC exposure disrupts Pavlovian–instrumental transfer (PIT). (**a**) Pavlovian conditioning of the general PIT protocol, represented as the average of magazine visits (MVs) performed during conditioned stimulus (CS), was similar between groups. For each group, mean MVs per min of the CS+ and mean MV per min of the CS − are plotted. (**b**) Instrumental conditioning of the general PIT protocol occurred at the same rate in both CONT and iuGC groups. Data are represented as the mean lever press per min for CONT and iuCG animals. (**c**) Baseline performance interval (BPI) of CONT and iuGC animals in the general PIT test session. For each group, the total number of lever presses performed during the 8 min of BPI is presented. (**d**) General PIT outcome is represented as the total number of lever presses performed during the CS+ and CS − periods. iuGC animals present a robust transfer impairment. (**e**) Pavlovian conditioning of the selective PIT paradigm was similar between groups. For each group, mean MVs per min of the CS period presentations and intertrial interval (pre-CS) period presentations are plotted. (**f**) Instrumental conditioning of the selective PIT paradigm revealed no differences between controls and iuGC animals. The number of lever presses per min performed in each day of the training is represented for each group. (**g**) BPI of CONT and iuGC animals in the selective PIT test session. For each group, the total number of lever presses performed during the BPI is presented. (**h**) iuGC animals present an impairment in selective PIT performance. The outcome of the selective PIT paradigm is shown as the total responses on the same lever or the different (dif) lever pressed, according to the CS presented. Same - lever pressing on the lever that originates the same reward as the CS presented; dif - lever pressing on the lever that originates a different reward as the CS presented. Graphs represent the total number of lever presses during the CS. All graphs are presented as mean ± s.e.m. CONT, control animals; CRF, continuous reinforcement; iuGC, *in utero* glucocorticoid exposed animals; RR, random ratio. $n = 8$–10 per group. $^{*}P \leqslant 0.05$, $^{***}P \leqslant 0.001$.

Briefly, free-floating sections were pretreated with 3% H_2O_2, rinsed in phosphate-buffered saline, blocked with 2.5% fetal bovine serum for 2 h at room temperature and incubated overnight at 4 °C with rabbit anti-c-fos polyclonal antibody. Afterwards, sections were washed and incubated with the secondary polyclonal swine anti-rabbit biotinylated for 1 h and processed with an avidin–biotin complex solution and detected with 0.5 mg ml^{-1} 3,3′-diaminobenzidine. Sections were washed and mounted on glass slides, air-dried, counterstained with hematoxylin and cover-slipped with Entellan.

Neurochemical analysis

Levels of catecholamines were evaluated by high-performance liquid chromatography, combined with electrochemical detection (HPLC/EC) as previously described.[13]

Three-dimensional dendritic analysis

Animals were transcardially perfused with 0.9% saline under deep pentobarbital anesthesia and processed as described previously.[23] In summary, brains were immersed in Golgi–Cox solution[24] for 14 days, processed and cut on a vibratome at 200 µm thick coronal sections. For each selected neuron, all branches of the dendritic tree were reconstructed at × 1000 magnification, using a motorized microscope (Zeiss, Thornwood, NY, USA) attached to a camera (Sony, Tokyo, Japan) and Neurolucida software (Microbrightfield, Williston, VT, USA). A three-dimensional analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield). Ten neurons were analyzed for each animal.

Stereological analysis

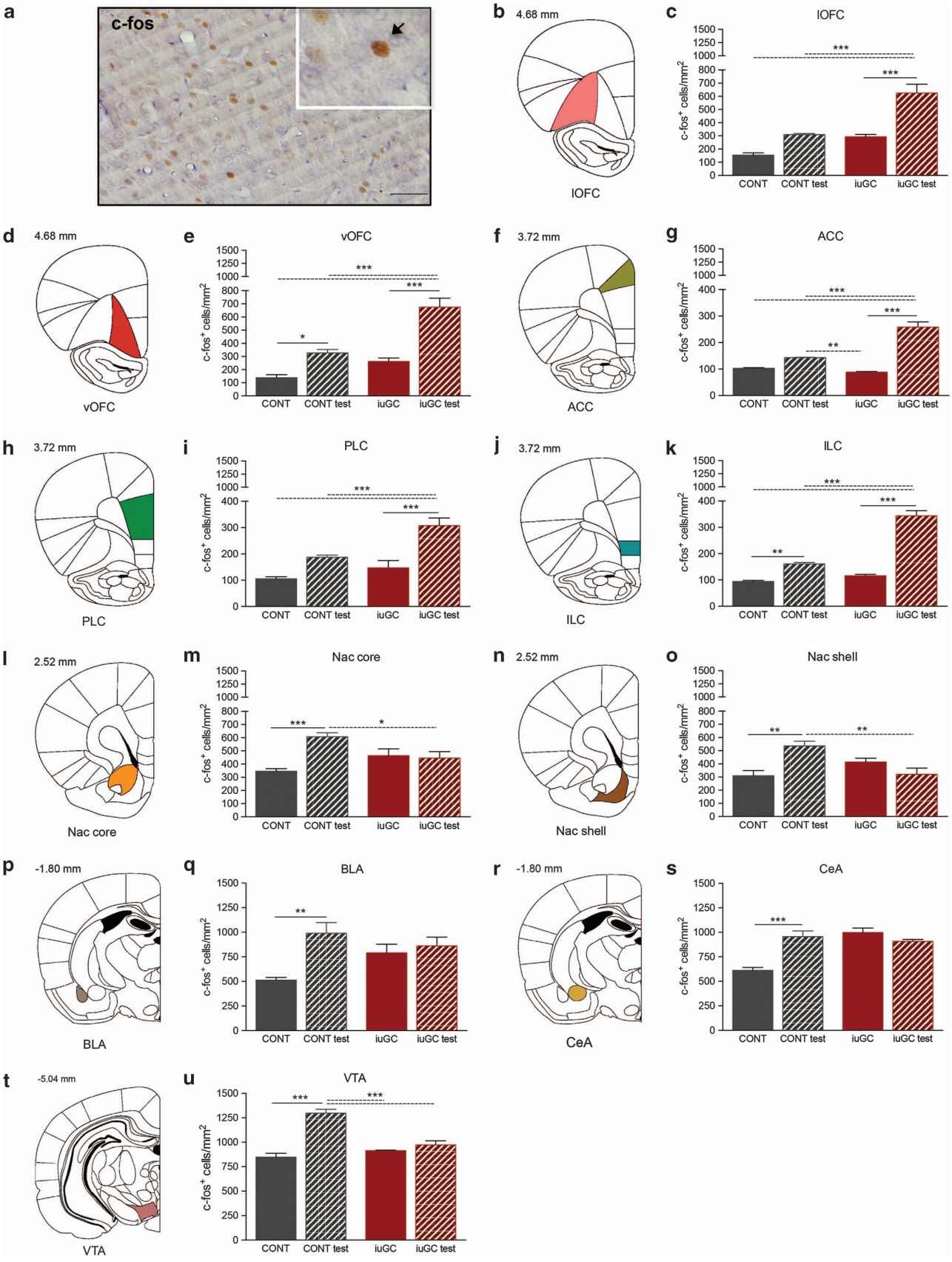
Perfused cerebral hemispheres were separated by a longitudinal cut in the midsagittal plane. The outline of the medial prefrontal cortex (mPFC)—infralimbic cortex (ILC), prelimbic cortex (PLC), anterior cingulate cortex (ACC)—and the orbitofrontal cortex (OFC)—dorsal OFC (dOFC) and ventral OFC (vOFC), was defined in each section using established landmarks.[22] Cavalieri's principle estimates of volumes and cell numbers were obtained using Integrator System software (Visiopharm, Copenhagen, Denmark) and a camera (PixeLINK, Ontario, Canada) attached to a motorized microscope (Olympus, Tokyo, Japan).

Drugs and treatment

All treatments started on the first day of the Pavlovian training and continued throughout all behavioral procedures. Levodopa (L-DOPA)/carbidopa (Sinemet, Merck, NJ, USA) was administrated orally at a dose of 24.0/6.0 mg kg^{-1} 3 h before behavioral procedures. Quinpirole hydrochloride (Biogen Scientifica, Madrid, Spain) was administered intraperitoneally at a dose of 0.15 mg kg^{-1} 30–40 min before the procedure. SKF82958 hydrobromide (Sigma, Seelze, Germany) was administered subcutaneously at a dose of 0.05 mg kg^{-1} 15 min before the procedure. All animals performed a shorter version of the selective PIT protocol described above - 4 days of Pavlovian training, 3 days of instrumental training (1 day of CRF, 1 day of RR5 and 1 day of RR10) and finally 1 day of PIT test.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 5.0 (La Jolla, CA, USA) and SPSS Statistics (Armonk, NY, USA). Parametrical and nonparametrical analyses were used when appropriate. Statistical comparisons are presented throughout the results section and in Supplementary Tables or in the legends of Supplementary Figures.



RESULTS

iuGC exposure impairs PIT in adulthood

Herein, we characterized, in the PIT protocol, an animal model of GC exposure at gestation days 18 and 19 (iuGC animals) that presents marked mesolimbic hypodopaminergia, anhedonia and drug-seeking behaviors.[11–13,25]

In the Pavlovian conditioning phase of the general PIT, both groups increased the number of MVs throughout the 10 days of training (Figure 1a, CONT: $F_{(1,144)} = 8.3$, $P = 0.013$; iuGC: $F_{(1,144)} = 11.2$, $P = 0.004$), with a significant effect of training day (CONT: $F_{(9,144)} = 7.9$, $P < 0.000$; iuGC: $F_{(9,144)} = 5.6$, $P < 0.000$). Also, results demonstrate that there was no significant interaction between the groups and day of training ($F_{(9,369)} = 0.2$, $P = 0.998$) or in the response to the cues ($F_{(1,360)} = 1.4$, $P = 0.174$). In the Pavlovian extinction test, animals from both groups conditioned to the CS+ (Supplementary Figure 1a). In the instrumental phase, both groups acquired the conditioning for lever pressing throughout the days of training (Figure 1b, $F_{(5,90)} = 134.6$, $P < 0.000$), at the same rate ($F_{(5,90)} = 1.2$, $P = 0.339$). In the test day, the BPI of CONT and iuGC animals did not differ (Figure 1c, $t_{18} = 0.2$, $P = 0.844$). While the CONT group showed an increase on the lever press when the CS+ was presented in comparison with the CS− (Figure 1d, $t_9 = 4.7$, $P = 0.001$) as expected; contrarily, the iuGC animals did not differ in the lever pressing between the CS+ and the CS− presentation periods ($t_9 = 1.1$, $P = 0.300$), indicating a disruption in the general PIT transfer. The impairment was further confirmed by the absence of interaction between the groups and the cues ($F_{(1,36)} = 1.4$, $P = 0.244$).

Besides the effect that cues paired with a reward can have in invigorating responses, they can also bias response selection,[26,27] a phenomenon evaluated by a selective version of the PIT paradigm, in which different rewards are paired with different cues. Pavlovian training for the selective PIT showed a significant increase in the MVs throughout the 9 days of training (Figure 1e; CONT: $F_{(1,104)} = 46.6$, $P < 0.000$; iuGC: $F_{(1,104)} = 28.2$, $P < 0.000$) with a strong effect of training day (CONT: $F_{(8,104)} = 1.8$, $P = 0.080$; iuGC: $F_{(8,104)} = 0.4$, $P = 0.001$). The analysis of interaction showed that both groups had the same performance through the days ($F_{(8,252)} = 0.001$, $P = 0.977$) and in response to the cues ($F_{(1,252)} = 0.155$, $P = 0.996$). In instrumental conditioning, both groups increased lever pressing throughout training (Figure 1f, $F_{(5,70)} = 118.0$, $P < 0.000$), with identical rate of acquisition ($F_{(5,70)} = 1.2$, $P = 0.320$). In the PIT transfer, the BPI of both groups did not differ (Figure 1g, $t_{14} = 0.4$, $P = 0.694$). On the same test, CONT animals showed evidence for outcome-specific transfer (Figure 1h, $t_7 = 5.3$, $P = 0.001$), whereas iuGC animals showed no discrimination between levers, presenting a marked impairment in the transfer ($t_7 = 0.36$, $P = 0.731$). The absence of response by iuGC group was further confirmed by the absence of interaction between the groups and the response to the cues ($F_{(1,28)} = 3.0$, $P = 0.097$).

iuGC exposure does not shift goal-directed actions to habit formation

Importantly, we have shown that stress biases decision-making strategies, potentiating the transition from goal-directed to habitual actions.[21,28] Moreover, it has been suggested that a Pavlovian CS can potentiate habitual responding more than it can potentiate goal-directed actions,[29] and that habits are particularly sensitive to general transfer effects as they are not associated with detailed sensory representations of the outcome.[30] Considering this, we decided to further explore the impact of prenatal GC exposure in the development of instrumental habit, using the operant behavior test. Animals increased lever pressing throughout training (Supplementary Figure 1b, $F_{(15,195)} = 89.7$, $P < 0.000$), with identical rate of acquisition between groups ($F_{(1,195)} = 0.8$, $P = 0.392$). To ascertain devaluation effects in action–outcome contingency, we analyzed two different periods: after moderate (early test) and extensive (late test) training. In both tests, we observed a decrease in lever pressing in the devalued condition versus valued condition (early test, Supplementary Figure 1c; CONT: $t_{12} = 4.1$, $P = 0.002$; iuGC: $t_{14} = 2.9$, $P = 0.124$; late test, Supplementary Figure 1d; CONT: $t_{11} = 4.6$, $P = 0.001$; iuGC: $t_{11} = 4.4$, $P = 0.001$), suggesting that iuGC animals acquire habitual responding at a similar rate as CONT individuals.

iuGC exposure impairs neuronal activation upon PIT performance

With the intent to dissect the neuronal circuitry involved in the impaired PIT response, we evaluated the neuronal activation pattern (using c-fos, Figure 2a) during PIT transfer.

Results from the behavioral performance of the selective PIT protocol showed that although both CONT and iuGC animals acquired similar Pavlovian conditioning (Supplementary Figure 2a; interaction between groups and days of training: $F_{(8,305)} = 0.466$, $P = 0.880$; interaction between groups and cue: $F_{(1,305)} = 0.969$, $P = 0.326$) and instrumental conditioning (Supplementary Figure 2b, $F_{(5,85)} = 0.05$, $P = 0.998$), the iuGC animals had a clear impairment on the PIT test (Supplementary Figure 2d, CONT: $t_8 = 3.0$, $P = 0.017$; iuGC: $t_8 = 0.6$, $P = 0.558$; interaction between groups and cue: $F_{(1,16)} = 1.0$, $P = 0.331$). The BPI of CONT and iuGC animals on PIT test day did not differ (Supplementary Figure 2c, $t8 = 1.5$, $P = 0.164$).

A marked neuronal activation after PIT performance was found in the mPFC and OFC. Results showed a significantly different pattern of activation between groups that performed PIT and groups that did not (lateral OFC: $F_{(1,15)} = 5.3$, $P = 0.037$; vOFC: $F_{(1,15)} = 7.2$, $P = 0.017$; ACC: $F_{(1,15)} = 40.4$, $P < 0.000$; PLC: $F_{(1,15)} = 3.8$, $P = 0.071$; ILC: $F_{(1,15)} = 13.5$, $P < 0.000$). As anticipated, *post hoc* analysis showed that several regions of the OFC, namely lateral OFC and vOFC, were recruited after PIT performance in CONT animals ($P = 0.062$, $P = 0.029$, respectively) and in iuGC animals (Figures 2b–e; $P < 0.000$). Similarly, mPFC subregions such as ACC, PLC and ILC were also activated in CONT animals ($P = 0.066$, $P = 0.059$, $P = 0.003$, respectively) and iuGC animals (Figures 2f–k; $P < 0.000$). These results are in accordance with previous data that

Figure 2. c-fos immunohistochemistry revealed that iuGC exposure leads to a differential neuronal activation pattern in iuGC animals after selective PIT performance. (**a**) Representative image of c-fos immunostaining in the PLC of an animal that performed the PIT test (black arrow indicates a c-fos$^+$ cell). PIT transfer recruited all prefrontal cortical regions of both groups but at different extents, with the activation being more pronounced in the iuGC animals compared with controls in the lOFC (**c**), vOFC (**e**), ACC (**g**), PLC (**i**) and ILC (**k**). In the limbic regions, PIT testing increased c-fos staining in control animals in all regions analyzed—NAc core (**m**), NAc shell (**o**), BLA (**q**), CeA (**s**) and VTA (**u**); whereas iuGC animals presented no activation upon testing. Representative images of coronal brain sections of (**b**) lOFC, (**d**) vOFC, (**f**) ACC, (**h**) PLC, (**j**) ILC, (**l**) NAc core, (**n**) NAc shell, (**p**) BLA, (**r**) CeA and (**t**) VTA are shown; numbers represent distance in millimeters to bregma. Average numbers ± s.e.m. are plotted. ACC, anterior cingulate cortex; BLA, basolateral amygdala; CeA, central amygdala; CONT, control animals; ILC, infralimbic cortex; lOFC, lateral OFC; iuGC, *in utero* glucocorticoid exposed animals; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; OFC, orbitofrontal cortex; PLC, prelimbic cortex; vOFC, ventral OFC; VTA, ventral tegmental area. nCONT = 5, niuGC = 5, nCONT test = 5, niuGC test = 4. $^*P \leqslant 0.05$, $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$. Scale bar, 50 μm.

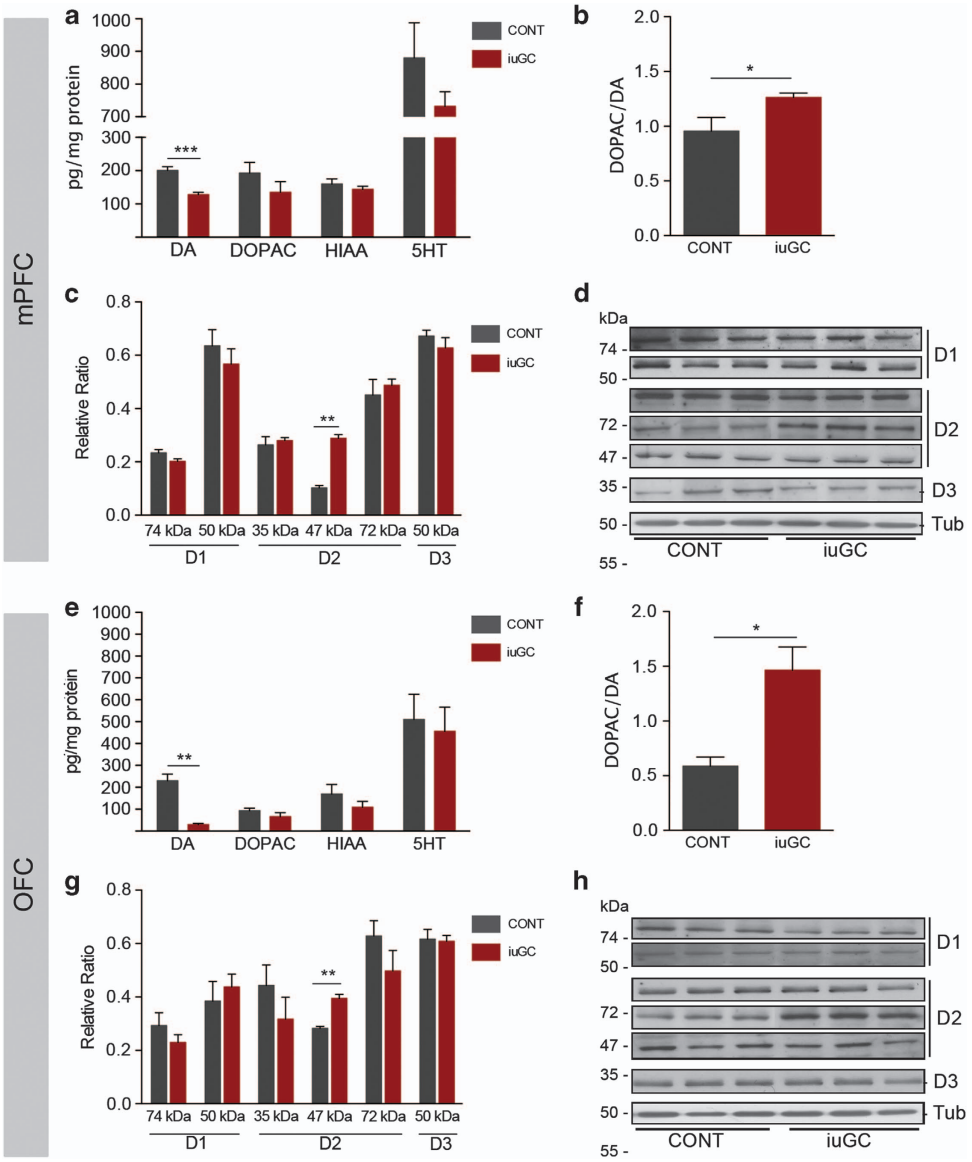


Figure 3. iuGC exposure affects cortical dopaminergic circuitry. (**a**) Dopamine (DA) was substantially reduced in the mPFC of iuGC animals in comparison with CONT animals in parallel with increased turnover (**b**), as measured by high-performance liquid chromatography with electrochemical detection. No differences in serotonin were found. (**c**) The levels of the nonglycosylated isoform of the dopamine receptor 2 (D2, ~47 kDa) were increased in the mPFC of iuGC animals, but no changes were observed regarding the D2 glycosylated isoform (~72 kDa), the putative D2 precursor (~35 kDa), the D1 glycosylated isoform (~74 kDa), the D1 nonglycosylated isoform (~50 kDa) or the D3 receptor (~50 kDa). (**d**) Representative immunoblot of D1 isoforms, D2 isoforms and D3 in the mPFC; tubulin was used as housekeeping protein. (**e**) In the OFC, we found reduced levels of DA in iuGC animals in comparison with CONT animals in parallel with an increased turnover of DA in this brain region (**f**). No differences in serotonin were found. (**g**) In the OFC, the nonglycosylated isoform of the D2 receptor (~47 kDa) was augmented in iuGC animals, whereas no changes were observed regarding the D2 glycosylated isoform (~72 kDa), the putative D2 precursor (~35 kDa), the D1 glycosylated isoform (~74 kDa), the D1 nonglycosylated isoform (~50 kDa) or the D3 receptor (~50 kDa). (**h**) Representative immunoblot of D1 isoforms, D2 isoforms and D3 in the OFC; tubulin was used as housekeeping protein. Average numbers ± s.e.m. are plotted. CONT, control animals; DOPAC, 3,4-dihydroxyphenylacetic acid; 5HIAA, 5-hydroxyindoleacetic acid; 5HT, serotonin; iuGC, *in utero* glucocorticoid exposed animals; mPFC, medial prefrontal cortex; OFC, orbitofrontal cortex. $n = 5$/group. $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$.

show the involvement of these regions in PIT.[16] Although iuGC animals did not present major differences in basal c-fos staining, iuGC animals that performed the PIT test presented a marked hyperactivation of these cortices in comparison with CONT animals (ACC, $P < 0.000$; PLC, $P = 0.003$; ILC, $P < 0.000$; lateral OFC, $P < 0.000$; vOFC, $P < 0.000$).

Results from the limbic regions - NAc core ($F_{(1,15)} = 13.5$, $P = 0.002$), NAc shell ($F_{(1,15)} = 16.5$, $P = 0.001$), basolateral amygdala ($F_{(1,15)} = 5.8$, $P = 0.029$), central amygdala ($F_{(1,15)} = 28.9$, $P < 0.000$) and VTA ($F_{(1,15)} = 27.8$, $P < 0.000$) also showed a significantly different pattern of activation between groups that have performed the PIT test or not. As anticipated, *post hoc* analysis showed that the NAc - core and shell (Figures 2l–o; $P = 0.001$, $P = 0.004$, respectively), the amygdala - basolateral amygdala and central amygdala (Figures 2p–s; $P = 0.005$, $P < 0.000$, respectively), and VTA (Figures 2t–u; $P < 0.000$) were strongly activated in CONT animals. On the contrary, no activation was observed in iuGC group, indicating a hypoactivation of the mesolimbic circuitry.

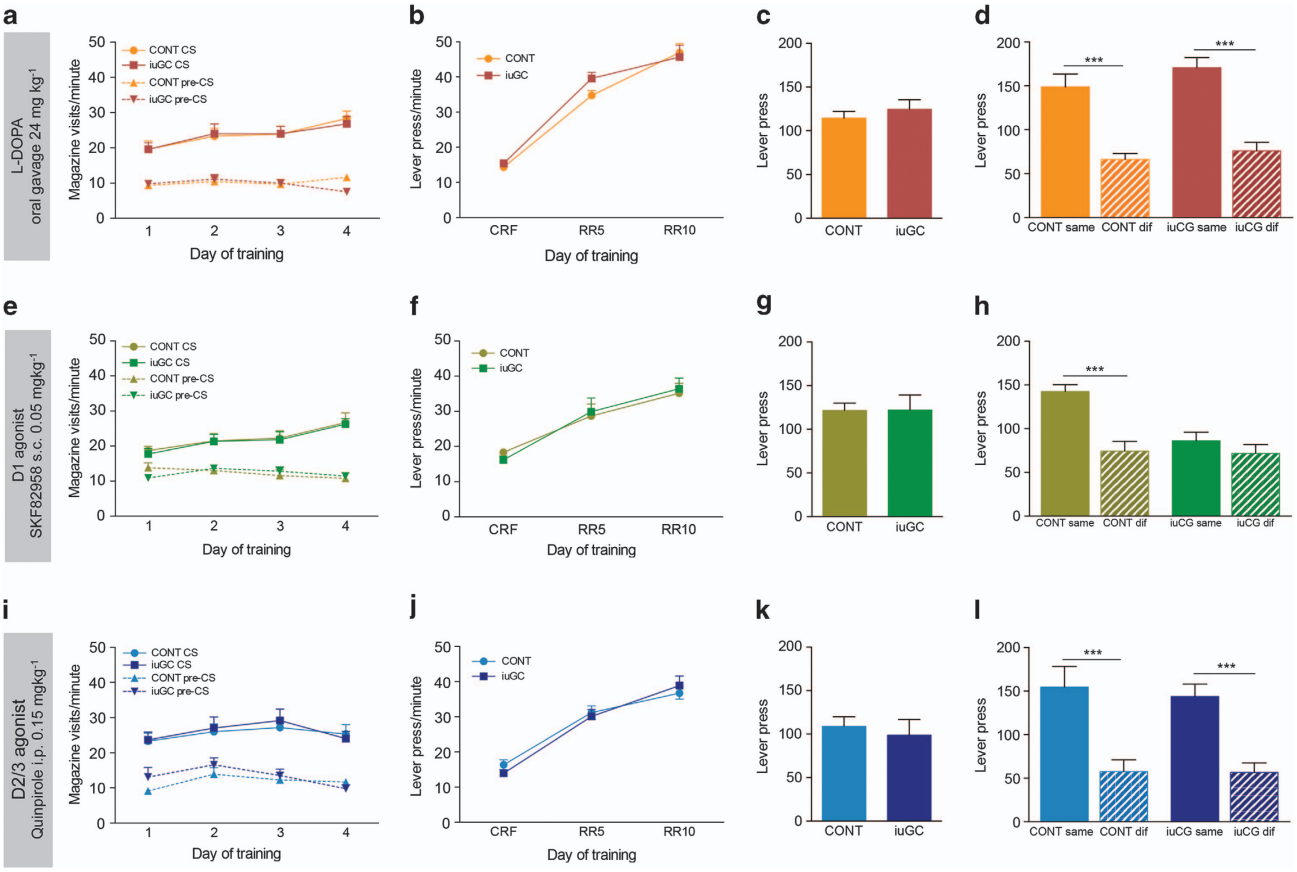


Figure 4. Modulation of dopaminergic transmission rescues impairment in PIT performance. Treatment with 24 mg kg^{-1} of L-DOPA, 3 h before behavior procedure did not alter the (**a**) Pavlovian conditioning and (**b**) the instrumental conditioning learning curves, or (**c**) the BPI. (**d**) L-DOPA treatment fully reverted the PIT impairment of iuGC animals. Administration of D2/D3 agonist, quinpirole at 15 mg kg^{-1} 30–40 min before behavior procedure, did not influence both (**e**) Pavlovian or (**f**) instrumental conditioning, as well as (**g**) the BPI. (**h**) D2/D3 agonist treatment fully reverted the PIT impairment in iuGC animals. Treatment with D1 agonist SKF82958 at 0.05 mg kg^{-1} 15 min before behavior procedure did not affect (**i**) Pavlovian and (**j**) instrumental conditioning or (**k**) the BPI. (**l**) The same treatment did not revert the PIT impairment observed in iuGC animals. Average numbers ± s.e.m. are plotted. CONT, control animals; CS, conditioned stimulus; iuGC, *in utero* glucocorticoid exposed animals; L-DOPA, levodopa. *$P \leqslant 0.05$, **$P \leqslant 0.01$, ***$P \leqslant 0.001$. $n = 8$ per group.

Dopaminergic dysfunction in iuGC animals

Several studies focused on the structural, morphological and neurochemical changes that iuGC exposure causes in limbic regions;[11,13,31] however, less is known about the cortical regions that also receive dopaminergic transmission arising from the VTA. In the current study we analyzed the neuronal number and volume of the OFC subregions - lateral OFC and vOFC, and the mPFC subregions - ACC, PLC and ILC (Supplementary Figure 3). In addition, we also performed three-dimensional morphological analysis of the pyramidal neurons of layer II/III of these regions. Interestingly, and despite slight differences in some of the mPFC and OFC subregions, it seems that iuGC exposure does not substantially affect the neuronal structure of these brain regions (Supplementary Figure 4).

To further evaluate the integrity of the above-mentioned regions, we quantified monoamine levels by HPLC/EC. DA levels were significantly reduced in the mPFC of iuGC animals (Figure 3a, $U = 0.0$, $P = 0.008$); this was accompanied by a significant increase in the DA turnover (Figure 3b, $U = 1.0$, $P = 0.016$). A similar pattern was found in the OFC (Figures 3e and f; DA: $U = 0.0$, $P = 0.008$; DA turnover: $U = 0.0$, $P = 0.016$). There were no major differences in serotonin levels in all the regions analyzed (Figures 3a and e).

We next quantified the expression levels of DA receptors in these brain regions by western blot analysis. D2 receptor (~47 kDa isoform) was increased in the mPFC (Figures 3c and d; $U = 0.0$, $P = 0.01$) and in the OFC (Figures 3g and h; $U = 0.0$, $P = 0.004$) of iuGC animals; these findings are in alignment with those previously reported for the NAc[13] and amygdala.[12] Conversely, we did not find changes in the D1 or D3 receptors in both the mPFC (Figures 3c and d) and the OFC (Figures 3g and h).

Manipulation of dopaminergic signaling improves PIT performance of iuGC animals

Since DA has a crucial role in reward-associated behaviors,[6] and in particular, in PIT performance,[16] we decided to normalize DA levels in the iuGC animals by performing a systemic treatment with the DA precursor L-DOPA. Administration of L-DOPA before training stages was effective in normalizing DA levels in the mesocorticolimbic circuit of iuGC animals (Supplementary Figure 5), and had no apparent effect on the performance on the Pavlovian conditioning, as both treated groups showed a significant increase in the MVs performed during the CS period compared with the pre-CS period (Figure 4a; CONT: $F_{(1,54)} = 28.6$, $P < 0.000$; iuGC: $F_{(1,54)} = 34.2$, $P < 0.000$). We further observed the absence of interaction between the treated groups and days of training ($F_{(3,136)} = 0.735$, $P = 0.533$) or the response to the cue ($F_{(1,136)} = 0.087$, $P = 0.768$). Also, the instrumental conditioning was identical between the groups (Figure 4b; $F_{(2,34)} = 0.820$, $P = 0.449$). On the test day, BPI of the CONT and iuGC animals was similar

(Figure 4c, $t_{17} = 0.8$, $P = 0.442$). Yet, L-DOPA treatment fully rescued the impaired PIT response of the iuGC animals (Figure 4d; CONT: $t_{18} = 5.1$, $P < 0.000$; iuGC: $t_{16} = 6.3$, $P < 0.000$), which was further confirmed by a significant interaction between treatment and the response to the cues ($F_{(1,34)} = 63.67$, $P < 0.000$).

Because there was sparse evidence for the functional role of D1 and D2 receptors in PIT performance, and considering our molecular data showing divergent results in their expression levels, we decided to treat animals with either the D1-specific agonist SKF82958 or the D2/3-specific agonist quinpirole. To avoid the motor effects of the drugs, we monitored the locomotor behavior of the animals after treatment (Supplementary Figures 5b and c).

In the selective PIT, D1 agonist-treated groups did not differ in the Pavlovian conditioning as both treated groups increased significantly the MVs performed during the CS period compared with the pre-CS period (Figure 4e; CONT: $F_{(1,30)} = 18.7$, $P = 0.002$; iuGC: $F_{(1,30)} = 52.6$, $P < 0.000$). The absence of an effect of the treatment in the conditioning was confirmed by a nonsignificant interaction between the groups and the days of training ($F_{(3,80)} = 0.7$, $P = 0.512$) or the response to the cues ($F_{(1,80)} =$,0.8 $P = 0.313$). The D1 agonist also had no effect in the instrumental conditioning of both the groups (Figure 4f; $F_{(2,20)} = 0.4$, $P = 0.666$). This treatment was not effective in reverting the PIT impairment observed in iuGC animals (Figure 4h; CONT: $t_{10} = 5.0$, $P = 0.035$; iuGC: $t_{10} = 1.0$, $P = 0.330$), with an absence of a significant interaction between the treated groups and the cue ($F_{(1,20)} = 2.3$, $P = 0.142$). However, the BPI of both groups in the PIT transfer was not different (Figure 4g, $t_{10} = 0.03$, $P = 0.973$). It is important to mention that the selected dosage of D1 agonist was biologically relevant as the animals were conditioned in a conditioned place preference paradigm (Supplementary Figure 6).

D2/3 agonist-treated animals did not present major changes in the Pavlovian conditioning as both CONT and iuGC animals treated with the agonist increased significantly the MVs performed during the CS period compared with the pre-CS period (Figure 4h; CONT: $F_{(1,54)} = 33.6$, $P < 0.000$; iuGC: $F_{(1,54)} = 19.9$, $P = 0.0004$). The absence of an effect of the D2/3 agonist treatment in the Pavlovian conditioning was further confirmed by the absence of interaction between the groups and the days of training ($F_{(3,136)} = 0.6$, $P = 0.597$) or the response to the cues ($F_{(1,136)} = 0.2$, $P = 0.660$). The D2/3 agonist treatment also failed to alter the instrumental conditioning of both the groups (Figure 4i; $F_{(2,34)} = 1.1$, $P = 0.330$). Importantly, D2/3 activation did not alter the BPI of CONT and iuGC animals ($t_{17} = 0.5$, $P = 0.632$), but it fully reverted the PIT impairment of iuGC animals (Figure 4k; CONT: $t_9 = 5.6$, $P = 0.0004$; iuGC: $t_8 = 11.9$, $P < 0.000$), which was confirmed by the significant interaction between the treatment and the response to the cues (Figure 4j, $F_{(1,34)} = 30.1$, $P < 0.000$).

Interestingly, the different treatments applied (L-DOPA, D1 agonist and D2 agonist) did not influence the baseline performance in the PIT test day ($F_{(2,44)} = 0.4$, $P = 0.696$).

Altogether, our results suggest that normalization of DA levels in the iuGC animals is crucial for the correct expression of PIT behavior, and this is dependent on the activation of D2/3, but not D1 receptors.

DISCUSSION

Herein, we show that iuGC animals present a significant impairment in the PIT performance that is dependent of DA levels. It is important to distinguish between general and selective PIT, as they are proposed to reflect distinct forms of incentive processing and to be mediated by somewhat different neural systems.[16,26,27] In general PIT, a Pavlovian cue generates an overall increase in the vigor of instrumental responding, independent of the specific sensory properties of the reward,[29,30,32] whereas in selective PIT, a CS elicits sensory-specific features of an outcome, biasing instrumental actions towards that outcome.[29,30,32] The disruption of both types of PIT suggests that iuGC exposure induces a general motivational deficit rather than alterations in bias action selection. Importantly, these findings are in accordance with human studies showing that childhood maltreatment is associated with blunted subjective responses to reward predicting cues in adulthood.[33] Evidence shows that injection of GCs after Pavlovian conditioning impairs transfer[34] and we recently demonstrated that chronic stress robustly decreases PIT performance.[20] This deficit is likely to occur as a result of programming effects of stress in dopaminergic neurons,[35,36] that ultimately leads to a decrease in the attribution of incentive salience.[11,37–39]

PIT is a complex behavior highly dependent on an intricate interaction between dopaminergic circuits of limbic (NAc, amygdala, dorsal striatum) and cortical (mPFC, OFC) structures.[16] The Pavlovian conditioning is highly dependent on limbic structures, particularly the NAc that is implicated in attaching motivational significance to Pavlovian stimuli.[40–42] Blockade of dopaminergic transmission in this region remarkably affects PIT behavior.[17,43,44] Yet, and despite a strong reduction in NAc dopaminergic signaling triggered by iuGC exposure, animals are able to acquire the Pavlovian conditioning, which may reflect compensation by other brain regions involved in this process, such as the central amygdala that encodes the affective value of the reward.[32] This is in line with studies showing that neurotoxic lesions of the NAc abolish PIT without affecting Pavlovian or instrumental conditioning separately.[45] The second phase of PIT encompasses instrumental conditioning that was apparently normal in the iuGC animals. This stage is mostly mediated by the dorsal striatum[46–48] with different functions ascribed to dorsolateral and dorsomedial subregions.[27,49] Importantly, it has been proposed that as behavior becomes habitual (dorsolateral subregion-dependent), it is also more susceptible for transfer of control.[50] Moreover, chronic stress can potentiate habit formation by inducing a hypertrophy of this region.[21] However, we found that iuGC exposure did not bias behavior towards habit, indicating a relatively preserved dorsal striatum.

Interestingly, the iuGC animals were unable to transfer, a phenomenon that can be the result of an abnormal neuronal activation of the regions that have an active role on PIT. For example, VTA lesions disrupt DA release in the NAc and produce an overall reduction in both general and selective PIT.[27] Thus, the observed hypoactivation of the VTA and NAc could (partially) explain the blunted response of the iuGC animals. On the contrary, as iuGC animals present hypotrophy and hypodopaminergia of cortical brain regions (mPFC and OFC), we also suggest that the hyperactivation observed during PIT testing reflects a compensatory adaptation. These changes could underlie PIT deficits as parallel phasic activation of mPFC and OFC neuronal subsets is required to integrate the transfer from Pavlovian incentives to instrumental actions,[51] contrary to the initial idea that each region acted on itself.[52–55]

The absence of transfer, with preservation of both Pavlovian and instrumental acquisitions, supports the premise that DA is crucial for the attribution of general incentive salience to reward-associated cues.[6,30] We found that normalization of DA levels was sufficient to fully rescue PIT disruption in the iuGC animals. It is well documented that boosting of DA by administration of amphetamines facilitates PIT performance.[56–58] Also, hyperdopaminergic mutant mice have higher incentive salience for sweet rewards.[59] Still, we failed to observe any detectable effect of L-DOPA in PIT of CONT animals, a discrepancy probably explained by an insufficient rise of DA levels with our treatment.

One other interesting finding supported by this work and our previous studies[12,13] was the selectivity of DA receptor D2 expression changes (putatively due to epigenetic alterations[13]) in the mesocorticolimbic system, particularly in the light of

evidence associating D2 receptor with reward-associated disorders that present altered incentive salience attribution such as addiction and binge eating[60] as well as motivation perception.[61] Moreover, it was shown that the D2 antagonist pimozide abolishes PIT,[17] and NAc microinjections of D2 antagonist raclopride reduce transfer.[43] However, to our knowledge, no studies have evaluated the effect of specific activation of DA receptors in PIT performance. Here, we found that systemic administration of quinpirole (D2/3 agonist) completely reverted PIT disruption in the iuGC animals, whereas a D1 agonist had no effect. These results indicate that iuGC-induced D2 dysfunction underlies PIT impairment, although we cannot fully exclude a role for D1 receptor in this process.

The observed reduced cue motivational drive fits with the marked anhedonia for natural rewards (food and sex) of iuGC animals.[25,31] However, it is somewhat opposing to the enhanced drug-seeking behavior observed in iuGC animals,[13] considering the evidence showing that individual propensity to attribute incentive salience to food cues is predictive of addictive behavior and cue-induced reinstatement.[62,63] Yet, one hypothesis is that incentive salience amplifies 'wanting' in ways that can be specific to one motivational target.[6] For example, drugs that act on the dopaminergic system such as amphetamines can be more desired than natural rewards for some individuals.[6,64] However, due to the intrinsic complexity of this behavior, we believe that additional studies are needed to comprehend the link between incentive salience attribution and the development of aberrant behaviors such as addiction.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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