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Dopaminergic Modulation of Affective and Social Deficits Induced by Prenatal Glucocorticoid Exposure

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Prenatal stress or exposure to elevated levels of glucocorticoids (GCs) can impair specific neurobehavioral circuits leading to alterations in emotional processes later in life. In turn, emotional deficits may interfere with the quality and degree of social interaction. Here, by using a comprehensive behavioral approach in combination with the measurement of ultrasonic vocalizations, we show that *in utero* GC (iuGC)-exposed animals present increased immobility in the forced swimming test, pronounced anhedonic behavior (both anticipatory and consummatory), and an impairment in social interaction at different life stages. Importantly, we also found that social behavioral expression is highly dependent on the affective status of the partner. A profound reduction in mesolimbic dopaminergic transmission was found in iuGC animals, suggesting a key role for dopamine (DA) in the etiology of the observed behavioral deficits. Confirming this idea, we present evidence that a simple pharmacological approach—acute L-3,4-dihydroxyphenylacetic acid (L-DOPA) oral administration, is able to normalize DA levels in iuGC animals, with a concomitant amelioration of several dimensions of the emotional and social behaviors. Interestingly, L-DOPA effects in control individuals were not so straightforward; suggesting that both hypo- and hyperdopaminergia are detrimental in the context of such complex behaviors.
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INTRODUCTION

Stress, or elevated levels of glucocorticoids (GCs), during critical developmental periods can program neuroendocrine and behavioral systems and increase the propensity for affective disorders later in life (Heim and Nemeroff, 2001; Heim *et al*, 2008; Seckl, 2008). Regardless of this evidence, synthetic GCs such as dexamethasone are often prescribed in pregnancies as a prophylactic treatment of intraventricular hemorrhage and respiratory distress syndrome associated with preterm delivery (NIH_Consens_Statement, 1994). Dexamethasone, a specific agonist of GC receptor (GR), is able to cross the placental barrier and is significantly more potent than endogenous corticosteroids (Seckl, 2004). Despite an obvious beneficial effect in fetal organ maturation, several preclinical studies show that synthetic GCs have a notorious deleterious impact on the developing brain (McArthur *et al*, 2005; Fukumoto *et al*, 2009; Rodrigues *et al*, 2010). In fact, it was shown that *in utero* GC (iuGC) exposure induces prominent neurochemical, structural, and molecular changes in several brain regions (Muneoka *et al*, 1997; Matthews, 2000; Leao *et al*, 2007; Nagano *et al*, 2008; Wyrwoll and Holmes, 2012), leading to long-lasting hyperanxiety, increased vulnerability to depression, and drug-seeking behavior (Welberg *et al*, 2001; Oliveira *et al*, 2006; Nagano *et al*, 2008; Roque *et al*, 2011; Rodrigues *et al*, 2012). The long-term consequences of prenatal GC exposure in humans are not so well known, however, evidence suggests that these operate in the same direction of preclinical studies. In fact, prenatal synthetic GC exposure impairs hypothalamic–pituitary–adrenal (HPA) axis reactivity (Davis *et al*, 2011; Alexander *et al*, 2012), and this may underlie the observed increased emotional reactivity of these children (Trautman *et al*, 1995).

Although the adverse effects of early-life exposure to GCs in emotional behavior are undeniable at least in animal models, its impact on social behavior remains largely unexplored. Herein, we used a wide range of behavioral paradigms that evaluated specific dimensions of emotional and social behaviors, which were further complemented with the assessment of ultrasonic vocalizations (USVs), a powerful tool to appraise the affective and/or motivational status of the animal (Brudzynski, 2009) and neurochemical analysis. There are two main types of calls in adult animals (despite several subtypes may exist): the high frequency calls (50 kHz), also known as positive affective USVs, that are emitted in the presence or anticipation of rewarding

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situations such as social interaction, exploratory activity, food, sex, and drugs; and the low frequency or negative calls (22 kHz), emitted in situations of danger and aversive stimuli or frustrating situations (Knutson *et al*, 1998; Burgdorf *et al*, 2000; Knutson *et al*, 2002; Wohr *et al*, 2005; Litvin *et al*, 2007; Burgdorf *et al*, 2008; Kim *et al*, 2010; Burgdorf *et al*, 2011). To the best of our knowledge, the combination of emotional/social behaviors with positive affective USV analysis has never been applied to evaluate the impact of prenatal GCs exposure on programming complex behaviors. Though the neurobiological underpinnings of emotional and social behaviors are complex and encompass several neurotransmitter systems and brain regions, herein we focused our analysis of biogenic amines in the nucleus accumbens (NAc) and amygdala, as variations of these neurotransmitters in both brain regions are known to modulate emotional behaviors (Martin *et al*, 2002; McLaughlin and Gobbi, 2012) and social interaction (Trezza and Vanderschuren, 2008a). Finally, and in light of the behavioral and neurochemical findings, we designed a pharmacological intervention designed to restore the normal activation of dopamine (DA) receptors to explore whether the behavioral alterations could be reverted.

MATERIALS AND METHODS

Animals and Treatments

Pregnant Wistar rats were individually housed under standard laboratory conditions (light/dark cycle of 12/12 h; 22 °C); food and water *ad libitum*. Subcutaneous injections of dexamethasone at 1 mg/kg (iuGC animals) or vehicle (control) were administered on days 18 and 19 of pregnancy at 1700 hours (light period). On postnatal day 21, progeny was separated according to prenatal treatment and gender. All manipulations were done in accordance with local regulations (European Union Directive 86/609/EEC) and NIH guidelines on animal care and experimentation.

Behavioral Tests

Behavioral evaluation was performed with male rats derived from at least three different litters at indicated ages. Tests were conducted during the active period of animals (2030–0230 hours).

Sucrose preference test (SPT) is commonly used to assess rodent anhedonia, and often is correlated with individual's degree of affective state and motivation. Twelve- to fourteen-week-old animals were presented with two bottles in their home cage, one bottle with water and the other with a 2% sucrose solution. Before testing, food and water-deprived animals are habituated for 1 day to the presence of two bottles and the sweet taste to reduce neophobia. After habituation, sucrose and water consumption was measured for 1 h for several days (bottle position switched daily). USVs were measured throughout days of exposure. After 1 week of interval, animals were tested in the forced swimming test (FST), a way to measure depressive-like behavior. Rats were placed in cylinders filled with warmed water. After a 5-min pretest session, animals were retested 24 h later (5 min test). At the end of each session, animals were placed on a heating pad before being returned to their home cages. A video camera placed in front of the cylinder was used to record sessions, and later scored by an investigator blind to the experimental details. Time of immobility (passiveness; defined as time spent either immobile or making righting movements to stay afloat), latency to immobility, and number of climbing attempts were scored. USVs were recorded during the procedure.

In another set of animals (14 weeks), we evaluated the emission of positive affective 50 kHz USVs as an index of natural reward anticipation to food (Burgdorf *et al*, 2000). Test was performed individually in food-restricted adult animals in a clean cage similar to their home cages ($43 \times 27 \times 19\,cm^3$, with approximately 3 cm of wood shavings covering the floor). Animals were placed in a novel and clean cage, for 3 min (baseline), and were exposed to cue for 2 min (white light situated 10 cm above the floor). After the light is turned off, animals receive a bowl full of food. On the 7th day, animals receive an empty recipient (extinction). USVs were recorded throughout all periods. Two weeks after the previous test, emission of USVs was assessed in an enriched environment. Animals were individually habituated to the test cage ($43.2 \times 43.2\,cm^2$ with transparent acrylic walls and white floor) with toys for 2 consecutive days for 10 min each. On the testing day, the animal was isolated from his partner for 3.5 h and then was placed individually in the test cage for 15 min; USVs were recorded during the protocol.

Using a different set of animals, we evaluated tumble and play behavior (Trezza and Vanderschuren, 2008b). Briefly, juvenile animals (39–50 days) were individually isolated for 3.5 h before testing. Pairs were then placed into the test cage, and play behavior and USVs were measured for 15 min. Pouncing (index of play solicitation, ie, when one of the animals is attempting to nose or rub the nape of the neck of the test partner), pinning (when one of the animals is lying with his dorsal surface on the floor with one animal on top of him), and social exploration (when they are sniffing any part of body, including the anogenital area of the test partner) were quantified by visual inspection of videos of the sessions. We used three types of pairs: (1) familiar pair (animals housed in the same cage), (2) unfamiliar pair (animals from the same experimental group, unknown to each other), and (3) unfamiliar and non-matched pair (two unknown animals from different experimental groups).

Because of potential confounding effects of hierarchical dominance and aggressive behaviors often seen in adult male animals, we used a slightly modified protocol to assess social interaction in adulthood (Wohr *et al*, 2008). Sixteen-week-old animals were evaluated regarding their communicative interaction, and posteriorly scored for social behavior. Briefly, individuals were removed from their home cage and placed into a novel cage; animals were allowed to see the partner. USVs were measured in the two cages for a period of 15 min. After this period, animals were reunited in their home cage, and social behavior (pouncing, pinning, and social exploration) and USVs were evaluated for another 15 min (using the same scoring as in the tumble and play test).

To assess locomotor activity upon L-3,4-dihydroxyphenylacetic acid (L-DOPA) treatment, we tested the animals in the open-field arena during their active period. Briefly,

animals were placed in the center of an arena (Med Associates), and their ambulation was monitored online over a period of 30 min. Total distance traveled was used as indicators of locomotor activity. Animals were submitted to oral gavage 4 h before the test with either water or L-DOPA (see below).

Analysis of USVs

An Ultrasound Microphone (Avisoft Bioacoustics) was placed about 20 cm above the floor in all experiments. Vocalization was recorded using an Avisoft Recorder (version 5.1.04) with the following settings: sampling rate: 250 000; format: 16 bit. For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (version 5.1.22; Avisoft Bioacoustic). This program was used to produce spectrograms of USVs by conducting a fast Fourier transform (256 FFT length, 100% frame, Hamming window filter, and 50% time window overlap). These spectrograms had a frequency resolution $\sim$1.2 kHz and a temporal resolution $\sim$0.4 ms.

A call detection of 50 kHz was provided by an automated threshold-based algorithm (threshold: -50 dB) and a hold time mechanism (hold time: 5 ms). A lower cut-off-frequency of 40 kHz was used to eliminate background noise. Calls were also inspected manually to ensure that, when necessary, USVs not detected automatically could be subsequently included in the automatic parameter analysis.

Biogenic Amine Determination

In a different set of 12–14-week-old animals, we evaluated biogenic amine levels. Naive, water- or L-DOPA-treated animals were anaesthetized, 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 $-80\,^{\circ}$C until use. Perchloric acid (0.2 N) was added to each sample, and after disruption and sonication (5 min on ice), samples were centrifuged at 5000 *g*. The resulting supernatant was filtered through a Spin-X high-performance liquid chromatography (HPLC) column (Costar) to remove debris.

Levels of DA, DOPAC, serotonin (5-HT), norepinephrine, epinephrine, and 5-hydroxyindoleacetic acid were measured by HPLC combined with electrochemical detection using a Gilson instrument, fitted with an analytical column (Supelco Supelcosil LC-18 3 μM, flow rate: 1.0 ml/min) as previously described (Rodrigues *et al*, 2012). Briefly, 150 μl supernatant aliquots were injected into the system, using a mobile phase of 0.7 M aqueous potassium phosphate (pH 3.0) in 10% methanol, 1-heptanesulfonic acid (222 mg/l), and Na-EDTA (40 mg/l). A standard curve using known concentrations of all amines was run each day.

Pharmacological Treatment

A new set of animals was used for L-DOPA experiments. Untreated, water-treated, and L-DOPA-treated animals were analyzed in all behavioral tests. L-DOPA/carbidopa (Sinemet) was crushed and suspended in miliQ water. The inactive ingredients of Sinemet are cellulose, magnesium stearate, starch, and D&C Yellow 10 dye. L-DOPA/carbidopa at a concentration of 24/6 mg/kg or water was administered by oral gavage daily by experienced researchers familiar to the animals in order to reduce stress effects of this procedure, 4 h before all the behavioral tests. One set of animals performed the anticipation to food test, and after 2 weeks was submitted to the SPT. An additional set of animals was used for the tumble and play protocol.

Statistical Analysis

Data are presented as means ± SEM. Data were analyzed using Graphpad Prism and SPSS, using *t*-test (FST, USVs—single measurements, social behavior of Figure 2, and HPLC) and ANOVA analyses (SPT, USVs in anticipation to food, enriched environment, and L-DOPA treatment effects—Figures 4d and 5). *Post-hoc* Bonferroni test was used when appropriate.

RESULTS

iuGC Animals Display Depressive-Like Behavior

Depressive behavior in rodents is often assessed by the FST and by measuring anhedonic behaviors (Treadway and Zald, 2011). We showed that iuGC animals present increased immobility time, decreased latency to immobility, and reduced climbing (Figure 1a; immobility time: $t=4.095$, $p=0.005$; latency: $t=2.207$, $p=0.038$; climbing: $t=3.387$, $p=0.0027$; and $n_{CONT}=12$, $n_{iuGC}=12$), consistent with increased depressive-like behavior. During this test, we observed no significant emission of negative vocalizations (data not shown).

In addition, we examined the hedonic status of these animals by performing the SPT. Contrary to control animals that increase their preference throughout the days, iuGC group presents a significant reduction in sucrose preference (Figure 1b; $F_{1,51}=13.56$, $p=0.0063$; $n_{CONT}=8$, $n_{iuGC}=9$). Interestingly, this behavior was associated with a trend for reduced number of positive 50 kHz calls in the last day of the test (Figure 1c; $t=1.201$, $p=0.2514$).

Moreover, we evaluated the anticipatory behavior to food and emission of 50 kHz calls in food-restricted animals. No significant differences in food consumption or in latency times to approach food were found (data not shown). Both control and iuGC groups increased the rate of 50 kHz USVs across days (Figure 1d; $F_{5,82}=5.83$, $p=0.0001$; $n_{CONT}=8$, $n_{iuGC}=8$); yet, the curves are significantly different, as iuGC animals present a lower number of positive USVs when compared with controls ($F_{1,82}=13.95$, $p=0.0003$). Although on day 1 differences are not notorious (Figure 1d′), at day 6 there is a significant reduction in the number of positive USVs in iuGC animals when compared with controls during the 2 min of cue exposure (Figure 1d″; $F_{1,23}=14.65$, $p=0.0009$). The emission of 50 kHz USVs was also measured during extinction period, ie, the time when the animal is presented with an empty recipient. As expected, control group emits less 50 kHz USVs during this period when compared with cue exposure period ($t=2{,}659$, $p=0.0187$), whereas iuGC animals do not present any differences (Figure 1e; $t=0.7442$, $p=0.4691$).

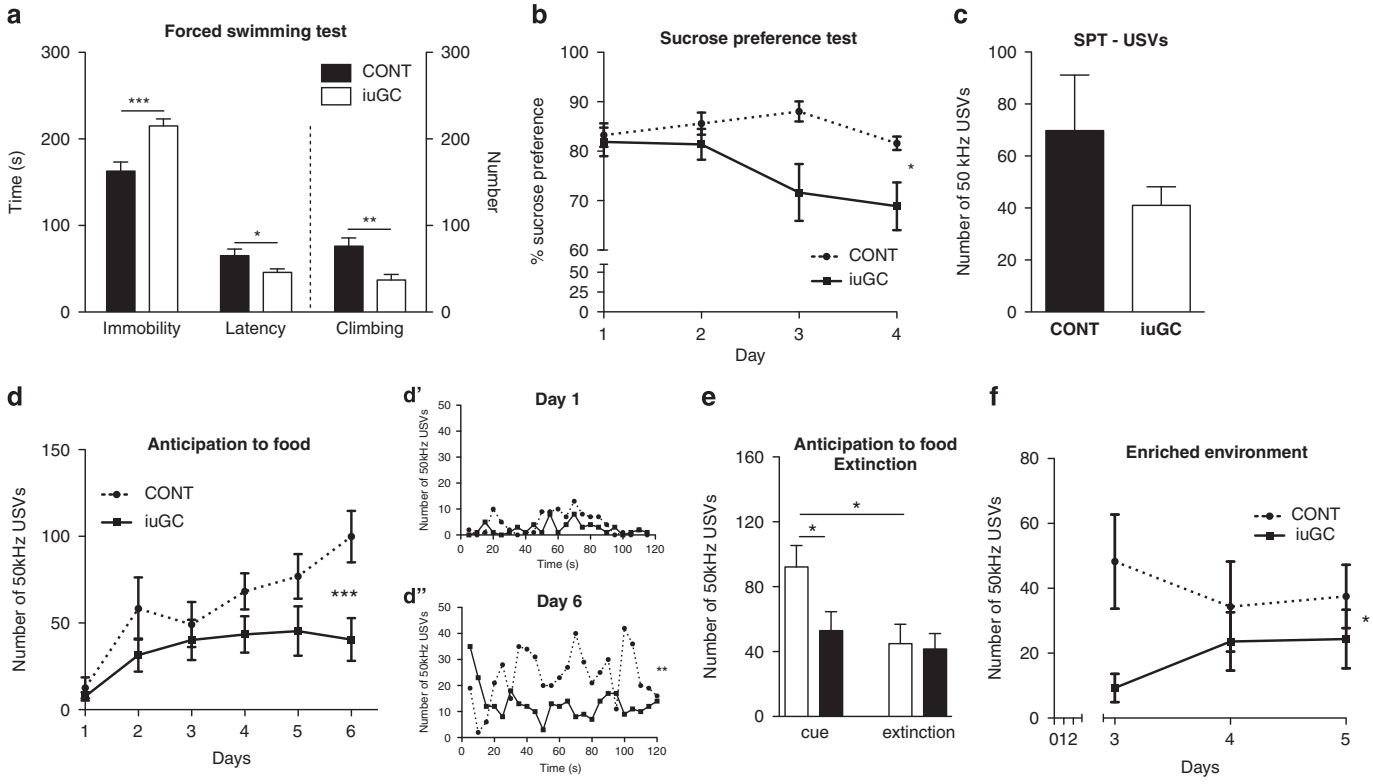

Figure 1 Prenatal *in utero* glucocorticoid (iuGC) exposure induces a persistent depressive-like behavior. (a) Forced swimming test. iuGC group presents increased immobility time and decreased latency to immobility and climbing attempts. (b) iuGC animals present decreased sucrose preference when compared with control group. (c) Trend for reduced 50 kHz vocalization emission during the first 5 min of sucrose preference test (SPT) in iuGC animals. (d) Decreased emission of positive affective 50 kHz vocalizations in anticipation to food. Food-restricted animals were exposed to a cue (light) predicting a bowl of food for 6 consecutive days. Main graph refers to the emission during the period of cue exposure. The pattern of call emission during the cue exposure period of days 1 and 6 is schematized in graphs d′ and d″. (e) Number of positive vocalizations during the cue exposure and extinction period. Although control animals decrease emission upon extinction, iuGC group does not present any differences. (f) iuGC animals emit less positive vocalizations during exposure to an enriched environment (first 5 min). CONT, control animals; USVs, ultrasonic vocalizations. $*p<0.05$, $**p<0.001$, and $***p<0.0001$; a: $n=12$; b, c: $n=8$–9; and d–f: $n=8$.

In agreement with these findings that are suggestive of decreased expression of pleasure towards rewarding events, we observed that iuGC animals emit less positive calls than controls, when placed in an enriched environment (Figure 1f; $F_{1,41}=5.58$, $p=0.0230$; $n_{CONT}=8$, $n_{iuGC}=8$). Interestingly, these differences are greater during the first day of exposure to enriched environment (Figure 1f).

Social Behavior is Impaired in iuGC Animals

In parallel, we analyzed social behavior of iuGC animals in different social paradigms. Rough tumble and play is one of the most rewarding behaviors in juvenile rats, and is positively correlated with the rate of 50 kHz emissions (Knutson *et al*, 1998; Burgdorf *et al*, 2008; Burgdorf *et al*, 2011). We evaluated this play behavior in three distinct conditions: (i) with the home cage partner, (ii) with an unfamiliar rat from the same experimental group, and (iii) with an unfamiliar rat from other experimental group (control–iuGC dyads).

After a period of 3.5 h isolation, 39–50 days-old animals were reunited in a clean cage; USVs were recorded and social behavior scored. When playing, iuGC pairs present less 50 kHz emissions in comparison with control pairs (Figure 2a; $t=2.853$, $p=0.0086$; $n_{CONT}=15$, $n_{iuGC}=12$); a similar picture is found in the case of unfamiliar pairs ($t=2.326$, $p=0.0268$; $n_{CONT}=18$, $n_{iuGC}=15$, $n_{CONT–iuGC}=24$). Interestingly, unfamiliar control–iuGC pairs present a significant reduction in the number of positive USVs in relation to control–control unfamiliar pairs ($t=2.916$, $p=0.0058$).

Besides monitoring USVs, we quantified the number of pouncing and pinning, two robust indicators of playfulness. Familiar iuGC pairs solicit (pouncing) and respond (pinning) significantly less than familiar control pairs (Figure 2b: $t=3.274$, $p=0.0023$; Figure 2c: $t=3.390$, $p=0.0017$; $n_{CONT}=20$, $n_{iuGC}=18$). A similar trend is observed in unfamiliar iuGC pairs when compared with unfamiliar control pairs. Interestingly, unfamiliar control–iuGC pairs pounce and pin significantly more than iuGC–iuGC pairs (pounce: $t=6.316$, $p<0.0001$; pin: $t=3.912$, $p=0.0003$; $n_{CONT}=18$, $n_{iuGC}=16$, $n_{CONT–iuGC}=24$), and, surprisingly, even more than control pairs for pouncing ($t=4.015$, $p=0.0002$).

As expected, all unfamiliar pairs presented reduced latency for the first occurrence of social contact when compared with familiar pairs (Supplementary Figure 1). No major differences were found between control and iuGC familiar pairs, though there is trend for increased latency in

both control–iuGC and iuGC–iuGC unfamiliar pairs (Supplementary Figure 1). In addition, we found that iuGC pairs present increased duration of social exploration when compared with control pairs (Figure 2; $t=2.236$, $p=0.0318$). All the three groups of unfamiliar pairs spend more time in social exploration than familiar ones (Figure 2d). Interestingly, unfamiliar control–iuGC pairs explore even more than control pairs ($t=5.166$, $p<0.0001$). In terms of individual responses, and in accordance with the previous data, we found that iuGC animals respond less to play solicitation than control animals (Figure 2e; $t=3.442$, $p=0.0014$).

Considering the results obtained with the control–iuGC pairs, we decided to further evaluate the individual response of each animal during play behavior. iuGC animals present decreased pouncing and pinning behavior when compared with control animals (Figure 2f; pouncing: $t=5.574$, $p<0.0001$; pinning: $t=2.0785$, $p=0.0442$; $n=23$). In accordance, iuGC animals present a significant reduction in play response (Figure 2f; $t=3.054$, $p=0.0038$). Hence, iuGC animals are not only less likely to initiate play but are also less responsive to it when solicited.

As iuGC-induced behavioral deficits seem to be long lasting (Oliveira *et al*, 2006; Roque *et al*, 2011), we decided to evaluate social behavior in adult animals (16 weeks). Besides appetitive situations, 50 kHz USVs are also emitted after short separation from conspecifics. Rats taken out from their home cage and individually exposed to a clean cage emit 50 kHz calls, and the home cage animal also does so (Wohr *et al*, 2008). This suggests an affiliative communicative function of 50 kHz USVs, namely to (re)establish or maintain social contact. In this experiment, one individual is placed in a new clean cage and the other remains in the home cage, during 15 min (animals can see each other). After this period, animals are reunited and social behavior was measured, using the same parameters as in the tumble and play. As depicted in Figure 2g, iuGC animals placed in the novel cage emit less communicative USVs when compared with controls ($t=2.452$, $p=0.050$; $n_{CONT}=8$, $n_{iuGC}=8$). In addition, when partners reunite, control pairs pounce and pin more than iuGC pairs (Figure 2h: $t=2.598$, $p=0.0408$; Figure 2i: $t=3.693$, $p=0.010$), a similar phenotype as the juvenile animals. Play response is markedly reduced in iuGC animals (Figure 2j; $t=3.147$, $p=0.0199$). Latency to first approach and duration of social exploration did not reveal any statistical differences, though there is a trend for increased latency and reduced social exploration in iuGC group (Supplementary Figure 1).

Dopaminergic Involvement in Emotional and Social Deficits

Considering the involvement of the mesolimbic dopaminergic pathway in emotional and social behaviors (O'Connell and Hofmann, 2011) but also in 50 kHz USVs emission (Brudzynski, 2009), we decided to further evaluate the impact of prenatal GCs on this circuit. Animals from the iuGC group presented reduced levels of DA both in the NAc and the amygdala (Figure 3a, $t=2.635$, $p=0.018$; $n_{CONT}=9$, $n_{iuGC}=10$; Figure 3b, $t=2.910$, $p=0.027$; $n_{CONT}=5$, $n_{iuGC}=5$; trend for a decrease in 5-HT in the amygdala of iuGC animals: $t=2.228$, $p=0.089$), as measured by HPLC. Importantly, no substantial differences were found in other monoamines, in confirmation of previous results (Oliveira *et al*, 2012; Rodrigues *et al*, 2012).

Having in mind the neurochemical results, we decided to determine if dopaminergic modulation could ameliorate the behavioral deficits observed in iuGC animals; to do so, we administered the DA precursor L-DOPA resuspended in water by oral gavage 4 h before each behavioral test. This simple treatment regimen resulted in concomitant increases in accumbens DA levels especially in iuGC group (Figure 3a; basal *vs* L-DOPA: controls: $t=1.398$, $p=0.1603$; iuGC: $t=2.269$, $p=0.044$; $n_{CONT\text{–}L\text{-}DOPA}=5$, $n_{iuGC\text{–}L\text{-}DOPA}=5$) to levels similar to those of control animals ($t=0.9828$, $p=0.3636$). A similar increase in amygdalar DA was found in iuGC animals, whereas no evident change was found in control group (Figure 3b; basal *vs* L-DOPA: controls: $t=0.7137$, $p=0.522$; iuGC: $t=4.875$, $p=0.0018$; $n_{CONT\text{–}L\text{-}DOPA}=5$, $n_{iuGC\text{–}L\text{-}DOPA}=5$).

Because of eventual stressful effects of oral gavage procedure, in all behavioral paradigms we evaluated both untreated/naive and water-treated animals. No major differences were found in the water-treated animals in comparison with naive groups.

L-DOPA treatment caused a mild stimulatory effect in locomotion, though no differences were observed between control and iuGC-treated groups (Supplementary Figure 2). In the FST, and identically to naive group, water-treated iuGC animals presented depressive-like behavior (Figure 4a; immobility: $t=4.711$, $p=0.0005$; latency: $t=3.395$, $p=0053$; $n_{CONT-H_2O}=8$, $n_{iuGC-H_2O}=6$). On the contrary, a prominent effect of L-DOPA treatment was found in the FST, because iuGC-treated animals behaved similarly as controls (Figure 4a; immobility: $t=0.3583$, $p=0.7243$; $n_{CONT\text{–}L\text{-}DOPA}=10$, $n_{iuGC\text{–}L\text{-}DOPA}=10$). Interestingly, L-DOPA treatment increased sucrose preference in both groups when compared with water treatment (Figure 4b; controls: $t=2.283$, $p=0.0356$; iuGC: $t=4.193$, $p=0.0011$; $n_{CONT}=8$, $n_{iuGC}=7$, $n_{CONT-H_2O}=11$, $n_{iuGC-H_2O}=8$, $n_{CONT\text{–}L\text{-}DOPA}=8$, $n_{iuGC\text{–}L\text{-}DOPA}=7$). The treatment reverted sucrose-preference deficit, indicative of anhedonic behavior, in iuGC animals (Figure 4b; $t=0.926$, $p=0.3536$), whereas no improvement was observed in water-treated animals ($p=0.0004$, $t=4.433$). Although L-DOPA treatment induced a reduction in the emission of positive USVs in control animals in relation to water-treated group, the opposite was found in iuGC animals (Figure 4c; $t=1.878$, $p=0.0951$ and $t=1.994$, $p=0.0741$, respectively).

In the anticipation to food paradigm, iuGC animals treated with L-DOPA presented higher number of positive calls throughout the days when compared with water-treated iuGC animals (Figure 4d; $F_{1,101}=65.59$, $p<0.0001$; $n_{CONT-H_2O}=14$, $n_{iuGC-H_2O}=11$, $n_{CONT\text{–}L\text{-}DOPA}=10$, $n_{iuGC\text{–}L\text{-}DOPA}=10$; to simplify the graph, curves from naive groups are not depicted). On the contrary, control animals did not present any statistical differences with the L-DOPA treatment ($F_{1,112}=0.74$, $p=0.3910$). In the extinction day, water-treated iuGC animals present a trend for reduced USV emission during cue exposure in comparison with controls (Figure 4e; $t=1.426$, $p=0.1916$, $n_{CONT-H_2O}=7$, $n_{iuGC-H_2O}=6$). Water-treated control animals emit less positive calls in the extinction period when compared with the cue exposure period (Figure 4e; $t=2.174$, $p=0.0525$),

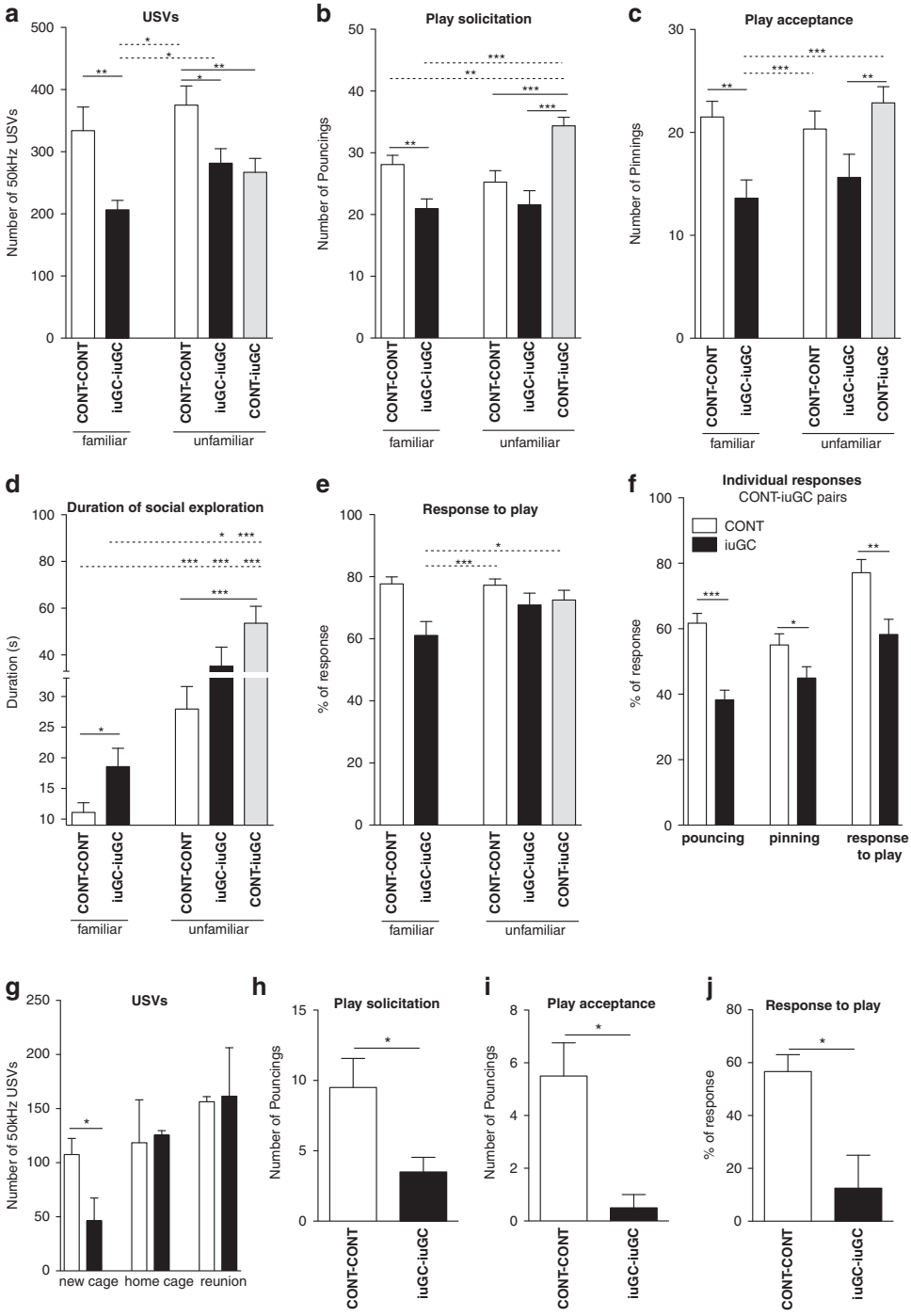

Figure 2 Prenatal *in utero* glucocorticoid (iuGC) exposure impairs social behavior. (a–f) Tumble and play behavior—juvenile period (39–50-day-old animals). (a) Number of positive affective ultrasonic vocalizations (USVs) during tumble and play protocol (first 5 min). Animals were tested with their home cage partner (familiar condition) and with unknown animals (unfamiliar condition). Both familiar and unfamiliar iuGC pairs emit less positive vocalizations when compared with their respective control pairs. The dyad control–iuGC also emits less positive calls than control pairs. (b) Pouncing as a measure of play solicitation behavior. In the case of familiar pairs, iuGC pairs request play less than controls, whereas mixed dyads (control–iuGC) present increased play solicitation behavior. (c) Play acceptance behavior. In the case of familiar pairs, iuGC pairs present a reduced number of pinning in comparison with controls, whereas mixed dyads (control–iuGC) present opposite profile. (d) The duration of social exploration is higher in non-familiar pairs when compared with familiar pairs, particularly in the mixed dyad. (e) Percentage of response to play solicitation is slightly decreased in familiar iuGC pairs. This is calculated by the number of times the animal requests to play divided by the number of times animal accepts to play. (f) Individual behavioral analysis of control–iuGC dyad revealed that iuGC animals pounce, pin, and respond to play significantly less in relation to controls. (g–j) Adult social behavior—communicative function (16-week-old animals). (g) Emission of positive vocalizations is decreased in isolated iuGC adult animals when compared with controls. No differences were found in the emission in the home cage, nor in the reunion period. iuGC animals present impaired adult social behavior because they display decreased play solicitation. (h) and acceptance (i), and a substantial reduction in the percentage of play acceptance (j) when compared with control group. CONT, control animals. $*p<0.05$, $**p<0.001$, and $***p<0.0001$; a–f: $n=15$–24 pairs; and g–h: $n=4$–8 pairs.

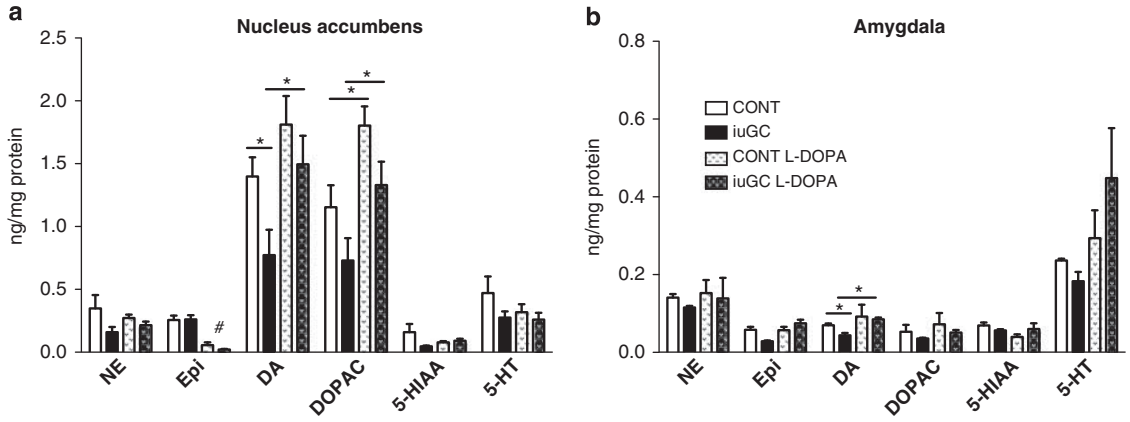

Figure 3 Hypodopaminergic status of the nucleus accumbens (NAc) and amygdala of *in utero* glucocorticoid (iuGC) animals as measured by high-performance liquid chromatography (12–14-week-old animals). (a) NAc biogenic amines profile showing a reduction in dopamine (DA) and a trend for reduced DOPAC levels, with no major differences in other neurotransmitters and metabolites. L-3,4-dihydroxyphenylacetic acid (L-DOPA) treatment 4 h before significantly increased DA levels in the NAc of iuGC animals, but only a slight increase was observed in control animals. An increase in DOPAC was found in both treated groups. (b) Decreased DA levels in the amygdala of iuGC animals when compared with controls. L-DOPA treatment increased DA levels in the amygdala of iuGC (but not control) animals. CONT, control animals; Epi, epinephrine; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; NE, norepinephrine. $^{*}p<0.05$; $n=5$–10.

and, conversely, iuGC animals did not present this discrimination capacity ($t=0.5482$, $p=0.5985$). Upon L-DOPA treatment, control animals present a trend for decreased emission in the extinction *vs* cue exposure period ($t=1.771$, $p=0.1019$), but iuGC animals present similar number of USVs ($t=1.049$, $p=0.3169$).

We also assessed social behavior upon L-DOPA treatment. Because of the large number of groups, and in order to simplify the presentation of the results, we separated the data of familiar and unfamiliar pairs. In the case of familiar pairs, L-DOPA had a notorious effect in diminishing the latency for the first social approach in both groups when compared with water-treated animals (Figure 5a; $F_{1,37}=19.65$, $p<0.0001$; $n_{CON}t=22$, $n_{iuGC}=17$, $n_{CONT-H_2O}=12$, $n_{iuGC-H_2O}=9$, $n_{CONT\text{-}L\text{-}DOPA}=11$, $n_{iuGC\text{-}L\text{-}DOPA}=10$). Still, L-DOPA-treated iuGC animals display increased latency when compared with treated control animals ($t=2.737$, $p=0.0131$).

L-DOPA increased the number of positive USVs in iuGC animals when compared with water-treated animals (Figure 5b; $t=3.077$, $p=0.0179$) with no effect in control group (Figure 5b; $t=0.9841$, $p=0.3642$). ANOVA analysis revealed an effect of L-DOPA treatment in this parameter ($F_{1,19}=5.62$, $p=0.0285$). The L-DOPA treatment had a significant effect in increasing the number of pouncing and pinning in both groups (Figure 5c, $F_{1,35}=22.90$, $p<0.0001$; Figure 5d, $F_{1,35}=10.17$, $p=0.003$). Treated control animals presented a 49% increase in pouncing ($t=2.606$, $p=0.0174$), whereas iuGC animals increased up to 119% ($t=4.110$, $p=0.0008$). Regarding pinning, both control and iuGC groups increased the numbers by 21% and 134%, respectively ($t=1.058$, $p=0.3021$; $t=3.378$, $p=0.0045$). In accordance, L-DOPA treatment significantly increased the percentage of play response (Figure 5e; $F_{1,34}=9.02$, $p=0.005$).

In the case of unfamiliar pairs, L-DOPA also diminished the latency for the first social approach when compared with non-treated pairs (Figure 5f; $F_{1,60}=6.11$, $p=0.0163$). Water-treated iuGC animals behaved similarly to naive group, presenting a trend for reduced vocalization emission during play (Figure 5g; $t=2.132$, $p=0.0704$; $n_{CON}t=7$, $n_{iuGC}=9$, $n_{CONT-iuGC}=6$, $n_{CONT-H_2O}=7$, $n_{iuGC-H_2O}=9$, $n_{CONT-iuGC-H_2O}=6$, $n_{CONT\text{-}L\text{-}DOPA}=8$, $n_{iuGC\text{-}L\text{-}DOPA}=8$, $n_{CONT\text{-}iuGC\text{-}L\text{-}DOPA}=8$). ANOVA analysis revealed no major effect of L-DOPA treatment in USV emission, but the difference in vocalization number found between control and iuGC-treated pairs disappeared (Figure 5g). L-DOPA enhanced pouncing behavior only in iuGC pairs (Figure 5h, 17% increase; $t=3.140$, $p=0.0072$). A deleterious effect of L-DOPA was found in the case of control–iuGC pairs (Figure 5h; $t=2.031$, $p=0.0551$).

Similarly, the treatment had a significant effect in increasing the number of pinning in controls (Figure 5i, 55% increase; $t=2.219$, $p=0.0396$) and iuGC pairs (Figure 5i, 81% increase; $t=3.140$, $p=0.00072$). No statistical differences were observed regarding the number of pinning in L-DOPA-treated control–iuGC pairs, though there was a tendency for a reduction ($t=1.4148$, $p=0.1624$). No major effect of L-DOPA treatment was found in the percentage of play response (Figure 5j; $F_{1,57}=2.47$, $p=0.1218$).

In the individual analysis of the control–iuGC pairs, ANOVA revealed that L-DOPA treatment had no significant effect in any of the parameters (Figure 5k: $F_{1,32}=0.92$, $p=0.3452$; Figure 5l: $F_{1,40}=0.00$, $p=1.00$; Figure 5m: $F_{1,41}=0.23$, $p=0.6326$; $n_{CON}t=23$, $n_{iuGC}=24$, $n_{CONT-H_2O}=17$, $n_{iuGC-H_2O}=17$, $n_{CONT\text{-}L\text{-}DOPA}=8$, $n_{iuGC\text{-}L\text{-}DOPA}=8$). However, *t*-test analysis revealed that there was a trend for increased pouncing and pinning in L-DOPA-treated iuGC group in comparison with water group (Figure 5k: $t=1.580$, $p=0.1338$; Figure 5l: $t=1.798$, $p=0.0873$). L-DOPA treatment ameliorated the percentage of play response of iuGC animals (Figure 5m; $t=2.283$, $p=0.0335$). Conversely, L-DOPA treatment had a deleterious effect in pinning behavior and percentage of play response in control group (Figure 5l: $t=1.798$, $p=0.0873$; Figure 5m: $t=3.091$, $p=0.0053$).

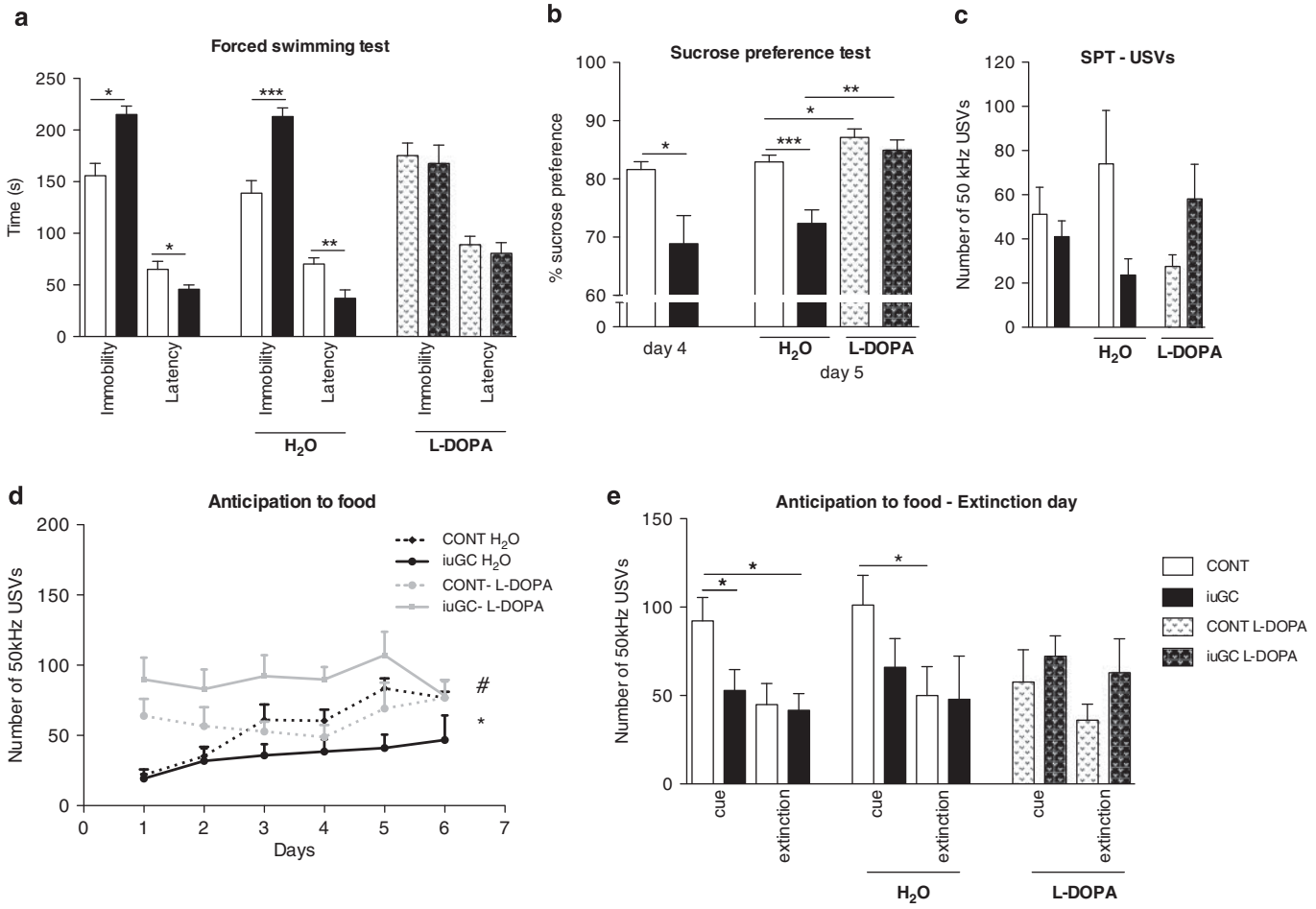

Figure 4 Normalization of dopamine (DA) levels by acute L-3,4-dihydroxyphenylacetic acid (L-DOPA) treatment (24 mg/kg by oral gavage; 4 h before behavioral tests) reverts depressive-like behavior of *in utero* glucocorticoid (iuGC) animals. (a) Upon L-DOPA treatment, iuGC animals are similar to controls in all parameters of the forced swimming test. (b) L-DOPA completely reverts the decreased sucrose preference of iuGC animals. (c) Ultrasonic vocalizations (USVs) emission in the sucrose preference test (SPT; day 5). L-DOPA-treated iuGC animals present a trend for increased positive vocalization emission during the first 5 min of test in comparison with water-treated group, whereas control animals present a diminishment. (d) In the anticipation to food paradigm, water-treated iuGC animals present reduced emission of vocalizations as expected (*). L-DOPA increased substantially the number of positive calls during cue exposure in iuGC animals (#). On the contrary, this treatment had a negative effect in control animals, because they slightly decreased the number of positive calls throughout the first days of exposure. (e) In the extinction day, after L-DOPA treatment, no differences are observed between groups, or between cue exposure and extinction periods. CONT, control animals. *$p<0.05$ and **$p<0.001$; a: $n=6$–10; b, c: $n=7$–11; and d–e: $n=10$–14.

DISCUSSION

Herein we confirmed that prenatal GC exposure alters emotional behavior, namely depressive-like behavior, in adulthood (Oliveira *et al*, 2006; Roque *et al*, 2011), as iuGC animals present increased immobility in the FST and decreased sucrose preference in parallel with a trend for reduced positive (50 kHz) USV emission. Although the finding that prenatal GC exposure increase vulnerability for depressive behaviors later in life has been consistently replicated in literature, both in humans and animal models, (Agid *et al*, 1999; Heim and Nemeroff, 2001; Gutman and Nemeroff, 2003; Darnaudery and Maccari, 2008; Heim *et al*, 2008; Mueller and Bale, 2008; Roque *et al*, 2011), its effects in social behavior are more controversial. Some authors have found less social play in juvenile rats after administration of either corticosterone or dexamethasone on the first days of life (Meaney *et al*, 1982), whereas others have found enhanced juvenile play (Kamphuis *et al*, 2004). Apart from differences in doses and exposure times, which may explain these discrepancies, our results show that iuGC exposure induces a wide spectrum of social behavior deficits at different ages.

Juvenile iuGC animals emitted less positive vocalizations and play behavior when interacting with their familiar partner. In the case of unfamiliar dyad, which involves some degree of anxiety, which is a critical factor considering the hyperanxious profile of iuGC animals (Oliveira *et al*, 2006), the behavior was remarkably different. As expected, the duration of social exploration was higher in unfamiliar when compared with familiar pairs, but this was especially evident in the control–iuGC dyad. Unfamiliar iuGC pairs present a trend for reduced play and emission of 50 kHz USVs in comparison with control dyads, again suggesting reduced pleasure in play behavior. Such findings are in accordance with reports showing that rats selectively bred for low levels of 50 kHz USVs exhibit alterations in early social motivation and time spent with conspecifics later in

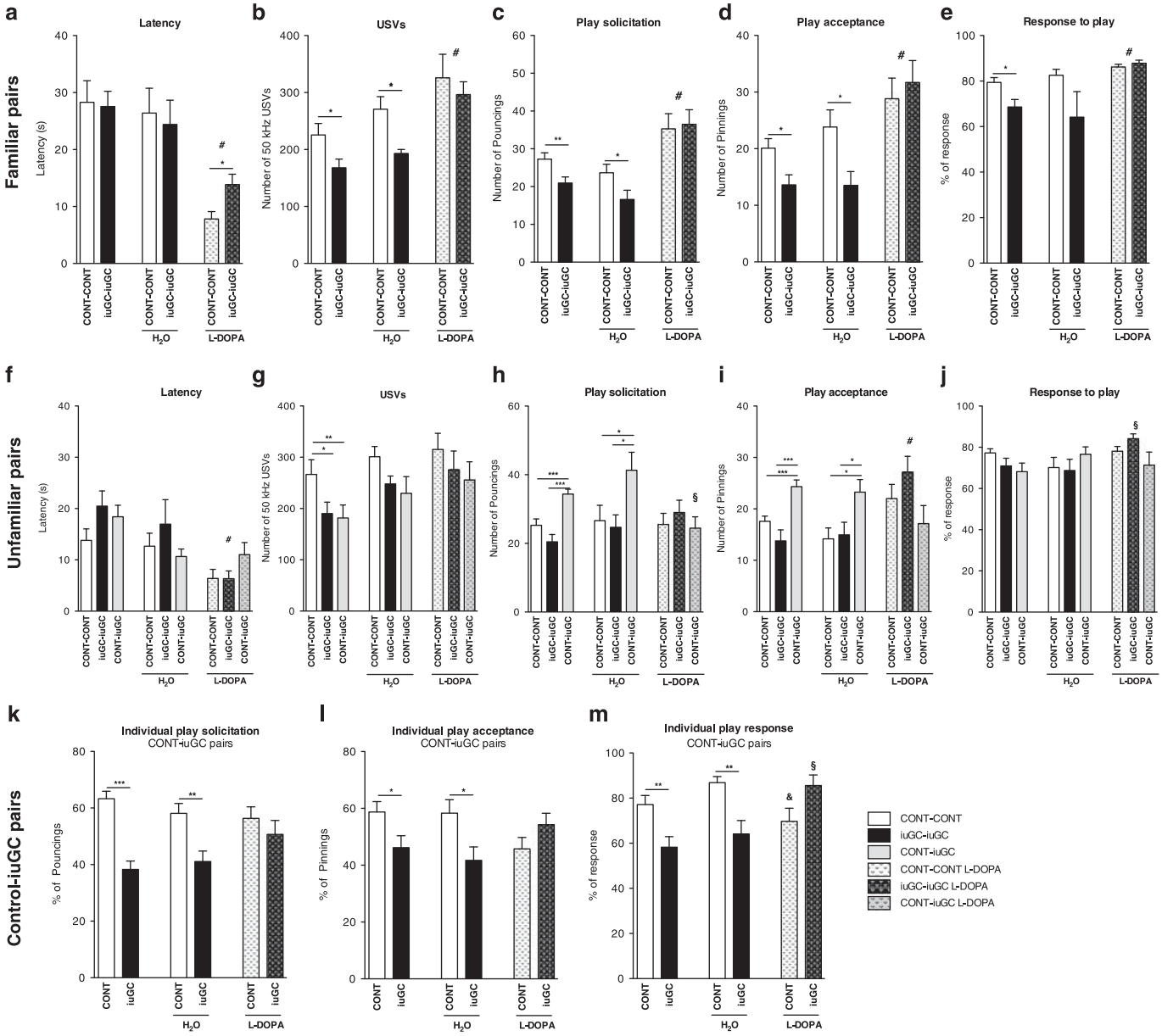

Figure 5 Effects of L-3,4-dihydroxyphenylacetic acid (L-DOPA) treatment (24 mg/kg by oral gavage; 4 h before behavioral tests) in social behavior. Animals were tested with their home cage partner (familiar pairs) and with unknown animals (unfamiliar pairs). Familiar pairs: (a) L-DOPA decreased substantially the latency for the occurrence of the first social contact in both groups when compared with water-treated animals (#); yet, *in utero* glucocorticoid (iuGC) pairs still present a slight increase in the latency when compared with controls. (b) There was an effect of L-DOPA treatment in increasing the number of positive USVs during play behavior in both groups (#), despite this effect was more pronounced in iuGC animals (§). L-DOPA had a remarkable boosting effect in pouncing (c), pinning (d), and percentage of play response (e) in both groups, though this effect was more pronounced in iuGC animals, which no longer present differences in comparison with controls. Unfamiliar pairs: (f) Decreased latency for the first social approach in all the L-DOPA-treated unfamiliar pairs (#). (g) No major effect of L-DOPA in the number of positive calls during play behavior, though there is a trend for increased vocalization emission in comparison with naive and water-treated animals. (h) L-DOPA had no major effect in pouncing behavior of control and iuGC pairs, whereas it decreased this parameter in control–iuGC pairs (§). (i) L-DOPA had an effect in pinning behavior (#). Although the treatment promoted pinning behavior in both control and iuGC groups, it had a detrimental effect in mixed pairs. (j) Minor effect of L-DOPA in improving percentage of play response in iuGC pairs but not in other groups. Individual analysis of control–iuGC pairs revealed that L-DOPA treatment normalized pouncing (k) and pinning (l) percentage of iuGC animals. L-DOPA had a detrimental effect in pinning behavior of the control animal. (m) The treatment improved noticeably the percentage of play response of iuGC individuals (§), and had a detrimental effect in individuals from control group (&). CONT, control animals. *$p<0.05$, **$p<0.001$, and ***$p<0.0001$; # refers to a significant effect of L-DOPA treatment (analysis of variance (ANOVA) analysis). $n=8$–22 pairs; unfamiliar: 7–18 pairs; and control–iuGC: 8–23 pairs.

life (Harmon *et al*, 2008; Burgdorf *et al*, 2009). Surprisingly, the opposite picture is found if the iuGC partner is a control animal, highlighting the idea that social behavior and vocalization emission is greatly dependent on the condition of the partner (Wohr and Schwarting, 2007; Wohr *et al*, 2008). Interestingly, in the case of control–iuGC dyad, there was no correlation between call emission and social play behavior, a finding that remains to be explored. Analysis

of individual behavior of these pairs revealed that iuGC animals pounce significantly less and present decreased play response when compared with control partner; unfortunately, we could not correlate these results with individual call emission due to the lack of discrimination of the technique.

In further support of the previous findings, in adulthood, isolated iuGC animals emit less positive calls than controls in the novel cage. When reunited, iuGC pairs present a marked reduction in play behavior, as indicated by the substantial decline in the number of pouncing and pinning when compared with controls, similar to what was found in juveniles. Altogether, these results prove that early-life GC exposure has a profound impact on social interaction throughout life. These findings are of extreme relevance in the context of neuropsychiatric conditions presenting impaired social behavior such as schizophrenia or autism, which have been suggested to have a neurodevelopmental basis, and stress as a triggering factor (Kinney *et al*, 2008).

Whereas in humans the 'positive affective state' is typically measured via self-report, in rodents can only empirically rely on facial or vocal displays and concomitant behavioral expression (Berridge *et al*, 2009; Burgdorf *et al*, 2011). In this work, the combination of an extensive behavioral characterization with the appraisal of USV emission provided a more discriminative assessment of the impact of prenatal exposure on GCs in both emotional and social behaviors. As an example, hedonic behavior is typically assessed by the preference toward alternative choices; however, it is important to distinguish between motivational anhedonia, ie, lack of motivation/drive to engage the pleasurable activity, and consummatory anhedonia, which is the lack of pleasure during the activity *per se* (Treadway and Zald, 2011; Der-Avakian and Markou, 2012). Herein, by measuring the rate of positive 50 kHz USVs in combination with the behavioral performance, we were able to demonstrate that iuGC exposure has an impact on both dimensions of reward behavior. First, iuGC animals emit less positive calls in anticipation to a rewarding event, which can be seen as a reduced index of their appetitive motivation (Burgdorf *et al*, 2011); this idea is further confirmed by the reduction in positive calls in the extinction stage in control but not in iuGC animals. Second, iuGC animals present diminished social interaction and sucrose preference, in parallel with a marked reduction in positive call emission, suggestive of consummatory anhedonia. Third, in the control–iuGC pairs, the differences are more pronounced in play solicitation than in acceptance, which hints a decreased motivation to engage in play in iuGC animals.

Importantly, the mesolimbic dopaminergic circuitry has been associated not only with rewarding events and hedonia (with contradictory functions ascribed—reviewed in Berridge, 2007) but also with the emission of the positive 50 kHz USVs (Burgdorf *et al*, 2000; Burgdorf *et al*, 2007; Brudzynski, 2009). In this perspective, it is important to refer that prenatal and adult GC exposure can interfere with the correct wiring of this system (reviewed in Piazza and Le Moal, 1996; Rodrigues *et al*, 2010). For example, iuGC induces a reduction in cell proliferation at postnatal day 3 (Leao *et al*, 2007), indicating a *quasi-immediate* deleterious effect of the synthetic GC. In addition, a significant reduction in tyrosine hydroxylase (TH)-positive cells was found (Leao *et al*, 2007; Rodrigues *et al*, 2012). In the same line of evidence, recent work suggests that in a susceptible genetic background, adolescent stress can induce hypermethylation of the TH gene, with a concomitant decrease in TH expression and DA levels (Niwa *et al*, 2013). Importantly, such TH epigenetic alterations are long lasting and GC dependent, because GR antagonism fully reverted the molecular and depressive-like behavior deficits of these animals (Niwa *et al*, 2013).

Yet, iuGC effects may also occur later in life, because adult animals present impaired HPA axis (Oliveira *et al*, 2006), which can modulate dopaminergic circuitry (Piazza and Le Moal, 1996), and that has been proposed to contribute for depression (Pariante and Lightman, 2008). In this context, it has been shown that in a model of social stress (induced by repeated aggression episodes), GR inactivation in dopaminoceptive neurons of the NAc (that project to ventral tegmental area and regulate the activity of DA-releasing neurons) completely abolished social aversion (Barik *et al*, 2013), suggesting a close link and interplay between GCs/stress and dopaminergic tone.

The demonstration of a hypodopaminergic status in the NAc (and amygdala) of iuGC animals is likely to underlie the inability of iuGC to express and/or feel pleasure when facing similar rewarding events as control animals. One hypothesis is that the reduction in NAc dopaminergic transmission decreases the drive toward natural rewards (Melis *et al*, 2005), which fits with the present observation of anhedonia and/or reduced motivation of iuGC animals toward food and social play. In further support of this idea, it was recently shown that optogenetic inhibition/excitation of midbrain DA neurons induces/relieves multiple independent depression symptoms caused by chronic stress (Tye *et al*, 2013). On the other hand, others have shown that phasic activation of the VTA-NAc pathway induced susceptibility to social-defeat stress, whereas inhibition of this pathway induced resilience (Chaudhury *et al*, 2013). To prove that DA is a key determinant of this phenotype, we decided to modulate the levels of this neurotransmitter using a simple pharmacological strategy. Systemic L-DOPA administration (Rodrigues *et al*, 2012) was effective in normalizing DA levels in the NAc and amygdala of iuGC animals, with no major side effects. This treatment fully reverted the depressive-like behavior of iuGC animals, because it diminished their increased immobility time in the FST test, and rescued sucrose-preference deficits, with a matching increase in positive USV emission. In accordance, L-DOPA exerted a boosting effect in positive call emission in iuGC animals in anticipation to food. This effect was not so clear in control animals; in fact, L-DOPA administration seemed to have a detrimental effect, because they present an awkward pattern of vocalization emission. This suggests that boosting DA in 'normal' animals is deleterious, in accordance with the theory that hyperdopaminergia contributes to aberrant representations of salient stimuli and/or altered reward learning (Uylings and van Pelt, 2002).

There is sparse evidence relating DA agonism/antagonism in social play behavior, but this neurotransmitter is fundamental for other types of social interaction. For example, increased DA in the NAc is associated with quality of maternal behavior in rodents (Champagne *et al*, 2004). Administra-

tion of DA antagonists blocks, whereas agonists induces, partner preference formation in female prairie voles (Wang *et al*, 1999; Gingrich *et al*, 2000). This mechanism appears to occur through D2-type receptors, which curiously are altered in iuGC animals (Rodrigues *et al*, 2012). Regarding play, one study showed that low dosages of apomorphine improve pinning and such effect is partially antagonized by DA antagonist haloperidol (Niesink and Van Ree, 1989). This is, to the best of our knowledge, the first report showing that L-DOPA/DA has a stimulating effect in several facets of social behavior. L-DOPA treatment reduced markedly the latency for the first social approach, and generally improved social play behavior in both groups, but intriguingly, L-DOPA effects were remarkably different between familiar and unfamiliar pairs. The promoting effect of L-DOPA was clearly more pronounced in the case of familiar dyads, especially in the iuGC pairs, that present a robust increase in all parameters of play behavior. The treatment effect in unfamiliar pairs was not so obvious, and even suggested a detrimental effect in control individuals, suggesting that other parameters may be contributing, a question that merits to be studied in the future.

Despite the beneficial effects of dopaminergic modulation, we cannot exclude the contribution of other neurotransmitters essential for social behavior, as for example oxytocin. In fact, there is growing evidence of an anatomical overlap and an intricate and reciprocal regulation between DA and oxytocin signaling in social behavior, and, less well studied, in emotional processes (Baskerville and Douglas, 2010). In this way, it would be interesting to study the cross-talk between these two neurotransmitters in this and other models of emotional and social dysfunction.

In summary, we show that prenatal GC exposure induces a long-lasting depressive-like trait together with deficits in social interaction. Normalization of DA levels of iuGC animals by acute L-DOPA administration had a prominent positive behavioral outcome in both emotional and social dimensions, highlighting dopaminergic (dys)function as a strategic target for developing new therapeutic approaches for conditions involving alterations in emotional and social behaviors.

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The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)