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

AJ Rodrigues[1,2,4], P Leão[1,2,4], JM Pêgo[1,2], D Cardona[1,2], MM Carvalho[1,2], M Oliveira[1,2], BM Costa[1,2], AF Carvalho[1,2], P Morgado[1,2], D Araújo[1,2], JA Palha[1,2], OFX Almeida[3] and N Sousa[1,2]

[1]*Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal;* [2]*ICVS/3B's–PT Government Associate Laboratory, Braga/Guimarães, Portugal and* [3]*Neuroadaptations Group, Max Planck Institute of Psychiatry, Munich, Germany*

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

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

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

Correspondence: Dr N Sousa, Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.
E-mail: njcsousa@ecsaude.uminho.pt
[4]These authors contributed equally to this work.
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Materials and methods

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Results

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

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

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

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

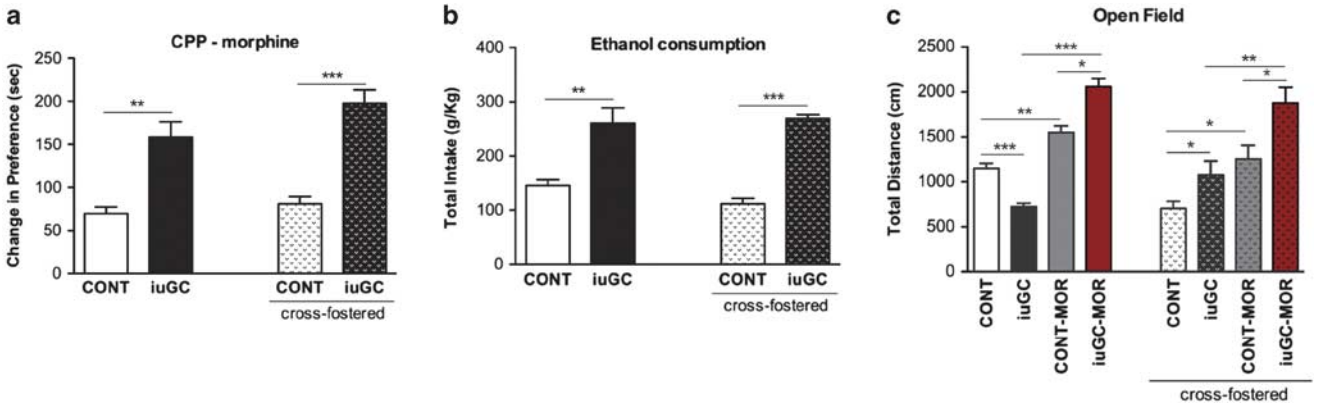
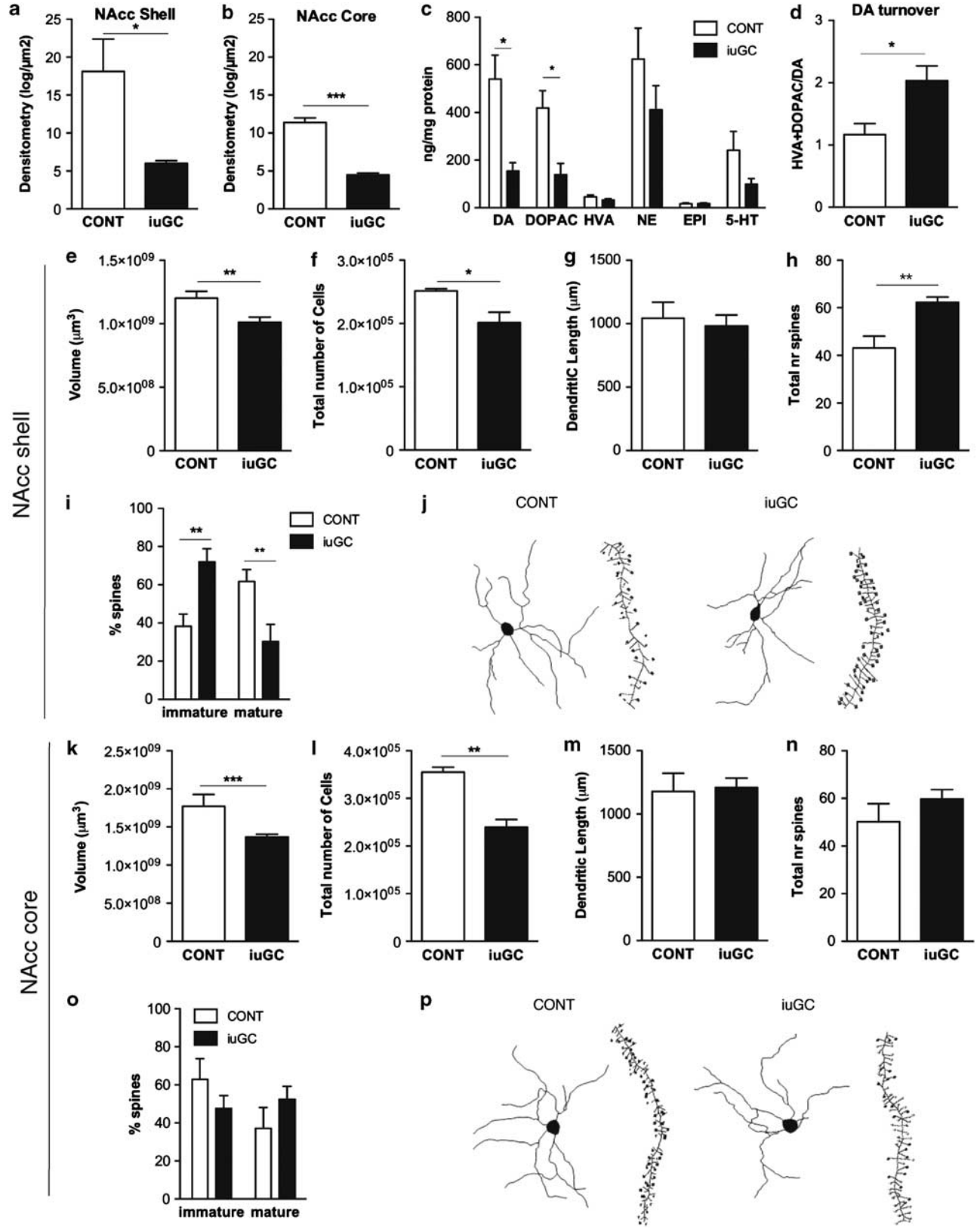


Figure 1 Prenatal *in utero* glucocorticoid (iuGC) exposure enhances drug-seeking behaviors. (**a**) In the contingent conditioned place preference paradigm (CPP), iuGC animals spend significantly more time in the morphine-associated compartment than controls. (**b**) In the non-contingent two-bottle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

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

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

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



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

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

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

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

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

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

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

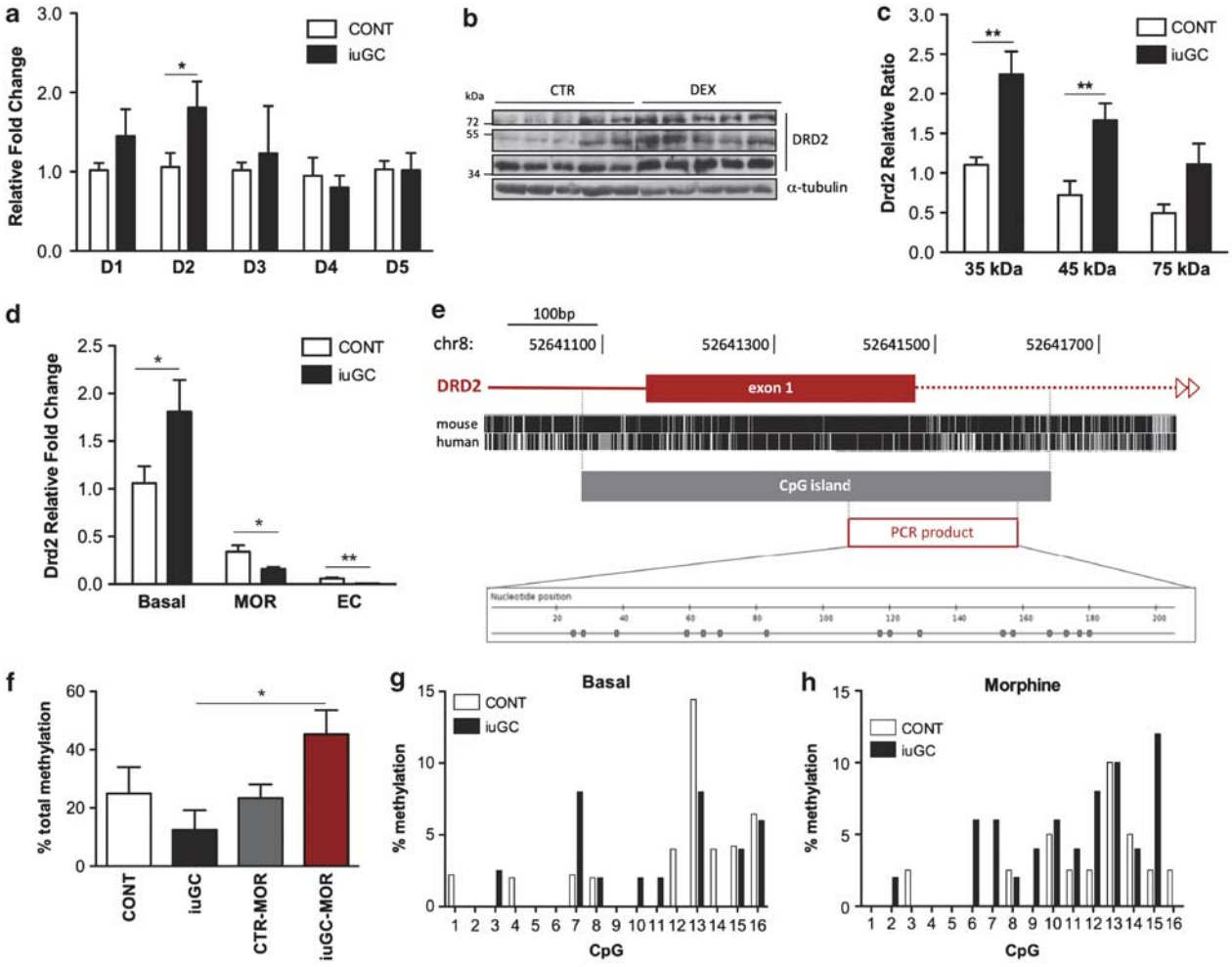


Figure 3 Impaired *dopamine receptor 2* (*Drd2*) response in *in utero* glucocorticoid-exposed (iuGC) animals under basal conditions and after exposure to substances of abuse. (**a**) *Drd2* mRNA expression was augmented in iuGC animals when compared with controls, but no changes were found in the expression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

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

Discussion

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

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

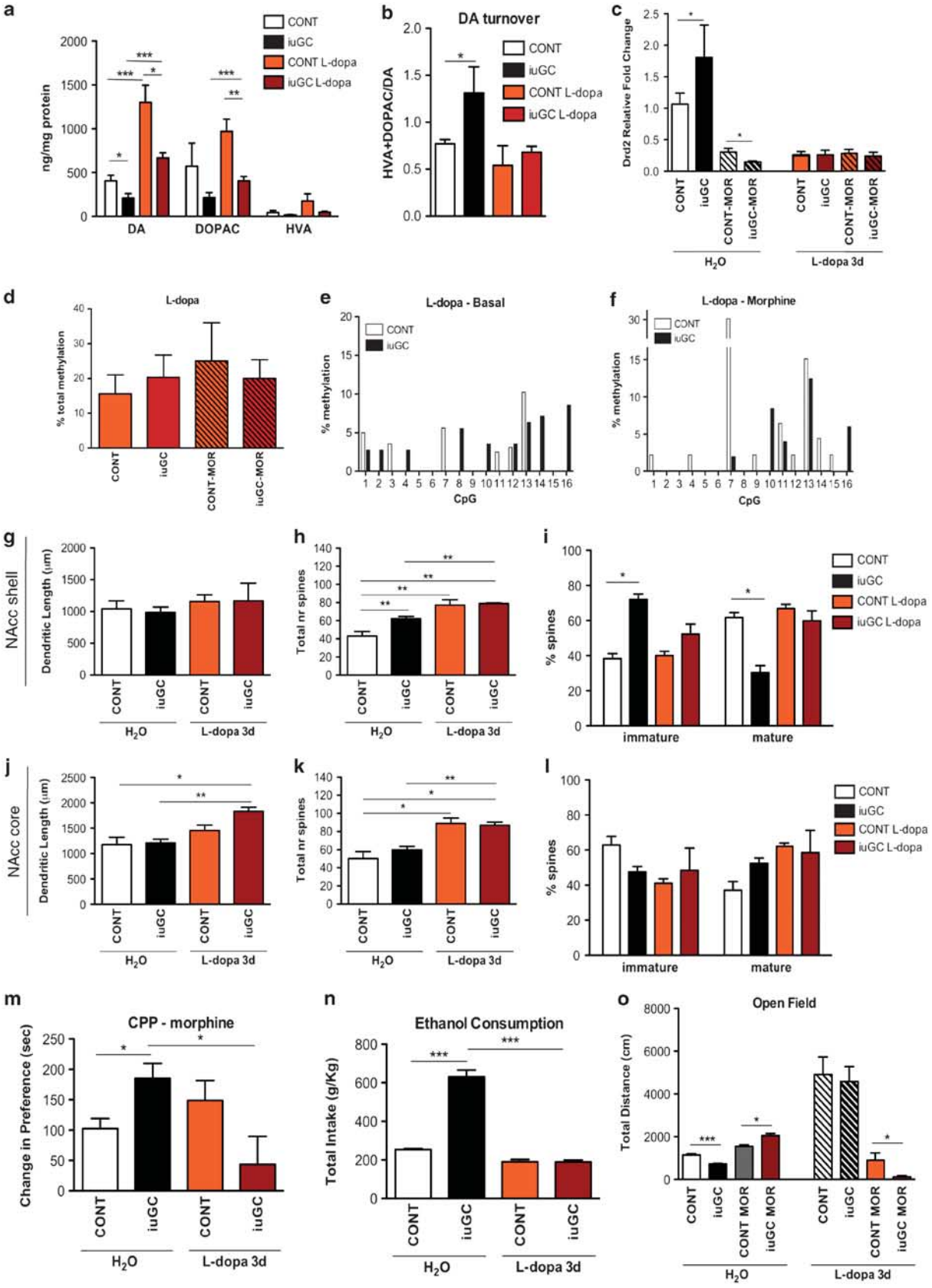
An intricate relationship between stress, the GC released in response to stress, and dopaminergic tone in the regulation of vulnerability to drug and substance abuse has been suggested.[1,5,14,26,41] Stress and drugs of abuse appear to activate dopaminergic synapses in a similar manner,[41] culminating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

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

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

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

Figure 4 Restoration of dopamine (DA) levels by L-dopa reverts the molecular, structural and behavioral phenotypes of *in utero* glucocorticoid (iuGC) animals. (**a**) Acute (3 days) treatment with L-dopa increased DA levels in the nucleus accumbens (NAcc) of both experimental groups; although iuGC animals still exhibited less DA than controls. In fact, iuGC animals given L-dopa presented DA levels similar to those of controls without treatment. (**b**) No differences were found in DA turnover after L-dopa treatment in iuGC animals. (**c**) *Dopamine receptor 2* (*Drd2*) expression was diminished after L-dopa treatment both in a basal situation and after morphine exposure (values normalized to controls given water). (**d**) L-dopa treatment did not change *Drd2* methylation status in a basal situation (**e**), but was able to revert the increased methylation in iuGC animals after morphine exposure (**f**). L-dopa supplementation had no significant effect on NAcc shell dendritic length (**g**), but triggered an increase in the number of spines, albeit similarly in control and iuGC animals, and reverted the altered ratio of mature to immature spines observed in iuGC animals (**h** and **i**). (**j**) In contrast, L-dopa treatment increased dendritic length in the NAcc core of both groups. An increase in the number of spines was also observed in both groups with no changes in the type of spines (**k** and **l**). (**m**) L-dopa treatment reverted the higher vulnerability of iuGC animals to morphine-induced CPP and also reverted the ethanol preference displayed by these animals (**n**). (**o**) In agreement, the higher locomotor pattern after morphine displayed by iuGC rats was completely reverted by L-dopa treatment. No differences were found in the locomotion between L-dopa treated control and iuGC animals in a basal situation. Data is presented as mean ± s.e.m. CONT, controls; MOR, after morphine injection 10 mg kg^{-1}; 3d: 3 days; $^{*}P<0.05$, $^{**}P<0.01$,$^{***}P<0.001$.


a
ng/mg protein
CONT
iuGC
CONT L-dopa
iuGC L-dopa
DA
DOPAC
HVA
b
DA turnover
HVA+DOPAC/DA
c
Drd2 Relative Fold Change
CONT-MOR
iuGC-MOR
H2O
L-dopa 3d
d
L-dopa
% total methylation
e
L-dopa - Basal
% methylation
CpG
f
L-dopa - Morphine
g
NAcc shell
Dendritic Length (µm)
h
Total nr spines
i
% spines
immature
mature
j
NAcc core
k
l
m
CPP - morphine
Change in Preference (sec)
n
Ethanol Consumption
Total Intake (g/Kg)
o
Open Field
Total Distance (cm)

both control and iuGC animals, *Drd2* methylation was observed to a greater extent in the iuGC group. This observation suggests that DNA methylation is not the sole mechanism involved in transcriptional repression of *Drd2* gene. Consistent with this, recent studies have demonstrated interdependence and cooperation between DNA methylation and histone modifications in the regulation of gene silencing and activation.[51] More extensive studies are needed to decipher the precise mechanisms underlying the 'epigenetic potential' of iuGC animals, namely the complex regulation of *Drd2* gene expression, which facilitates adaptation to specific physiological states and demands.

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

Conflict of interest

The authors declare no conflict of interest.

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