

Resilience to stress and sex-specific remodeling of microglia and neuronal morphology in a rat model of anxiety and anhedonia

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ABSTRACT

Prenatal exposure to stress or glucocorticoids (GC) is associated with the appearance of psychiatric diseases later in life. Microglia, the immune cells of the brain, are altered in stress-related disorders.

Synthetic GC such as dexamethasone (DEX) are commonly prescribed in case of preterm risk labour in order to promote fetal lung maturation. Recently, we reported long-lasting differences in microglia morphology in a model of *in utero* exposure to DEX (iuDEX), that presents an anxious phenotype. However, it is still unclear if stress differentially affects iuDEX males and females.

In this work, we evaluated how iuDEX animals of both sexes cope with chronic mild stress for 2 weeks. We evaluated emotional behavior and microglia and neuronal morphology in the dorsal hippocampus (dHIP) and *nucleus accumbens* (NAc), two brain regions involved in emotion-related disorders. We report that males and females prenatally exposed to DEX have better performance in anxiety- and depression-related behavioral tests after chronic stress exposure in adulthood than non-exposed animals. Interestingly, iuDEX animals present sex-dependent changes in microglia morphology in the dHIP (hypertrophy in females) and in the NAc (atrophy in females and hypertrophy in males). After chronic stress, these cells undergo sex-specific morphological remodeling. Paralleled to these alterations in cytoarchitecture of microglia, we report inter-regional differences in dendritic morphology in a sex-specific manner. iuDEX females present fewer complex neurons in the NAc, whereas iuDEX males presented less complex neuronal morphology in the dHIP. Interestingly, these alterations were modified by stress exposure.

Our work shows that stressful events during pregnancy can exert a preserved sex-specific effect in adulthood. Although the role of the observed cellular remodeling is still unknown, sex-specific differences in microglia plasticity induced by long-term stress exposure may anticipate differences in drug efficacy in the context of stress-induced anxiety- or depression-related behaviors.

1. Introduction

Early life adversity, namely prenatal exposure to stress or glucocorticoids (GC), induce persistent effects in the brain, which may underlie increased risk for developing neuropsychiatric diseases later in life, such

as anxious-like phenotype and increased susceptibility to depression in both animal models and humans (Heim and Nemeroff, 2001; Welberg and Seckl, 2001; Oliveira et al., 2012; Rodrigues et al., 2012; Borges et al., 2013; Drozdowicz and Bostwick, 2014). Anxiety is often comorbid with other psychiatric disorders, namely depression. Sex differences in

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clinical manifestation, morbidity, and prevalence of these psychiatric disorders are well established (Bekbbat and Neigh, 2018). Among biological factors playing a role in psychiatric disorders, emerging evidence points to the relevance of glial cells in the etiology of psychiatric disorders, as well as possible drug targets (Manev et al., 2003; Di Benedetto and Rupprecht, 2013).

In rodents, the behavioral changes associated with elevated levels of GC during development, namely anxiety and depressive-like behavior, are paralleled by important deleterious effects on neuronal morphology (Sousa et al., 1998; Sousa and Almeida, 2002; Leao et al., 2007; Oliveira et al., 2012), and spine density (Oliveira et al., 2012; Rodrigues et al., 2012; Tanokashira et al., 2012; Soares-Cunha et al., 2014) in several brain regions implicated in anxiety-like behavior, including the hippocampus (HIP) (Sousa et al., 1998; Li et al., 2014), the amygdala and the bed nucleus of stria terminalis (Oliveira et al., 2012). *In vitro* studies showed that exposure to GC leads to a differential modulation of neuronal morphology, characterized by dendritic atrophy and axonal hypertrophy (Pinheiro et al., 2018).

Besides neurons, GC also affects microglial cells (Caetano et al., 2017; Duarte et al., 2019). These immune cells colonize the brain early in development in a sex-specific manner (Ginhoux et al., 2010, 2013; Schwarz et al., 2012) and are crucial in immune surveillance and dynamic maintenance of homeostasis throughout the lifespan (Tremblay et al., 2011). In addition, they are implicated in adult neurogenesis and in the integration of these new neurons into neuronal circuits (Wake et al., 2013). Microglia express glucocorticoid receptors (GRs) and respond to GC (Sierra et al., 2008), emerging as prime candidates to mediate the effects of GC in the brain. In accordance, in a model of *in utero* exposure to dexamethasone (iuDEX), that presents prominent anxious behavior, we described that the pattern of microglia morphology impacts on anxious behavior and cognition in a sex-specific manner (Caetano et al., 2017; Duarte et al., 2019). We also reported that the pharmacologic blockade of adenosine A_{2A} receptors ($A_{2A}R$) is able to reshape microglia morphology in the prefrontal cortex (PFC) and in the dorsal hippocampus (dHIP), with behavioral normalization, an effect that is also sex-dependent (Caetano et al., 2017; Duarte et al., 2019).

Considering that exposure to GC during neurodevelopment is associated with anxiety-like behavior and sex-specific morphologic features of microglia, and that microglia has been implicated in the pathogenesis of depression (Yirmiya et al., 2015; Bollinger et al., 2016), we now investigated if microglia/neuronal changes may occur in parallel with increased vulnerability to stress-induced depressive behavior in both sexes. To do so, we subjected iuDEX animals to a short-term protocol of unpredictable chronic mild stress (uCMS) for two weeks, in order to induce a depressive-like phenotype. We evaluated eventual sex dimorphism in behavior and plastic changes in the morphology of microglia and neurons of the *nucleus accumbens* (NAc) and dHIP, two brain regions that have been associated with emotion-related disorders (Francis and Lobo, 2017; Liu et al., 2017). For example, dysfunction of the NAc has been associated with anhedonia, a cardinal symptom in this condition (Bessa et al., 2013). The HIP plays an important role in both cognitive function and emotional processing (Fanselow and Dong, 2010; Strange et al., 2014), and it is also one of the key brain structures involved in the regulation of the stress response (Herman et al., 2005).

2. Methods

The timeline of all procedures is shown in Fig. 1A.

2.1. Animals

Pregnant Wistar Han rats (Charles River Laboratories, L'Arbresle, France) were individually housed and kept under standard laboratory conditions (light/dark cycle of 12/12 h, lights on from 08:00 a.m. to 08:00 p.m.; 22 °C, relative humidity of 55%); *ad libitum* access to food and water. Subcutaneous injections of DEX (iuDEX, 1 mg/kg) or vehicle

(Control) were administered on gestation days 18 and 19 at 3:00 p.m. (light period). On postnatal day (PND) 21, the progeny was separated according to prenatal treatment and sex and house grouped until PND90. Health monitoring was performed according to FELASA guidelines. All procedures were carried out in accordance with European Union regulations (Directive, 2010/63/EU). Animal facilities and the people directly involved in animal experiments were certified by the Portuguese regulatory entity Direção-Geral de Alimentação e Veterinária (DGAV). All protocols were approved by the Ethics Committee of the Life and Health Sciences Research Institute and by DGAV (#19074).

2.2. Unpredictable chronic mild stress (uCMS)

At PND90, rats were randomly divided in four experimental groups: a group of animals not exposed to iuDEX or uCMS (Control); a group of animals prenatally exposed to DEX and not exposed to uCMS (iuDEX); a group of animals exposed to uCMS protocol (Stress); a group of animals prenatally exposed to DEX and exposed to uCMS (iuDEX + Stress). A validated uCMS protocol previously described (Willner, 2005; Alves et al., 2017) was applied for 2 weeks. The uCMS protocol consisted of a variety of mild stressors, as confinement to a restricted space, placement in a tilted cage (30°), housing on damp bedding, exposure to noise, overnight illumination, food or water deprivation followed by exposure to inaccessible food/water, overcrowding, exposure to strobe lights, switch of cagemates and reversed light/dark cycle for 48 h every 7 days. Rats in the stress group were randomly exposed to 2–4 stressors every day for 2 weeks (Supplementary Fig. 1). Control animals were pair-housed, daily handled and given access to water and standard chow diet *ad libitum*. Body weight was monitored weekly.

2.3. Behavioral analysis

At the end of the uCMS protocol, behavioral tests were performed to evaluate anxiety, anhedonia and depressive-like behavior. The Elevated Plus Maze (EPM) and Forced Swimming Tests (FST) were conducted during the light period of the light/dark cycle (9:00 a.m.–5:00 p.m.); the Sucrose Preference Test (SPT) was performed during the dark period, from (9:00 p.m.–10:00 p.m.). Rats were allowed to habituate to the test room prior to the beginning of the experiments.

2.3.1. Elevated-plus maze (EPM)

To assess anxiety-like behavior, the EPM test was used. At PND105, animals were placed for 5 min in the center of a plus-shaped platform elevated 72.4 cm above the floor and illuminated by a dim light. The behavioral apparatus (ENV-560; Med Associates Inc., St. Albans, VT, USA) consisted in two open arms (50.8 cm × 10.2 cm) and two closed arms (50.8 cm × 10.2 cm × 40.6 cm). The ratio of time spent in the open arms/total time spent in the open and in close arms was calculated as an index of anxiety-like behavior.

2.3.2. Sucrose preference test (SPT)

The SPT is widely used to assess anhedonia and is often related with individual's degree of affective state and motivation. Baseline sucrose preference (SP) was determined immediately before the start of the uCMS protocol (three independent trials). Before each trial, rats were food and water deprived for 12 h. For testing, two pre-weighed bottles, containing water or a 2% (m/v) sucrose solution were presented to individually housed animals for 1 h. After uCMS, at PND106, rats were tested for SP that was calculated according to the formula: $SP = (\text{sucrose intake} / (\text{sucrose intake} + \text{water intake})) \times 100$, as previously described (Bekris et al., 2005).

2.3.3. Forced swimming test (FST)

Immobility time, a marker of a passive coping strategy (one of the components of depressive-like behavior) was assessed through the FST. Rats were individually placed in transparent glass cylinder (62 cm

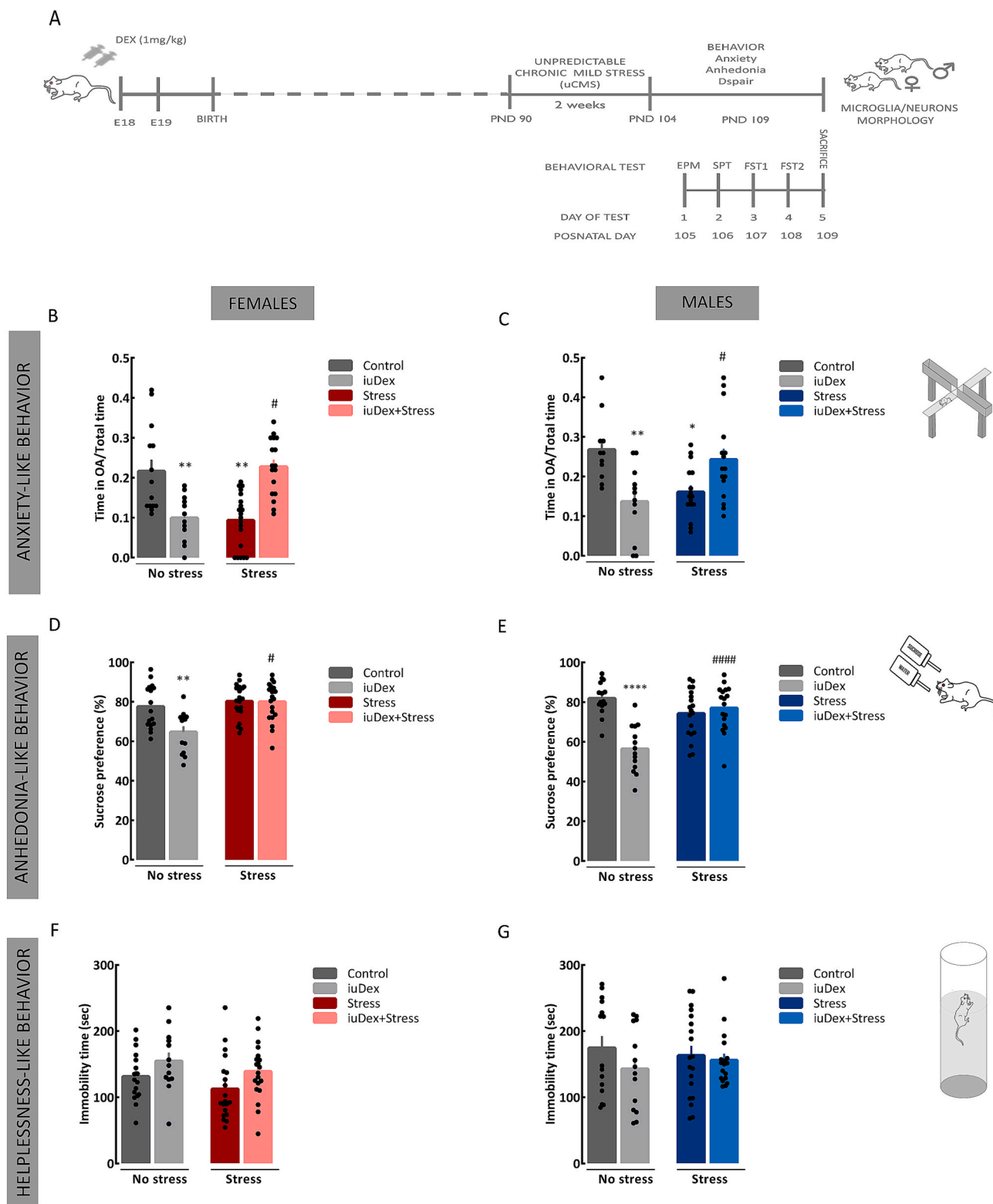


Fig. 1. Prenatal exposure to DEX preserved from short-term chronic stress effects: anxiety- and anhedonic-like behavior. (A) Schematic drawing of the animal model and chronic mild stress protocol: pregnant female Wistar rats were injected with DEX (1 mg/kg/day, sc) or saline on days 18 and 19 of gestation. At postnatal (PND) 90 female and male offspring were exposed to a short-term protocol of chronic mild stress for 2 weeks. (B, C) Time spent in open arms per total time in the elevated plus maze (EPM) test, was assessed to evaluate anxiety-like behaviour of females and males. (D, E) Anhedonic-like behaviour assessed by the preference for sucrose in the sucrose preference test (SPT) in females and males. (F, G) Depressive-like behaviour assessed by the total time of immobility in the forced swimming test (FST) for females and males. Results are presented as the mean \pm SEM of 11–17 animals; * $p < 0.05$, comparing with control, # $p < 0.05$, comparing with iuDex calculated using a two-way ANOVA followed by Bonferroni’s multiple comparisons test.

height and 25.4 cm diameter) filled with water (25 °C; 50 cm depth) for 5 min. The test was conducted 24 h after a 5-min pre-test session. This test was performed at PND107-108. Trials were video-recorded, and the total time animals spent immobile (immobility time) was measured. Immobility time was registered and used as a measure of depressive-like behavior.

2.4. Immunohistochemistry and 3D morphometric analysis of microglia

At the end of the experimental procedures, 24 h after FST, animals were deeply anaesthetized with sodium pentobarbital (20%; Eutasil®, Sanofi, Gentilly, France) and transcardially perfused with 0.9% saline. Brains were removed, post-fixed in 4% paraformaldehyde (PFA), cryoprotected in 30% sucrose overnight, and then embedded in Optimal Cutting Temperature compound (OCT, ThermoScientific, Waltham, MA, USA), snap-frozen and stored at -80 °C. Coronal sections (50 µm) of the hippocampal dentate gyrus (DG) (stereotaxic coordinates of interaural 5.20 mm and bregma -3.80 mm) and NAc (stereotaxic coordinates of interaural 10.20 mm and bregma 1.2 mm) were further stained to assess microglia cells. Briefly, free-floating sections were blocked with 5% bovine serum albumin (BSA) and 0.1% Triton X-100 for 2 h at room temperature (RT) and incubated with rabbit anti-Iba-1, microglia/macrophage marker (1:1000, WAKO, Osaka, Japan) antibody for 48 h at 4 °C. Though we used Iba-1 as a marker of microglia cells, it is important to refer that this marker is also expressed in other types of immune cells. Though there is no evidence of compromised blood brain barrier in iuDEX or uCMS models, there is no absolute evidence that other immune cells are not present in the brain, so Iba-1 staining should be interpreted with caution.

Afterwards, sections were washed and incubated with the appropriate secondary antibody (donkey anti-rabbit, 1:1000, Invitrogen, Waltham, MA, USA) and 4',6-diamidino-2-phenylindole (DAPI, 1:5000) for 2 h at RT. Sections were washed and mounted on gelatinized slides, using glycergel (DAKO mounting medium). Images of 10 random microglial cells from each animal from dHIP and NAc were acquired with a laser scanning confocal microscope LSM 710 META connected to ZEN Black software (Zeiss Microscopy, Oberkochen, Germany), using a 63× objective lens (oil immersed, Plan-Apochromat 63×/1.40 Oil DIC M27). Microglial cells were reconstructed using the NeuroLucida software (MBF Bioscience, Williston, VT, USA). Morphometric data related to branch analysis were extracted by the NeuroLucida Explorer software (MBF Bioscience). The parameters analyzed were the total number of processes in each cell, the number of processes per branch order, the total length of processes and the length of processes per branch order, considering processes of order 1 those emerging directly from the cell body, processes of order 2 those arising from processes of order 1, and so forth (Caetano et al., 2017).

2.5. Neuronal morphology

To assess the dendritic morphology of granule neurons of the DG and medium spiny neurons of the NAc, the Golgi-Cox staining was performed. Animals, 24 h after FST, were transcardially perfused with 0.9% saline and brains removed and immersed in a Golgi-Cox solution (1:1 solution of 5% potassium dichromate and 5% mercuric chloride diluted 4:10 with 5% potassium chromate) for 14 days. Afterwards, brains were transferred to a 30% sucrose solution and cut on a vibratome. Coronal sections (200 µm thick) were collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides, alkalized in 18.7% ammonia, developed in Dektol (Kodak, Rochester, NY, USA), fixed in Kodak Rapid Fix, dehydrated and xylene cleared. For each selected neuron, dendritic branches were reconstructed at ×1000 (oil) magnification, using a motorized microscope (Axioplan 2; Carl Zeiss, LLC, USA) and NeuroLucida software (MBF Bioscience, Williston, VT, USA). Three-dimensional analysis of the reconstructed neurons was performed using the NeuroExplorer software (MBF Bioscience). Structural analysis

included the total dendritic length and the 3D arrangement of neuronal branching using the Sholl analysis (number of dendritic intersections with concentric circles positioned at radial intervals of 20 µm). For each animal, 10 neurons from dHIP and NAc per animal were analyzed and neurons from the same animal were averaged. Observers were blinded to the experimental condition of each subject.

2.6. Corticosterone levels measurement

Corticosterone levels in blood serum were measured using an ELISA kit (Abcam, Cambridge, UK), according to the manufacturer's instructions. Absorbance at 450 nm was determined using a microplate reader and corticosterone concentration (ng/ml) was extrapolated from a standard curve. Blood sampling (tail venipuncture) was performed during the diurnal nadir (8:00 a.m.-9:00 a.m.) and diurnal zenith (8:00 p.m.-9:00 p.m.). For ELISA, we only used one technical replicate and prioritized the number of biological samples. The lowest level of detection of the ELISA was 43.18 ng/ml.

2.7. Estrous cycle analysis

Smears were obtained by vaginal cytology collected at the end of behavioral tests and stained using the hematoxylin-eosin method. Three cell types (nucleated epithelial cells, cornified epithelial cells and leukocytes) were counted to define the reproductive cycle (estrous), which is defined by the prevalence of each cell type: proestrus (nucleated), estrus (cornified), metestrus (all types in same proportion) and diestrus (leukocytes) (Westwood, 2008). Images were acquired with a light microscope Leica DM 4000 B (Leica, Wetzlar, Germany) with a ×10 objective lens (Plan 10×/0.25PH1).

2.8. Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). All statistical analysis was performed using Graphpad Prism 6.0 and results were considered significant for $p \leq 0.05$. Normality tests were performed for all data. Two-way analysis of variance (ANOVA) test was used for evaluating the main effects of iuDEX and stress exposure. Bonferroni's correction was used for *post hoc* tests.

3. Results

3.1. Prenatal exposure to DEX preserved from short-term stress effect: anxiety-like behavior and anhedonia

In the present study we used a validated model of anxiety-like behavior - iuDEX - in order to explore the effect of chronic stress in terms of behavior and microglia and neuronal morphology. We aimed to understand how iuDEX males and females that present anxiety-like behavior cope with a short-term protocol of stress in terms of behavior. We tested all groups in the EPM to assess anxiety-like behavior; and in the SPT and FST to assess anhedonic- and passive coping response, respectively.

For the EPM test, we observed a significant interaction between the iuDEX and stress exposure in females and males (two-way ANOVA; females: $F_{(1, 60)} = 43.88, p < 0.0001$; males: $F_{(1, 51)} = 19.33, p < 0.0001$). In line with previous results for EPM (Oliveira et al., 2006; Caetano et al., 2017; Duarte et al., 2019), iuDEX females and males displayed anxiety-like behavior with a significant decrease in the time spent in the open arms of the EPM compared to sex-matched controls (post hoc analysis; females: $t = 4.20, p = 0.0005$; males: $t = 3.55, p = 0.005$). As anticipated, uCMS induced an anxiety-like behavior in both sexes (post hoc analysis; females: $t = 4.94, p < 0.0001$; males: $t = 3.08, p = 0.020$). Notably, the hyperanxious behavior in iuDEX female and male animals was no longer present after stress exposure in adulthood in both sexes (post hoc analysis; females: $t = 4.46, p = 0.0002$; males: $t = 3.168, p =$

0.01; Fig. 1B and C). Analysis of the number of open arm entries revealed that, in both sexes, iuDEX showed a lower number of entries into open arms compared with control animals (post hoc analysis; females: $t = 4.11$, $p = 0.0004$; males: $t = 2.45$, $p = 0.04$); iuDEX females and males that were exposed to uCMS in adulthood have an increased number of entries into open arms compared to iuDEX animals (post hoc analysis; females: $t = 4.34$, $p = 0.0011$; males: $t = 2.43$, $p < 0.04$; Supplementary Fig. 2A and B). No differences were found in the number of entries in the closed arms in both sexes (Supplementary Fig. 2C and D). In the analysis of total arm entries, which could be an indicator of locomotor activity, we did not observe differences between groups in both sexes (Supplementary Fig. 2E and F).

In terms of anhedonia, evaluated using the SPT, a significant effect of iuDEX (two-way ANOVA; females: $F_{(1, 66)} = 7.36$, $p = 0.009$; males: $F_{(1, 62)} = 16.78$, $p = 0.0001$) and stress exposure (two-way ANOVA; females: $F_{(1, 66)} = 13.75$, $p = 0.0004$; males: $F_{(1, 62)} = 5.51$, $p = 0.022$), as well as an iuDEX-Stress interaction (two-way ANOVA; females: $F_{(1, 66)} = 6.84$, $p = 0.011$; males: $F_{(1, 62)} = 26.09$, $p < 0.0001$) were observed. iuDEX females and males presented anhedonic-like behavior in comparison to control animals, with a significant decrease in the percentage of sucrose preference compared to sex-matched controls (post hoc analysis; females: $t = 3.51$, $p = 0.0049$; males: $t = 6.15$, $p < 0.0001$). The uCMS protocol did not affect the anhedonic phenotype in both males and females (post hoc analysis; females: $t = 0.80$, $p > 0.9999$; males: $t = 1.98$, $p = 0.311$). Conversely, iuDEX animals of both sexes that were exposed to uCMS in adulthood, no longer presented anhedonia compared to iuDEX animals that were not exposed to stress protocol (post hoc analysis; females: $t = 4.309$, $p = 0.0003$; males: $t = 5.192$, $p < 0.0001$; Fig. 1D and E).

Neither control females nor males exposed to the short protocol of uCMS presented alterations in passive coping response (Fig. 1F and G), as assessed by the FST, in line with our previous results (Oliveira et al., 2006; Caetano et al., 2017; Duarte et al., 2019).

The reduction in body weight gain and a disruption in the pattern of corticosterone levels are well-known consequences of exposure to chronic stress. However, the effect of stress in animals prenatally exposed to DEX in these biological parameters is still unknown.

Animal weight was monitored longitudinally throughout the experiment in order to determine the weight gain of rats in all conditions. No differences were found in weight gain in females and males prenatally exposed to DEX when compared with age-matched controls. However, we observed a significant effect of stress in females (two-way ANOVA; $F_{(1, 144)} = 18.38$, $p < 0.0001$) and males (two-way ANOVA; $F_{(1, 130)} = 32.90$, $p < 0.0001$) (Fig. 2A and B). We also detected a reduced body weight in iuDEX males compared to controls at baseline (post hoc analysis; males: $t = 4.78$, $p < 0.0001$) with no differences in females

(Supplementary Fig. 3A and B).

At the end of uCMS, corticosterone levels were measured in the serum. We did not observe any effect of DEX *per se* in corticosterone levels in both sexes in both periods of the day. However, control females and males exposed to stress presented a disruption in the diurnal nadir pattern of corticosterone with an increase of the corticosterone serum levels (post hoc analysis; females: $t = 4.629$, $p = 0.0003$; males: $t = 5.640$, $p < 0.0001$) and a decrease in zenith diurnal levels (post hoc analysis; females: $t = 3.139$, $p = 0.0203$; males: $t = 3.302$, $p = 0.0128$). Remarkably, in the diurnal nadir pattern, we found a trend for normal corticosterone levels in iuDEX exposed to stress in both sexes that did not reach statistical significance (post hoc analysis; females: $t = 0.4121$, $p > 0.9999$; males: $t = 0.6249$, $p > 0.9999$). Interestingly, both iuDEX females and males exposed to stress secreted lower levels of corticosterone (post hoc analysis; females: $t = 3.984$, $p = 0.0019$; males: $t = 4.549$, $p = 0.0003$), suggesting an imbalance in the HPA axis activity caused by stress (Fig. 3A and B).

The estrous cycle analysis was performed in females to verify if stress could affect the hormonal cycle. All females were distributed for the 4 phases of the estrous cycle, being 24 (in a total of 55) in metestrus phase on the day of euthanasia (Supplementary Table 1; Supplementary Fig. 4). In this study, we did not correlate the estrous cycle with the behavior, but estrous cycle does not impact on behavior as previously described by us and other authors (Schwarz et al., 2012; Caetano et al., 2017).

3.2. Sex-specific remodeling of microglia in iuDEX model and impact of short-term stress

We next aimed to evaluate how stress impacted microglia morphology in the iuDEX model. For that, we performed a detailed morphometric analysis of microglia in female and male adult rats in two different brain regions, the dHIP (Fig. 4A, F) and the NAC (Fig. 4K, P). In dHIP, iuDEX females presented an increase of the total number and total length of microglia ramifications when compared to control animals (post hoc analysis; number: $t = 8.487$, $p < 0.0001$; length: $t = 6.913$, $p < 0.0001$), as previously described by our group (Duarte et al., 2019) (Fig. 4B, C, D, E; Supplementary Fig. 5A; Supplementary Table 2). In males, no differences in microglia cells were observed in iuDEX animals when compared with controls (Fig. 4G, H, I, J; Supplementary Fig. 5B; Supplementary Table 3). Importantly, stress *per se* did not affect microglia morphology in both sexes. Interestingly, stress induced a normalization of the phenotype of iuDEX females (post hoc analysis; number: $t = 6.668$, $p < 0.0001$; length: $t = 5.330$, $p < 0.0001$ (Fig. 4B, C, D, E).

In the NAC, we observed opposite differences between sexes. In line

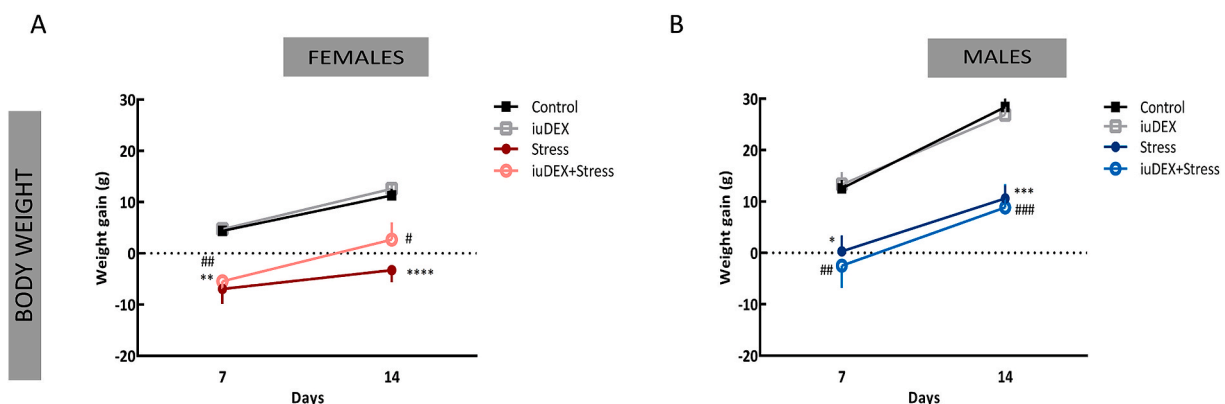


Fig. 2. Short-term stress induces a dysregulation of body weight gain. Pregnant female Wistar rats were injected with DEX (1 mg/kg/day, sc) or saline on days 18 and 19 of gestation. At postnatal (PND) 90 female and male offspring were exposed to a short-term protocol of chronic mild stress for 2 weeks. (A, B) Body weight gain of female and male rats prenatally exposed to DEX and exposed to stress in adulthood. Results are presented as the mean \pm SEM of 11–17 animals. * $p < 0.05$, comparing with control, # $p < 0.05$, comparing with iuDEX calculated using a two-way ANOVA followed by Bonferroni's multiple comparisons test.

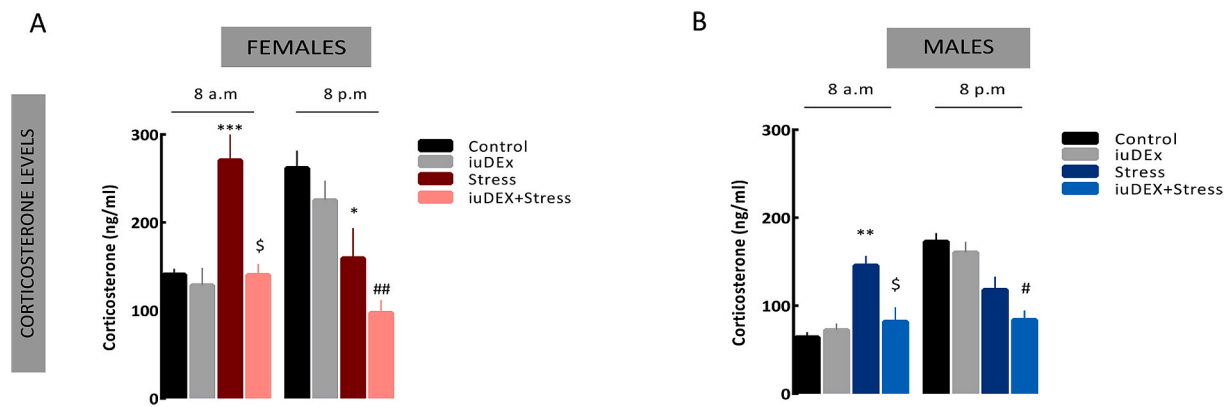


Fig. 3. Short-term stress induces a dysregulation of circadian corticosterone secretion pattern. Pregnant female Wistar rats were injected with DEX (1 mg/kg/day, sc) or saline on days 18 and 19 of gestation. At postnatal (PND) 90 female and male offspring were exposed to a short-term protocol of chronic mild stress for 2 weeks. (A, B) Corticosterone serum levels measured at 8:00 a.m. and 8:00 p.m. (circadian peaks of production) by ELISA in female and male rats prenatally exposed to DEX and exposed to a stress in adulthood. Results are presented as the mean \pm SEM of 10–12 animals (corticosterone); * p < 0.05, comparing with control, # p < 0.05, comparing with iuDEX calculated using a two-way ANOVA followed by Bonferroni's multiple comparisons test.

with what we already observed in the PFC (Caetano et al., 2017), iuDEX females exhibited a general decrease in the total number and length of microglia processes (post hoc analysis; number: $t = 4.347$, $p = 0.0002$; length: $t = 4.672$, $p < 0.0001$) (Fig. 4 L, N; Supplementary Fig. 6A; Supplementary Table 4). Conversely, iuDEX males presented an increase in all parameters (post hoc analysis; number: $t = 5.042$, $p < 0.0001$; length: $t = 3.695$, $p = 0.0020$) (Fig. 4 Q, S; Supplementary Fig. 6B; Supplementary Table 5). Microglia remodeling associated with stress was similar to DEX, namely, atrophy in females (post hoc analysis; number: $t = 4.918$, $p < 0.0001$; length: $t = 4.912$, $p < 0.0001$) and hypertrophy in males (post hoc analysis; number: $t = 2.587$, $p = 0.0656$; length: $t = 2.722$, $p = 0.0450$). Furthermore, uCMS in combination to iuDEX (both stimuli together) normalized DEX-induced atrophy in females (post hoc analysis; number: $t = 2.919$, $p = 0.0253$; length: $t = 2.645$, $p = 0.0558$) (Fig. 4 L, M, N, O) and the DEX-induced hypertrophy in males (post hoc analysis; number: $t = 3.546$, $p = 0.0034$; length: $t = 1.683$, $p = 0.5706$) (Fig. 4 Q, R, S, T). We also analyzed the soma size and we did not observe any differences in the dHIP and in the NAC in terms of area and perimeter of the soma (Supplementary Fig. 7).

3.3. Prenatal exposure to DEX is associated with sex-specific remodeling of neurons, an effect that is modulated by short-term stress

Previous studies have demonstrated the importance of structural changes of neurons in dHIP and NAC in the pathophysiology of depression (Bessa et al., 2013; Patricio et al., 2015). Based on these observations, it became important to understand the impact of stress in neuronal complexity in the iuDEX model.

We performed a detailed three-dimensional morphometric analysis of Golgi-impregnated neurons in the dHIP (Fig. 5A and B) and in NAC (Fig. 5G and H) in both sexes. In dHIP, the neuronal morphology was not altered in any condition analyzed in females (Fig. 5C and D). In males, we observed an effect of iuDEX (two-way ANOVA; $F_{(1, 12)} = 5.804$, $p = 0.0330$) and a significant effect between iuDEX-stress exposure (two-way ANOVA; $F_{(1, 12)} = 38.91$, $p < 0.0001$). iuDEX induced atrophy in neurons with a significant decrease in their total dendritic length in males when compared with controls (post hoc analysis; $t = 6.114$, $p = 0.0003$). Notably, uCMS *per se* induced a significant decrease in total dendritic length (post hoc analysis; $t = 5.205$, $p = 0.0013$). This atrophic effect was reverted in iuDEX males exposed to stress in adulthood (post hoc analysis; $t = 3.617$, $p = 0.0212$). In Sholl analysis we also observed a significant interaction between variables (two-way ANOVA; $F_{(33, 144)} = 2.012$, $p = 0.0026$) (Fig. 5E and F).

Concerning the NAC (Fig. 5G and H), and similar to microglia cytoarchitecture, we observed sex-dependent alterations in medium

spiny neurons. Females that were prenatally exposed to DEX exhibited a dendritic remodeling characterized by a shortening of dendritic length (post hoc analysis; $t = 7.976$, $p < 0.0001$) (Fig. 5I). Contrary to females, iuDEX males presented a hypertrophy of neurons (post hoc analysis; $t = 4.868$, $p = 0.0023$) (Fig. 5K). Importantly, stress *per se* did not induce alterations in neurons morphology in females but induced a hypertrophy in males (post hoc analysis; $t = 3.611$, $p = 0.0215$). These structural changes in both sexes were reversed when iuDEX animals were exposed to stress in adulthood (post hoc analysis; females: $t = 7.969$, $p < 0.0001$; males: $t = 4.429$, $p = 0.0049$) (Fig. 5I, K). In addition, sholl analysis revealed less complex neurons in iuDEX females (two-way ANOVA; $F_{(3, 121)} = 5.306$, $p = 0.0018$ and more complex neurons in iuDEX males (two-way ANOVA; $F_{(3, 139)} = 10.71$, $p < 0.0001$) (Fig. 5J, L).

4. Discussion

In this work, we show that the exposure to DEX during pregnancy induces major effects on adult behavior in both sexes and that these behavioral alterations are accompanied by sex-specific structural changes in microglia and neurons in the dHIP and NAC.

Previously, we showed that iuDEX is associated with an anxious phenotype and impacts on microglia remodeling in a sex-specific manner. We observed microglia hypertrophy in males and atrophy in females in the PFC, an important brain region in the genesis of anxiety-related behavior (Caetano et al., 2017). In addition, iuDEX females presented hypertrophy of microglial cells in the dHIP, with impaired recognition memory and the loss of neuronal synchronization between dHIP and PFC (Duarte et al., 2019). This sexual dimorphism in remodeling of microglial cells was translated into a sex-selective resistance to a therapeutic intervention, since chronic treatment with a selective $A_{2A}R$ antagonist (modulator of microglia and an experimental anxiolytic drug) normalized anxiety-like behavior and microglia alterations in iuDEX males in the PFC, with a recovery of cognitive deficits; as well as microglia morphology in iuDEX females in the dHIP (Caetano et al., 2017; Duarte et al., 2019). However, it is still unclear if/how stress in adulthood differentially affects iuDEX males and females.

Interestingly, we now show that in response to stress in adulthood, iuDEX rats manage better with this challenge, improving their performance in anxiety-like and anhedonic-like behaviors. Rats prenatally exposed to DEX and later subjected to a chronic mild stress protocol developed some type of stress resilience, similar to stress inoculation models (Santarelli et al., 2014; Hsiao et al., 2016). Literature strongly suggests that prenatal adverse events can enhance vulnerability for anxiety- and depressive-like phenotypes in adulthood (Lupien et al., 2009; Weinstock, 2017), including the iuDEX model presented in this

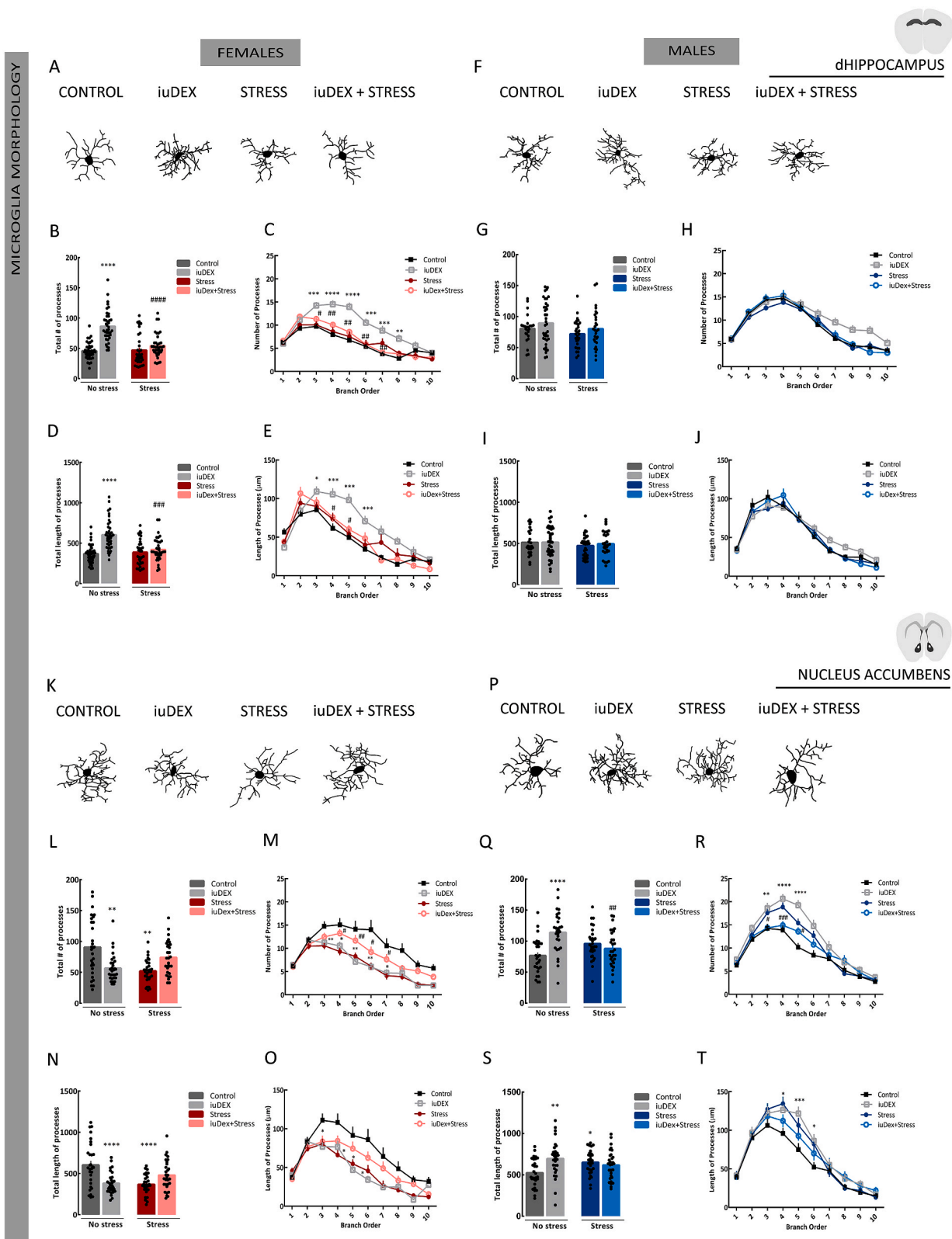


Fig. 4. Sex-specific remodeling of microglia at dHIP and NAC upon iuDEX and short-term chronic stress at adulthood in females and males. Pregnant female Wistar rats were injected with DEX (1 mg/kg/day, sc) or saline on days 18 and 19 of gestation. At postnatal (PND) 90 female and male were exposed to a short-term protocol of chronic mild stress for 2 weeks. Microglial morphometric structure was manually reconstructed in the NeuroLucida software based on 3D images of Iba-1 stained microglia (A, F, K, P) according to the morphological parameters: total number of cells (B, G, L, Q), number of processes per branch (C, H, M, R), total length of microglial processes (D, I, N, S) and length of processes per branch (E, J, O, T) in dHIP and NAC. Results are presented as the mean \pm SEM of 40–50 cells from 4 to 5 animals; * $p < 0.05$, comparing with control, # $p < 0.05$, comparing with iuDEX calculated using a two-way ANOVA followed by Bonferroni's multiple comparisons test.

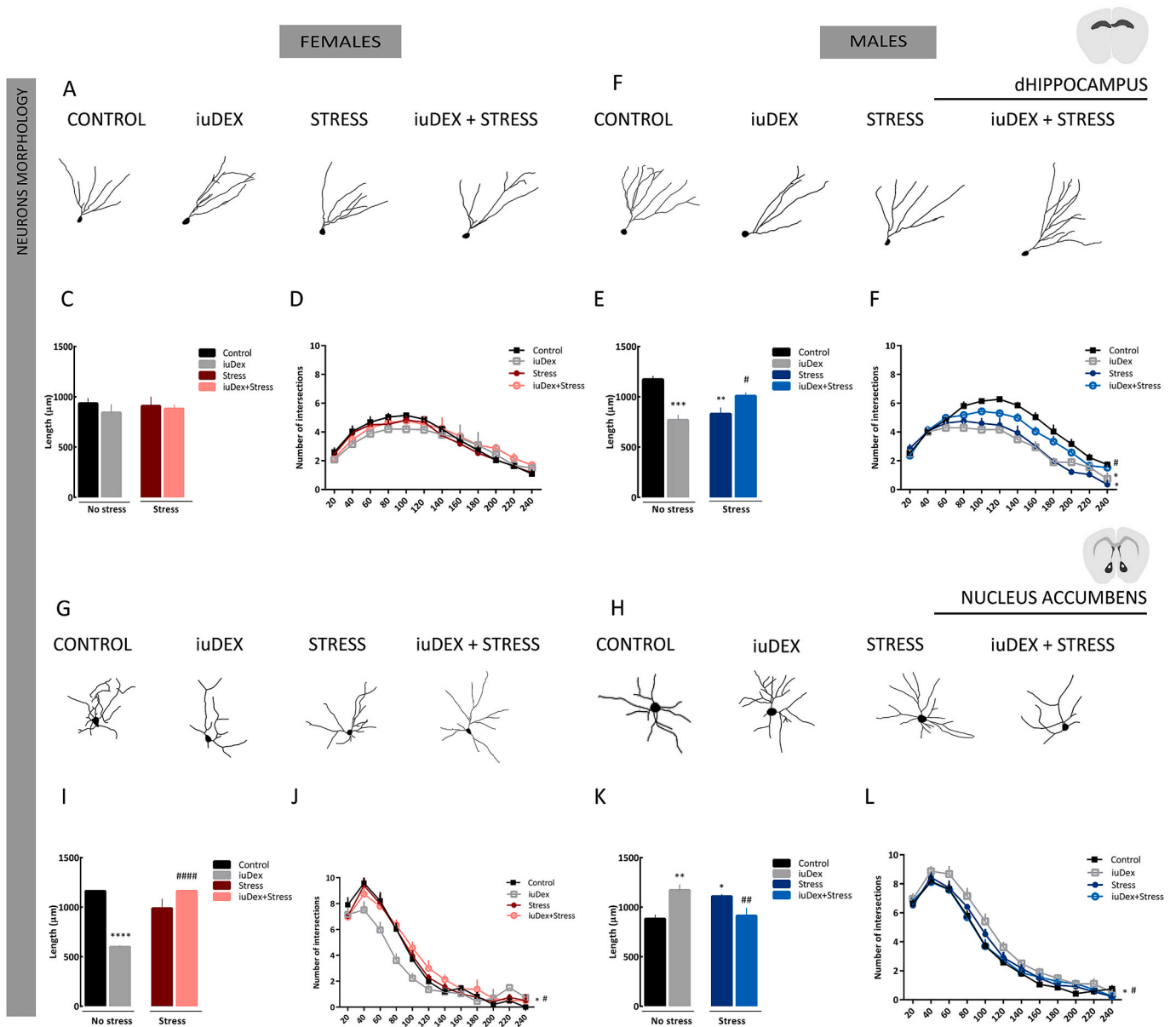


Fig. 5. Sex- and brain-specific remodeling of neurons at dHIP and NAc upon iuDEX and short-term chronic stress at adulthood. Pregnant female Wistar rats were injected with DEX (1 mg/kg/day, sc) or saline on days 18 and 19 of gestation. At postnatal (PND) 90 female and male offspring were exposed to a short-term protocol of chronic mild stress for 2 weeks. Representative manual reconstruction of Golgi-impregnated granule neurons at dHIP (A, B) and spiny medium neurons at NAc (G, H). Total dendritic length of neurons in the dentate gyrus at dHIP (C, E) and of neurons at NAc (I, L). Sholl analysis of dendritic distribution of neurons in dHIP (D, F) and in NAc (J, L). Results are presented as the mean ± SEM of 30–40 cells from 3 to 4 animals; *p < 0.05, comparing with control, #p < 0.05, comparing with iuDEX calculated using a two-way ANOVA followed by Bonferroni’s multiple comparisons test.

work. Paradoxically, iuDEX exposure adaptations also appear to confer some degree of protection to the effects of adult chronic stress. This phenomenon is reminiscent of a stress adaptive response or hormesis (Bateson et al., 2004), i.e., prenatal exposure to a certain adverse agent (GC) results in a protective effect to that same agent later in life (adult chronic stress), which is translated into an improved organism response (to stress in this case). In the case of the fetus, this adaptive response is hypothesized to allow individuals to cope better with the harsh conditions of the environment after birth, which is evolutionary advantageous (Reynolds, 2013). However, when there is a mismatch between the adaptive response and the environment this can be detrimental, as in the case of iuDEX animals raised in non-stressful conditions, that manifest anxious- and depressive-like behaviors. So, iuDEX exposure can therefore be both a friend or a foe, depending on the postnatal environmental conditions. In humans there are also evidences that experiencing a

moderate number of serious negative life events may contribute to a propensity for coping well when subjected to stress conditions (Seery et al., 2013; Domhardt et al., 2015). These effects can be reconciled with other studies which predict that adversity restricted to early life can trigger the acquisition of stable active coping strategies in adulthood (Daskalakis et al., 2012; Nederhof and Schmidt, 2012). However, the type and the duration of the stressor influences stress response and sex differences could be mediated in a complex way, since the gonadal hormones can exert both organizational and activational influences in response to stress (Arnold, 2009). One important consideration is that most of the studies perform uCMS for 6 weeks (Bessa et al., 2013; Morais et al., 2014; Patricio et al., 2015; Alves et al., 2018), but we decided to use a shorter uCMS protocol (2 weeks) in order to evaluate different susceptibility to depression, without reaching the “ceiling effect” of stress exposure.

It is important to note that iuDEX induces a decrease in body weight only in males, but not in body weight gain during the uCMS protocol. Other studies using this model already described that iuDEX males present reduced weight at birth that remained significantly reduced until young adults (Oliveira et al., 2006). This differences in males could reflect in a worse performance in behavioral tests, namely in locomotor activity. However, we did not observe any alteration in locomotor activity in all groups that would provide more convincing support that the decrease in body weight in iuDEX affect the behavioral tasks.

There is increasing evidence supporting a role of glial cells in the etiology of neuropsychiatric disorders (Hodes et al., 2015; Rial et al., 2015). We previously reported that the pattern of microglia morphology, which is sex-specific, impacts anxiety- and cognition-like behaviors (Caetano et al., 2017; Duarte et al., 2019). Working on the premise that microglia morphology is implicated in the response to stress conditions and in the establishment of a depressive-like phenotype (Kreisel et al., 2014), we also explored the effect of chronic stress in microglia morphology in a model presenting anxiety-like behavior in two brain regions involved in the pathophysiology of depression, the NAc and the HIP. The HIP is a known stress-responsive brain area, which is also associated with depression (Patricio et al., 2015; Liu et al., 2017). Indeed, the DG appears to be highly vulnerable to the deleterious effects of stress, since it affects the generation of new granule neurons (Pham et al., 2003), which have been recognized as relevant players in the development and improvement of behavioral deficits, namely cognitive and emotional, that underlie neuropsychiatric disorders (Snyder et al., 2011; Mateus-Pinheiro et al., 2013). However, if the microglial alterations occur in parallel with mood- and anxiety-related deficits in the dHIP, following chronic exposure to stress in a model of prenatal exposure to DEX was still unknown. The major alterations observed in microglia morphology in the dHIP occur in females. Remarkably, in this brain region we also observed a normalization of microglia DEX-induced hypertrophy after exposure to the stress protocol.

Since this model also presents anhedonia in both sexes, we tried to unveil if anhedonia was paralleled with structural alterations in microglia morphology in the NAc, which is recognized as a key component in the neural circuitry of reward (Gorwood, 2008). In addition, we evaluated whether DEX-induced alterations are influenced by stress in adulthood. We report, for the first time, that iuDEX significantly altered the morphology of microglia in a sex-specific manner in the NAc. Similar to what happens in the PFC, iuDEX promoted a hyper-ramified state in males, which has been described as characteristic of stress-related conditions (Hinwood et al., 2013; Kreisel et al., 2014). Notably, iuDEX females in the NAc presented microglia with less and shorter processes as we already observed in mPFC (Caetano et al., 2017). The NAc and mPFC form a functional PFC–striatal circuit that is critical for the control regulating reward-seeking processes and cognitive functions (Goeders and Smith, 1983). Besides, mPFC is a core region in the control of anxiety (Calhoun and Tye, 2015). Importantly, our results suggest that the pattern of microglia morphology is similar in these two regions upon iuDEX exposure with impact in anxiety and anhedonia. Interestingly and in accordance with our observations in behavior after stress, we observed a normalization of the hypertrophy in males and of the atrophy in females induced by DEX.

When microglial cells are primed in early life stages, they could show an exacerbated response to challenges later in life, that could explain these alterations in their morphology in the dHIP and NAc (Lenz and Nelson, 2018). These differences between sexes probably result from the when subjected to stress conditions in brain regions with a sex-dependent complexity typified by more complex cells in females than in males (Schwarz et al., 2012; Lenz and McCarthy, 2015). Moreover, the existence of sex- and brain region-specific morphologic differentiation of microglia must be conserved among species, since we observed the same dichotomy in a mice model (Simoes-Henriques et al., 2019).

The observed dimorphic pattern of microglia highlights the

importance of considering these cells in therapeutic strategies considering sex differences. These alterations in microglia may mediate functional neuroimmune sex disparities. The dynamic morphology of microglia is closely related to their functional state, however further studies using additional markers of microglia cells and other tools to modulate microglia function are needed to infer about the potential changes in the function of microglia. Considering that microglia are in constant baseline motility (Franco-Bocanegra et al., 2019), allowing microglia to effectively explore the environment in order to clear cellular debris and remodel extracellular matrix (Nimmerjahn et al., 2005; Garden and Moller, 2006), alterations in microglia morphology between sexes would anticipate functional changes. Past evidence has shown that early life stress exposure increased microglia motility in the adult mice brain (Takatsuru et al., 2015), which may indicate that these cells could have been recruited to support other brain regions that were affected by the stress protocol.

In parallel, we also performed an analysis of neuronal morphology in the dHIP and NAc in order to clarify if the morphologic alterations observed in microglia were paralleled with alterations in neuronal morphology. The crosstalk microglia-neurons is important to maintain homeostasis and highlights the microglia involvement in regulating synapse formation and maturation, reshaping brain wiring and behavior (Paolicelli et al., 2011; Ji et al., 2013; Lim et al., 2013; Parkhurst et al., 2013; Cristovao et al., 2014). iuDEX males presented an atrophy of granule neurons of the dHIP. Some studies already described dendritic atrophy after chronic stress, namely in hippocampal CA3 pyramidal neurons (Magarinos and McEwen, 1995; Bessa et al., 2009; Patricio et al., 2015). Those studies also showed that some antidepressants can revert alterations in the dendritic arborization modifications of the dorsal dentate granule cells of the HIP to pre-stress levels (Bessa et al., 2009; Patricio et al., 2015). In spite of this, no differences were observed in the morphology of granule cells in females. Sex differences in the brain include regional volume differences due to differential cell death, neuronal and glial genesis, dendritic branching and synaptic patterning that could culminate in differences in neural morphology or different responses between sexes (McCarthy et al., 2015). Nevertheless, the majority of studies reporting the effect of stress in granule cells only studied males, indicating that there is still much to be studied regarding sex differences in this context.

Chronic stress is known to induce anhedonia, impacting on the fine structure in the NAc neurons (Willner, 2005; Christoffel et al., 2011; Bessa et al., 2013). Here, we observed that, similar to what we observed in microglia, both sexes showed opposite patterns of dendritic remodeling. Many studies have suggested a role for the NAc in depression, in which anhedonia is a cardinal symptom (Liu et al., 1992; Di Chiara et al., 1999; Rada et al., 2003; Gorwood, 2008). Additionally, studies described that prenatal administration of synthetic GC delays maturation of neurons, myelination, glia and vasculature in the offspring, altering neuronal structure and synapse formation (Seckl, 2008) that could have an impact in the morphology and behavior later in life. Our results add important new data to support this view, since we showed that prenatal exposure to DEX induces a neuronal atrophy in males and a hypertrophy in females in parallel with an anhedonic-like behavior in both sexes. Remarkably, in this brain region we also observed a normalization of neuronal morphology in both female and male iuDEX offspring after stress protocol. In line with our previous results, in which we reported a sex-specific remodeling of microglia in the PFC associated with anxiety-like behavior (Caetano et al., 2017), here we were able to associate anhedonia with microglial and neuronal dimorphic morphological differences.

The exposure to GCs can cause a profound impact on neuronal structure and function, which culminates in impairments of behavior (Lucassen et al., 2014). Moreover, remodeling of microglia could also affect other cells, namely neurons, with impact in behavior (Paolicelli and Ferretti, 2017). It is important to refer that, in the NAc, stress (iuDEX or uCMS) induce the same type of remodeling in neurons and microglia,

but is different between males and females. Contrasting patterns of cellular plasticity across the brain have already been described for neurons in response to stress conditions (Chattarji et al., 2015); however, the regional morphologic adaptation of microglia to stress mediators, namely GC, was not yet well described. With our results, we demonstrated that brain regions of each sex adapt differently when prenatally exposed to DEX.

Altogether, these findings provide further evidence that GCs and chronic stress cause brain region- and sex-specific alterations in microglia and neurons responses, accompanied with behavioral and cognitive alterations. To date, we still cannot establish a direct relation between the behavioral and neurobiological underpinnings, however, we can speculate an association between behavioral phenotypes and changes in microglia and neurons morphology. Males and females presented a cellular plasticity that is sex-specific and results in similar behavioral phenotypes. Taken together, these findings illustrate the complex nature of microglia and neurons morphology, demonstrating the dynamic nature of stress induced morphological alterations in both sexes. Microglia and neurons, therefore, may play different roles in the male and female brain, implicating them in sex-specific effects of adversity.

5. Conclusion

The results of the present study show that stressful events during pregnancy have an impact on behavior in both sexes, and animals were preserved from stress effects in adulthood by prenatal stress that are paralleled by marked, but reversible alterations in the morphology of neurons and microglia. The present characterization of microglia and neuronal morphology in iuDEX model and after uCMS constitutes one step ahead in understanding the role of microglia and neurons in the etiology of neuropsychiatric disorders. Prenatal exposure to DEX induced anhedonia, in association with fine structural alterations in neurons and microglia morphology in the NAc in a sex-specific manner. Importantly, the later behavioral and morphological adaptations were normalized upon a short-term protocol of uCMS. Although the role of cellular remodeling is still unknown, sex-specific differences in microglia plasticity induced by long-term stress may anticipate differences in drug efficacy, as previously observed by our group and others. Our data raises the possibility that alterations in neuronal and microglia morphology in the dHIP and NAc represent highly relevant plastic events due to stress that may lead to the observed behavioral manifestations of enhanced emotionality.

Author contribution section

RG designed the experiments with CS-C, AJR and CAG, performed the experiments, and wrote the manuscript. CSC, AVR and BC help to perform behavioral experiments. All authors discussed the results, contributed to and approved the final manuscript. CAG and AJR supervised RG, contributed to the design of the experiments, and revised the manuscript.

CRediT authorship contribution statement

Rita Gaspar: Methodology, Validation, Formal analysis, Investigation, Visualization, Writing - original draft. **Carina Soares-Cunha:** Methodology, Investigation, Validation. **Ana Verónica Domingues:** Methodology, Investigation, Validation. **Bárbara Coimbra:** Methodology, Investigation, Validation. **Filipa I. Baptista:** Validation, Writing - review & editing. **Luísa Pinto:** Methodology, Writing - review & editing. **António F. Ambrósio:** Funding acquisition, Writing - review & editing. **Ana João Rodrigues:** Conceptualization, Methodology, Supervision, Writing - review & editing. **Catarina A. Gomes:** Conceptualization, Methodology, Supervision, Writing - review & editing.

Declaration of competing interest

The authors report no conflicts of interest.

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Appendix A. Supplementary data

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