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ORIGINAL ARTICLE

Adenosine A_{2A} receptor regulation of microglia morphological remodeling-gender bias in physiology and in a model of chronic anxiety

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Developmental risk factors, such as the exposure to stress or high levels of glucocorticoids (GCs), may contribute to the pathogenesis of anxiety disorders. The immunomodulatory role of GCs and the immunological fingerprint found in animals prenatally exposed to GCs point towards an interplay between the immune and the nervous systems in the etiology of these disorders. Microglia are immune cells of the brain, responsive to GCs and morphologically altered in stress-related disorders. These cells are regulated by adenosine A_{2A} receptors, which are also involved in the pathophysiology of anxiety. We now compare animal behavior and microglia morphology in males and females prenatally exposed to the GC dexamethasone. We report that prenatal exposure to dexamethasone is associated with a gender-specific remodeling of microglial cell processes in the prefrontal cortex: males show a hyper-ramification and increased length whereas females exhibit a decrease in the number and in the length of microglia processes. Microglial cells re-organization responded in a gender-specific manner to the chronic treatment with a selective adenosine A_{2A} receptor antagonist, which was able to ameliorate microglial processes alterations and anxiety behavior in males, but not in females.

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INTRODUCTION

Anxiety disorders are a common form of psychopathology, impairing and predisposing to other debilitating psychiatric conditions throughout life. Developmental risk factors, such as the exposure to glucocorticoids (GC) above physiological levels, associated with early life stress or certain GC therapies during pregnancy and childhood, contribute to the pathogenesis of mood disorders.[1,2] Exposure to high levels of GC in specific developmental windows causes deleterious effects on neurons, including impairment in neuronal migration,[3] alterations in spine formation[4,5] and neuronal survival, number and morphology.[4,6–10] Some of these changes occur in brain regions implicated in anxiety, namely the hippocampus,[8,9] the amygdala and the bed nucleus of stria terminalis,[4] suggesting that they may contribute to the observed hyperanxious phenotype and altered fear responses.[4,8]

Considering the immunomodulatory role of GC, it is not surprising that animals prenatally exposed to GC present a distinct immunological fingerprint,[11] raising the question whether the 'programming' effects of GC are due to a direct, exclusive impact on neurons, or result also from changes in glial immune cells, i.e. microglia. Microglia are equipped with GC receptors (GR)[12] and respond to GC,[13] emerging as prime candidates to mediate the effects of GC in the brain. Microglia control dendritic/synaptic elimination[14] and synapse formation and maturation,[15–18] globally regulating the number and function of synapses and animal behavior.[19,20] The efficiency of microglia functions depends on the dynamic extension and retraction of their processes, which work as homeostatic sensors of the brain parenchyma.[21,22] Thus, abnormalities in the morphology of microglial processes, such as increased branching,[23] decreased length of cellular processes[24] or reduced process displacement toward an injury site,[25] have been related with pathological conditions. Glial cells have been proposed to be particularly affected in mood-related conditions.[26] Accordingly, in a model of chronic stress, increased branching of microglial cells was described in the prefrontal cortex (PFC),[23] a core region in the control of stress and anxiety, along with amygdala, bed nucleus of stria terminalis and ventral hippocampus.[27–29] Microglia dynamics is controlled by several modulatory systems, such as adenosine A_{2A} receptors ($A_{2A}R$),[25,30–32] which are also involved in the control of fear and anxiety.[33–38] Given their role in brain development and neuropsychiatric disorders, microglia are indeed positioned to have a crucial role in the prenatal etiology of brain disorders. Moreover, the existence of gender differences in microglia brain colonization, number and secretion of inflammatory mediators[39]

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may provide a mechanistic basis for the gender bias found in symptoms and prevalence of several mood disorders, although these cells have been mostly examined in males.

We now investigated the short- and long-term effects of prenatal exposure to the synthetic GC dexamethasone (DEX) on microglia morphology in the PFC. Morphological analysis of microglia was assessed by using a three-dimensional (3D) reconstruction method, bringing an added value to the conventional, bidimensional cellular analysis, particularly defective in the rigorous characterization of microglia processes and branching degree. Such morphological analyses were correlated with corticosterone circulating levels and behavioral performance in anxiety-related tests. Finally, we evaluated the impact of blocking $A_{2A}R$ in microglia morphology, behavior, neuro- and biochemical changes in both genders.

MATERIALS AND METHODS

Animals and pharmacological treatments

Animal manipulation was done according to the European Union guidelines (Directive 2010/63/EU) on animal care and experimentation. Sample size was estimated based on previous studies already published using the same animal model and the same behavioral analysis.[40]

Pregnant Wistar rats were injected with saline or DEX (1 mg kg^{-1}, subcutaneous) at gestational days 18 and 19.[4,40] Adults from the offspring were treated with saline or with the selective $A_{2A}R$ antagonist, SCH58261 (0.1 mg kg^{-1} day^{-1}, intraperitoneal) for 21 days preceding postnatal day (PND) 90, a protocol of chronic administration previously described as anxiolytic in adult rodents subjected to stress protocols.[37]

Animals were randomly allocated to experimental groups and into cages. Adult offspring from the pregnant rats injected with saline that presented an anxious behavior were excluded from the behavioral analysis and this was pre-established before the beginning of the study. The investigators that performed the behavioral tests and the morphometric evaluation of microglia, as well as the respective analysis, were blinded to the experimental condition of the animals.

Behavior tests

Open field test. The open field (OF) test was carried out in an arena (white floor and transparent acrylic walls, 43.2 × 43.2 cm; Med Associates, St Albans, VT, USA) under bright white light. The animals were placed in the center of the arena and the locomotor activity (velocity and total distance travelled) was monitored online (beam infrared arrays) for 5 min.

Elevated plus maze test. The elevated plus maze (EPM) test was used to assess anxious-like behavior. Animals were placed in the center of a black polypropylene plus-shaped platform (Med Associates) with two open arms (50.8 × 10.2 cm) and two closed arms (50.8 × 10.2 × 40.6 cm), located 72.4 cm above the floor in a dark room. The number of entries into each of the arms and the time spent therein were measured (for 5 min) with an infrared photo beam system and analyzed with specific software (MedPCIV, MedAssociates, Georgia, VT, USA). Data were processed to obtain the ratio of time spent in the open arms versus total time and the number of entries into each arm of the maze.

Forced swimming test. Learned helplessness (one of the components of depressive-related behavior) was assessed by the forced swim test (FST). Twenty four hours after a pre-test session (10 min), the animals were individually placed inside a transparent cylinder (50 cm) filled with water at 25 °C for 5 min, to measure the time spent in immobility and the latency to immobility. Experiments were recorded with a camera installed on the top of the cylinder and the videos analyzed blindly on the software 'Observador' (University of Athens, Medical School, Department of Pharmacology).

Estrous cycle analysis

Smears were obtained through vaginal cytology collected at the end of behavioral assessment and stained using the haematoxylin-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 the same proportion) and diestrus (leukocytes).[41] Images were acquired with a light microscope Leica DM 4000B (Leica, Wetzlar, Germany) with a ×10 objective lens (Plan 63x/0.25 PH1).

Corticosterone determination

Blood samples from adult (PND 90) rats were collected (puncture of the tail vein) at 0800 hours (diurnal nadir) and 1100 hours (diurnal zenith) in the day preceding death and processed to isolate serum, where corticosterone (CORT) levels were measured using the Corticosterone ELISA Kit (Abcam, Cambridge, UK), according to the manufacturer's instructions.

Immunohistochemistry and 3D morphometric analysis of microglia

Animals were transcardially perfused with saline and 4% paraformaldehyde (PFA), brains were fixed in 4% PFA, transferred to a solution of 30% sucrose in PBS and stored at −80 °C until further processing. PFC criosections (50 μm; interaural 12.20 mm and bregma 3.20 mm; Paxinos and Watson, 1998) were blocked/permeabilized (5% BSA, 0.1% Triton X-100, 2 h, room temperature, RT) and incubated with the primary antibody (rabbit anti-Iba-1, 1:1000, 48 h at 4 °C, WAKO, Osaka, Japan); after washing, sections were incubated with the secondary antibody (donkey anti-rabbit, 1:1000, 2 h, RT; Invitrogen, Waltham, MA, USA) and with 4',6-diamidino-2-phenylindole (DAPI, 1:5000, 10 min, RT). Sections were mounted on gelatinized slides using DAKO glycergel mounting medium. Images of 10–15 random microglial cells per condition per animal were acquired with a laser scanning confocal microscope LSM 710 META connected to ZEN software (Zeiss, Oberkochen, Germany) using a ×63 objective.

After importing *Z*-stacks to the Neurolucida software (MBF Bioscience, Williston, VT, USA), microglia were manually drawn along acquired planes, granting a 3D image of each cell. Morphometric data (number and length of cellular processes and Sholl analysis) were then extracted by the Neurolucida Explorer software (MBF Bioscience).

Western blot

PFC extracts from PND 1, 7 and 90 were digested in RIPA (radioimmunoprecipitation) assay buffer supplemented with complete miniprotease inhibitor cocktail tablets and phosphatase inhibitors (Roche, Penzberg, Germany) and homogenized using a tissue grinder. Homogenates were centrifuged at 16 100 *g* for 10 min at 4 °C for supernatant collection. The bicinchoninic acid (BCA) protein assay was used to determine the concentration of total protein in each sample (Thermo-Scientific, Waltham, MA, USA). After sample denaturation (70 °C, 5 min) an electrophoresis was performed using a 10% resolving gel (120 V, 60 min, RT). Proteins were then electrotransferred (1 A for 120 min at 4 °C) to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked in a solution of 5% low-fat dry milk in Tris-buffered saline with Tween 20 (TBS-T, pH 7.6, 1 h at RT; Merck, Darmstadt, Germany) before overnight incubation (4 °C) with the primary antibodies (mouse anti-$A_{2A}R$, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA; rabbit anti-GR, 1:1000; Santa Cruz Biotechnology) and 1 h at RT with secondary antibodies (goat anti-mouse, 1:5000; BioRad; goat anti-rabbit, 1:10 000; GE Healthcare, Little Chalfont, UK) prepared in 1% low-fat dry milk diluted in TBS-T. Membranes were washed and incubated up to 5 min with enhanced chemofluorescent (ECF, GE Healthcare) or enhanced chemiluminescence (ECL, Advantsa, Menlo Park, CA, USA). Detection was done on Thyphoon FLA 9000, GE Healthcare (for ECF) and ImageQuantTM LAS 500, GE Healthcare (for ECL). All membranes were re-probed for glyceraldehyde 3-phosphate dehydrogenase as a loading control.

Microglial cell culture and pharmacological treatment

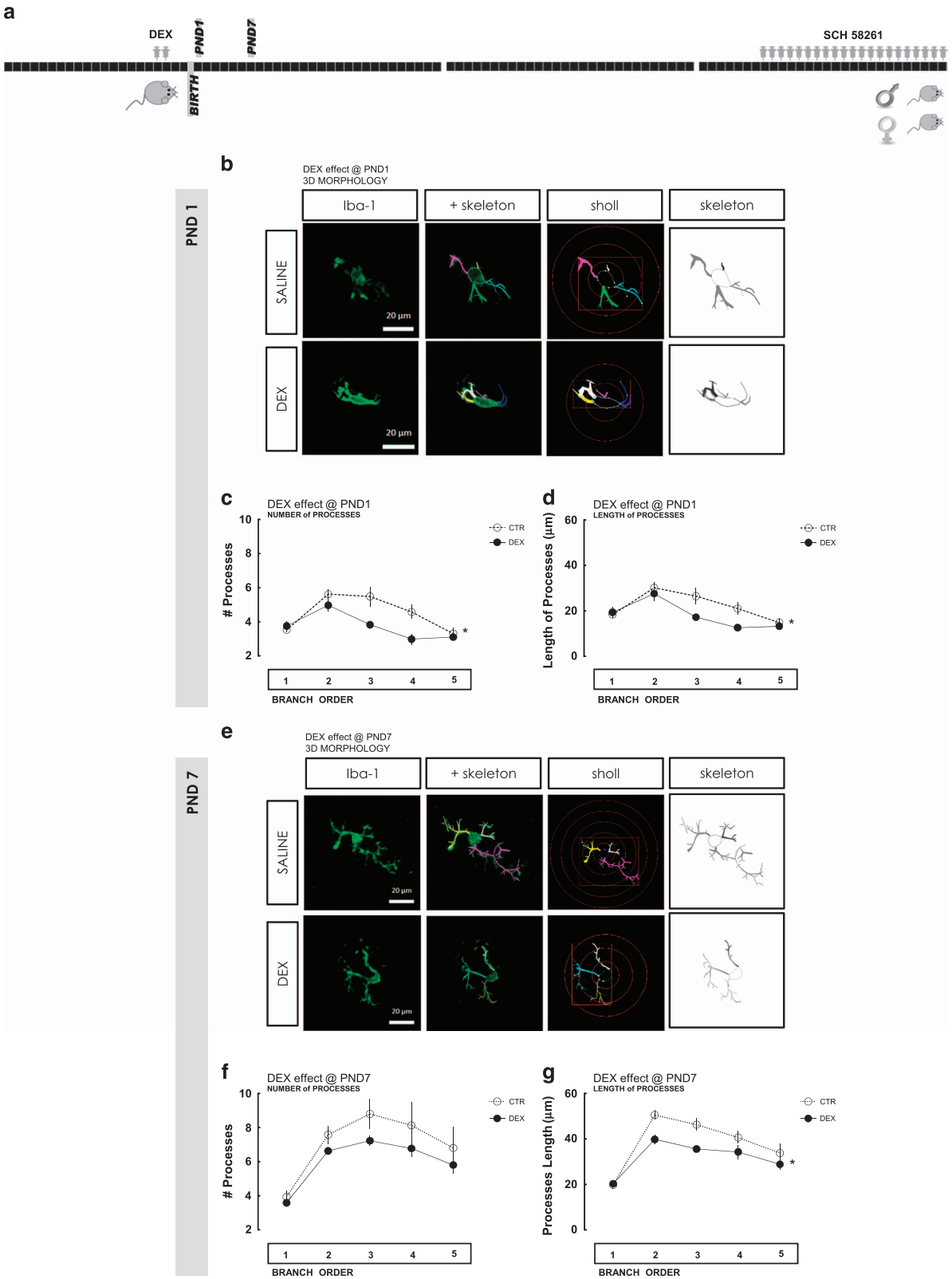
The murine microglial cell line, N9 (a kind gift from professor Claudia Verderio), was grown in Roswell Park Memorial Institute medium (RPMI, Gibco, Invitrogen) supplemented with 5% heat inactivated fetal bovine serum (Gibco), 1% penicillin-streptomycin (Gibco), 23.8 mM sodium bicarbonate and 30 mM glucose, pH 7.2. All reagents were from Sigma (Sintra, Portugal), unless otherwise stated. Cells were maintained at 37 °C under a humidified atmosphere containing 5% CO_2 and 95% O_2. After trypsinization, cells were plated for immunocytochemistry (24-well plates; 1×10^4 cells/well) and Western blot (six-well plates at 2.5×10^5 cells/well).

To evaluate the effect of DEX on microglia $A_{2A}R$ and GR density, cells were exposed to different concentrations (0.1, 1, 10 μM) of DEX (Acros Organics, Geel, Belgium) for different time periods (3, 6, 24 and 48 h).

Western blot and immunocytochemistry

For Western blot analysis, N9 cells were lysed and further processed in the same conditions described for PFC extracts.

For immunocytochemistry, cells were fixed with 4% PFA (20 min, RT; Sigma) and then washed with PBS, blocked/permeabilized by incubation with a solution of 5% BSA and 0.1% Triton X-100 in MilliQ H_2O (2 h, RT with mild agitation). Incubation with primary antibodies, rabbit anti-Iba-1 (1:1000; WAKO) or goat anti-$A_{2A}R$ (1:200; Santa Cruz Biotechnology) took place overnight at 4 °C and for 2 h at RT with the secondary donkey anti-rabbit (1:1000; Invitrogen) and donkey anti-goat (1:1000; Invitrogen) antibodies. After washing with PBS, DAPI (1:5000 in MilliQ H_2O; Invitrogen) was incubated for 10 min at RT to stain the nuclei. Following washes,



coverslips were mounted on slides using DAKO glycergel mounting medium (DAKO, Glostrup, Denmark). Images of microglial cells were randomly obtained with a laser scanning confocal microscope LSM 710 META connected to ZEN software by a 63× objective lens (oil immersed, Plan-Apochromat 63×/1.40 Oil DIC M27).

Data analysis

The statistical analysis was carried out in GraphPad Prism. Values are presented as mean ± s.e.m. Variance was similar between the experimental groups statistically compared. Comparison between two independent means was done by a Student's *t*-test. To assess differences between four groups, a one-way analysis of variance was used, followed by a Tukey's *post hoc* test, to compare all groups to each other. For the Sholl analysis of reconstructed microglial cells, a repeated measures analysis was performed. Differences were considered significant at $P < 0.05$. Statistical analysis was performed using absolute values, that is, before normalization procedures.

RESULTS

Prenatal administration of DEX induces short-term changes in microglia morphology in the prefrontal cortex

Quantitative assessment of microglia morphology (3D morphometry) in the PFC of animals prenatally exposed to DEX (Figure 1a) was performed at PND 1 and 7, periods of morphological differentiation of microglia. The main difference observed between PND 1 and 7 in animals prenatally treated with saline (control animals) was an increase in the number and length of cellular processes above the first order (not directly emerging from the cell soma) at PND 7 (Figure 1b–d versus e–g). Prenatal DEX triggered short-term rearrangements of microglia morphology, characterized by a general decrease in the number and length of processes, that is evident at PND1 (Figure 1b–d and Supplementary Information (Supplementary Table 1 and Supplementary Figure 1A), and still observed at PND7 (Figure 1e–g and Supplementary Table 2 and Supplementary Figure 1B). These differences were only observed in processes from the second order or above.

Prenatal administration of DEX induces long-term gender-specific changes in microglia morphology in the prefrontal cortex

We also performed a detailed morphometric study of microglia in adult male and female rats (PND 90), to clarify if DEX-induced effects in the first postnatal week were transient or could persist until adulthood. We observed that DEX treatment resulted in long-lasting changes in microglia morphology, but these alterations are oppositely regulated in females and males. Females that were exposed to DEX exhibited a general decrease (Figure 2a–c, filled versus open circles and Supplementary Table 3 and Supplementary Figure 2), whereas males displayed an increase (Figure 2d–f, filled versus open circles and Supplementary Table 4 and Supplementary Figure 3) in the number and length of microglia processes.

A_{2A}R blockade differentially affects microglia morphology in females and males

Since A_{2A}R pharmacological blockade controls microglial processes,[25] we administered the selective A_{2A}R antagonist, SCH58261 (0.1 mg/kg/day intraperiatoneal) for 21 consecutive days before PND 90 (Figure 1a) and evaluated microglia morphology. A_{2A}R blockade was unable to normalize DEX-induced re-organization of female microglial cells (Figure 3a–c and Supplementary Table 3 and Supplementary Figure 2), but induced a mild de-ramification of male microglial cells, being effective in partially recovering the morphology of male microglia (Figure 3d–f and Supplementary Table 4 and Supplementary Figure 3). Importantly, the blockade of A_{2A}R *per se*, that is, in animals that were not exposed to DEX, caused a significant reduction in the number and length of microglial processes in females (Figure 3a–c and Supplementary Table 3 and Supplementary Figure 2), while A_{2A}R blockade did not modify microglial cells in males (Figure 3d–f and Supplementary Table 4 and Supplementary Figure 3).

A_{2A}R chronic blockade exerts gender-specific anxiolytic effects

The sequence of the behavioral tests performed is schematically represented in Figure 4a. Confirming previous reports,[4,40] in the EPM test, rats prenatally exposed to DEX displayed anxious-related behavior, as gauged by the reduced time spent in the open arms in both genders (ratio of time in open arms/ total time in control females: 0.4 ± 0.03, $n = 6$; $P < 0.05$ vs DEX females: 0.2 ± 0.04, $n = 8$; Figure 4b; control males: 0.2 ± 0.04, $n = 6$; $P < 0.05$ vs DEX males: 0.05 ± 0.04, $n = 6$; Figure 4d).

To test the ability of A_{2A}R to ameliorate DEX-induced anxious behavior, males and females were treated with the A_{2A}R selective antagonist, SCH58261, administered for 21 consecutive days (0.1 mg kg^{-1} day^{-1}) before PND 90. The hyperanxious behavior was not reverted in DEX females chronically treated with the A_{2A}R selective antagonist (SCH+DEX: 0.2 ± 0.03, $n = 12$; $P > 0.05$ vs DEX: 0.2 ± 0.2, $n = 8$; Figure 4b), although A_{2A}R blockade reverted the anxious behavior in males (SCH+DEX: 0.2 ± 0.05, $n = 4$; $P < 0.05$ vs DEX: 0.05 ± 0.04, $n = 4$; Figure 4d). Notably, SCH58261 *per se* was anxiogenic in females (SCH: 0.2 ± 0.03, $n = 12$, $P < 0.05$ vs CTR: 0.4 ± 0.03, $n = 6$; Figure 4b), but not in males (SCH: 0.2 ± 0.06, $n = 4$; $P > 0.05$ vs CTR: 0.2 ± 0.04, $n = 4$; Figure 4d).

Neither females (Supplementary Figure 4A, C) nor males (Supplementary Figure 4E, F) prenatally exposed to DEX presented alterations in the learned helplessness behavior, evaluated as the immobility time and the latency to immobility in the FST. All the animals were tested in the OF, which excluded effects of the treatments on locomotor activity (Supplementary Figure 5A, B, D, E). Additionally, treatment with prenatal DEX, with SCH58261 in adulthood or with both did not affect progressive weight gain (Supplementary Figure 5C, F). The stage of the reproductive cycle was also evaluated in females to verify if hormonal oscillations could bias the behavioral performance: no correlation was found in the EPM (Figure 4c) or in the FST (Supplementary Figure 4B, D). As previously described in the literature,[39] our data do not show an influence of the estrous cycle on the morphology of microglial cells.

Prenatal DEX and A_{2A}R chronic blockade alter the endogenous levels of corticosterone in a gender-specific manner

We also measured endogenous levels of serum corticosterone (CORT). Control animals exhibit the expected oscillation in nadir and zenith diurnal levels of CORT,[42] which were elevated at 8 pm

Figure 1. Prenatal administration of DEX induces short-term changes in microglia morphology in the prefrontal cortex. (**a**) Schematic drawing of the animal model and pharmacological treatment: pregnant female Wistar rats were injected with DEX (1 mg kg^{-1} day^{-1}, sc) or saline on days 18 and 19 of gestation. Animals from the offspring were chronically treated with SCH58261 (0.1 mg kg^{-1} day^{-1}, ip) or saline for 21 consecutive days before PND 90. Analysis was performed at PND 1 and 7. (**b–d**, **e–g**) Microglial morphometry assessed through manual reconstruction of Iba-1 stained microglia (green) from animals at PND 1 (**b–d**) and PND 7 (**e–g**) in the Neurolucida software. (**b**, **e**) Iba-1 immunoreactivity (Iba-1), stained microglia merged with manual reconstruction (+ cytoskeleton); Sholl analysis representation (Sholl) and isolated manual reconstruction (skeleton) at PND 1 (**b**) and PND 7 (**e**). (**c**, **d**, **f**, **g**) Number and length of microglial processes resulting from the morphometric analysis of reconstructed cells from the PFC, compared between DEX- or saline-treated animals at PND 1 (**c**, **d**) and PDN 7 (**f**, **g**), according to branch order. Results are presented as the mean ± s.e.m. of 4–5 animals; $^*P < 0.05$, comparing with control (for individual branch orders), calculated using an unpaired Student's *t*-test. DEX, dexamethasone; PFC, prefrontal cortex; PND, postnatal day.

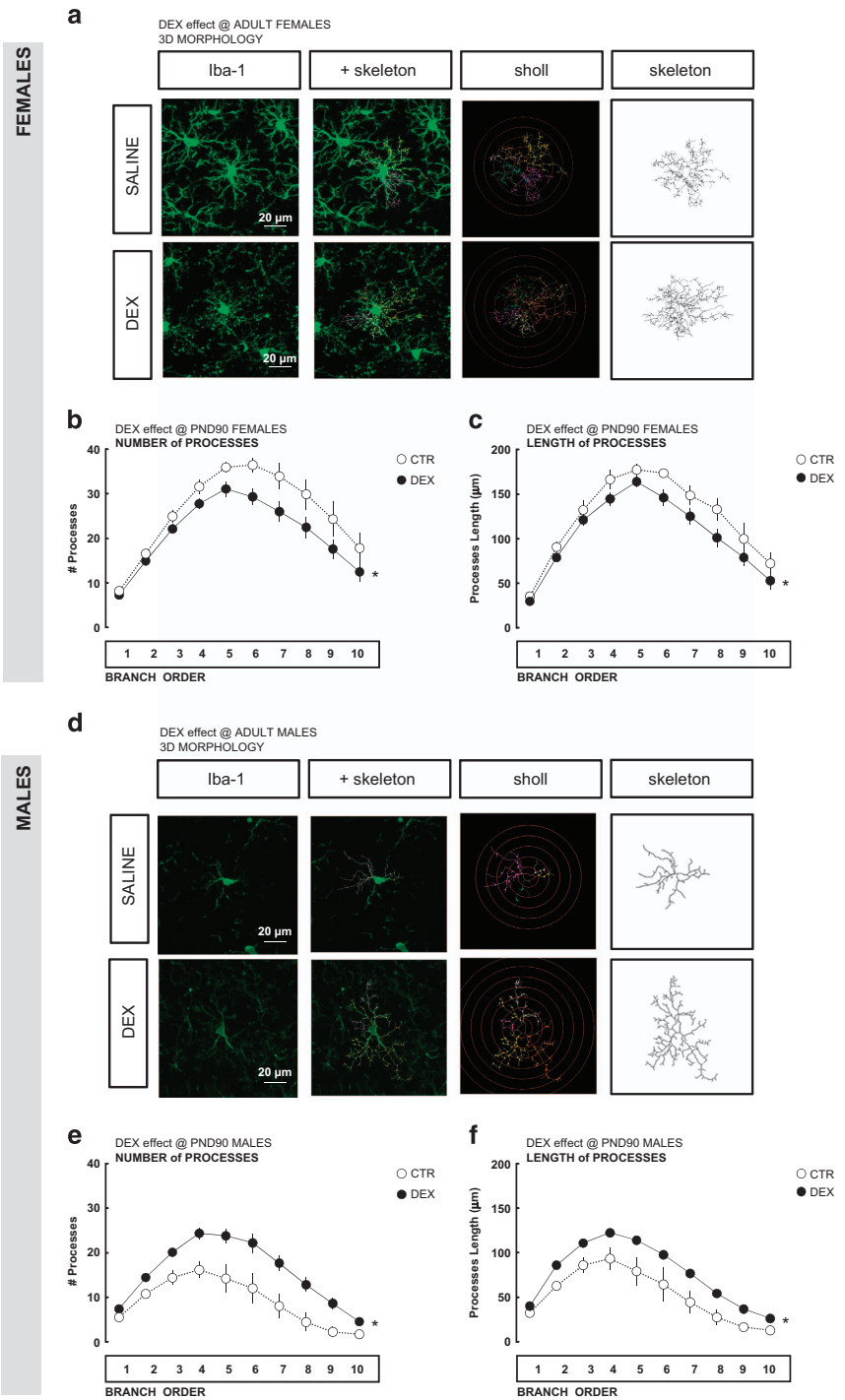


Figure 2. Prenatal administration of DEX induces long-term changes in microglia morphology in the PFC of males and females. Animals from the offspring of saline- or DEX-treated pregnant rats were chronically treated with SCH58261 (0.1 mg kg^{-1} day^{-1}, ip) or saline for 21 consecutive days before PND 90. (**a, d**) Microglial morphometric structure was manually reconstructed in the Neurolucida software based on 3D images of Iba-1 stained microglia (green). Representative images of Iba-1 stained microglia include: Iba-1 immunoreactivity (Iba-1), Iba-1 stained microglia merged with manual reconstruction (+ skeleton); Sholl analysis representation (Sholl) and isolated manual reconstruction (skeleton) of microglia from females (**a**) and males (**d**). (**b, c, e, f**) Number and length of microglial processes resulting from the morphometric analysis of reconstructed cells from the PFC at PND 90 of females (**b, c**) and males (**e, f**), compared between treatments according to branch order. Results are presented as the mean ± s.e.m. of 4–6 animals; *$P < 0.05$, comparing with control, calculated using an unpaired Student's *t*-test. 3D, three-dimensional; DEX, dexamethasone; PFC, prefrontal cortex; PND, postnatal day.

comparing with levels measured at 8 am (data not shown). In females, prenatal DEX decreased serum CORT levels at 0800 hours (Figure 5a: 82.5 ± 6.6% of control, $n = 8$, $P < 0.05$). $A_{2A}R$ blockade *per se* did not alter CORT levels (Figure 5a: 99.5 ± 7.4% of control, $n = 12$, $P > 0.05$) and was not able to revert DEX-induced changes in CORT levels (Figure 5a: SCH+DEX: 77.1 ± 6% of control, $n = 10$, $P > 0.05$). Male CORT levels were not altered in any condition tested (Figure 5b).

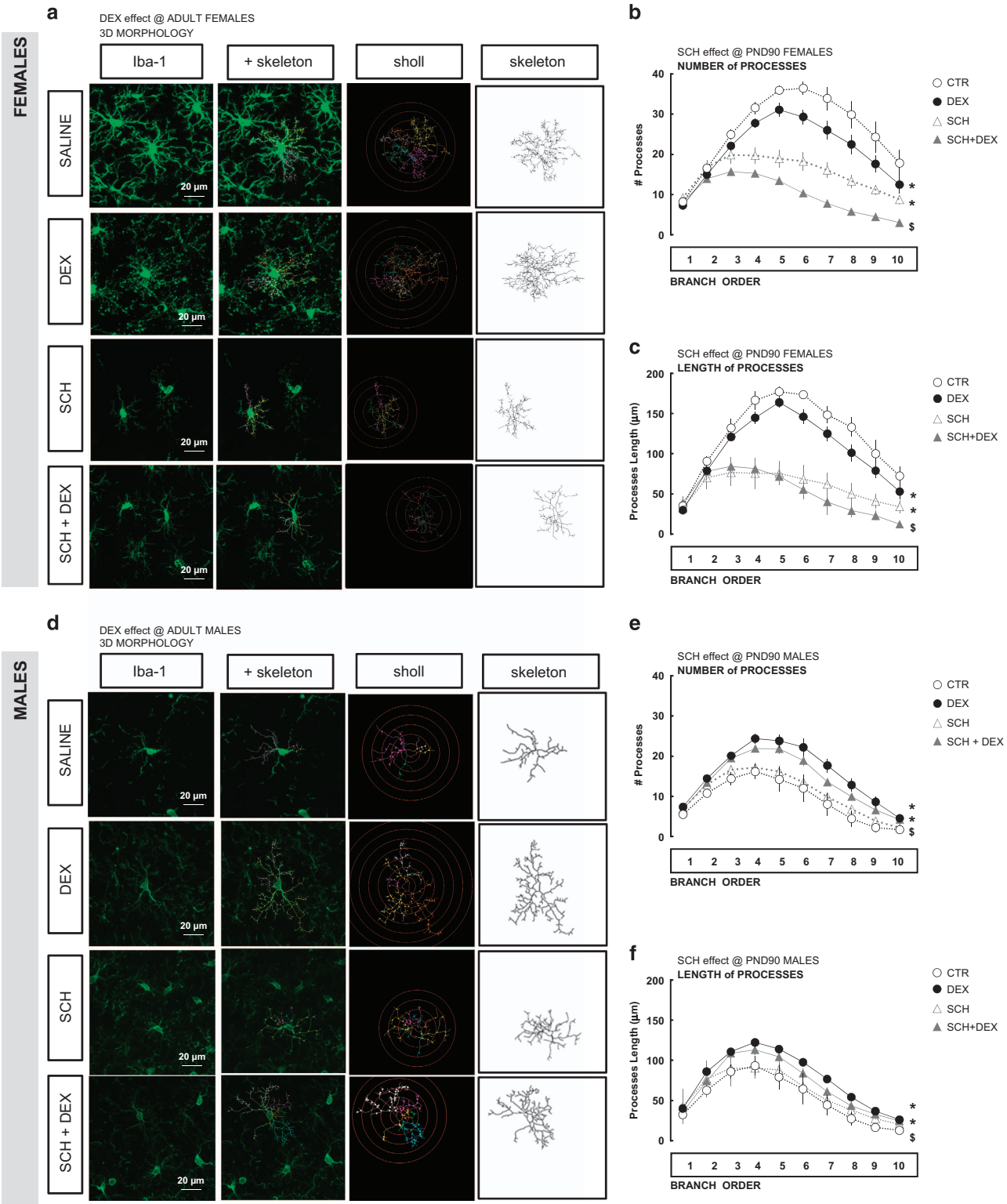


Figure 3. A_{2A}R blockade differentially affects microglia morphology in females and males. Animals from the offspring of saline- or DEX-treated pregnant rats were chronically treated with SCH58261 (0.1 mg kg $^{-1}$day^{-1}, ip) or saline for 21 consecutive days before PND 90. (**a**, **d**) Microglial morphometric structure was manually reconstructed in the Neurolucida software based on 3D images of Iba-1 stained microglia (green). Representative images of Iba-1 stained microglia include: Iba-1 immunoreactivity (Iba-1), stained microglia merged with manual reconstruction (+ cytoskeleton); Sholl analysis representation (Sholl) and isolated manual reconstruction (skeleton) of microglia from females (**a**) and males (**d**). (**b, c, e, f**) Number and length of microglial processes resulting from the morphometric analysis of reconstructed cells from the PFC at PND 90, compared between treatments, according to branch order. Results are presented as the mean ± s.e.m. of 4–7 animals; $^{*}P < 0.05$, comparing with control, $^{\$}P < 0.05$, comparing with DEX, calculated using a one-way ANOVA followed by a Tukey's multiple comparisons test. 3D, three-dimensional; ANOVA, analysis of variance; DEX, dexamethasone; PFC, prefrontal cortex; PND, postnatal day.

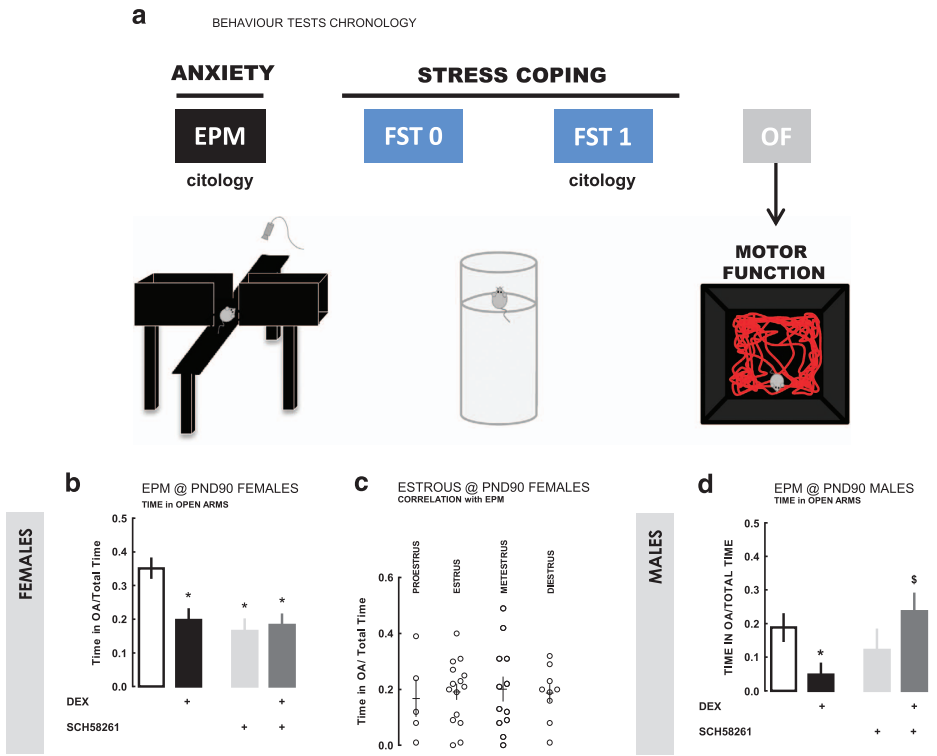


Figure 4. A_{2A}R chronic blockade exerts gender-specific anxiolytic effects. Pregnant female Wistar rats were injected with DEX (1 mg kg^{-1} day^{-1}, sc) or saline on days 18 and 19 of gestation. Animals from the offspring were chronically treated with SCH58261 or saline (0.1 mg $kg^{-1}$$day^{-1}$ ip) for 21 consecutive days before PND 90. (**a**) Chronology of the behavioral tests performed. (**b**, **d**) Time spent in open arms per total time of the EPM test, performed to evaluate anxiety-related behavior of females (**b**) and males (**d**). (**c**) Females estrous cycle determined in the end of EPM experiments, to evaluate its impact on anxiety. DEX, dexamethasone; EPM, elevated plus maze; FST, forced swim test; OF, open field; PND, postnatal day.

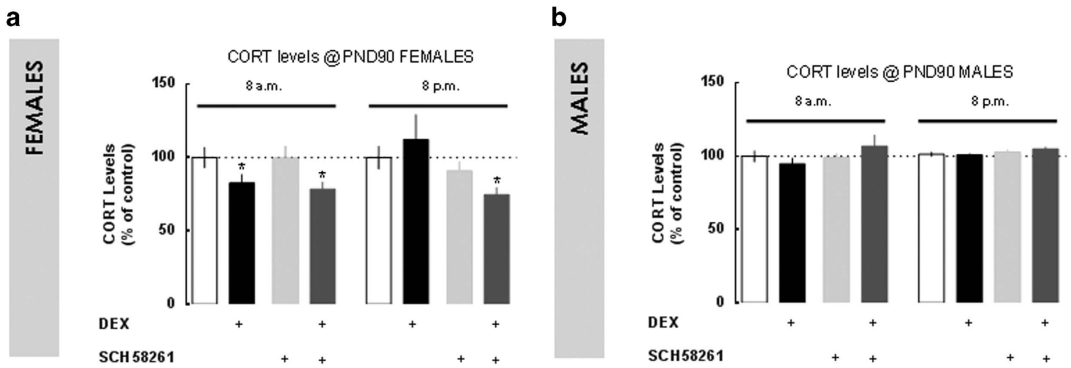


Figure 5. Prenatal DEX and A_{2A}R chronic blockade alter the endogenous levels of corticosterone in a gender-specific manner. (**a, b**) Corticosterone (CORT) serum levels measured at 0800 and 2000 hours (diurnal peaks of production) by ELISA, to assess the endogenous levels of corticosteroids of females (**a**) and males (**b**). Results are presented as the mean ± s.e.m. of 8-12 animals and as a % of control levels of CORT at 8:00 am (females: 200.9 ± 1.5 ng ml^{-1}, $n = 12$; males: 58.4 ± 0.1 ng ml^{-1}, $n = 8$) and at 8:00 pm (females: 227.5 ± 1.8 ng ml^{-1}, $n = 12$; males: 66.7 ± 0.2 ng $ml,^{-1}$ $n = 8$); *$P < 0.05$, comparing with control, calculated using a one-way ANOVA followed by a Tukey's multiple comparisons test. ANOVA, analysis of variance; DEX, dexamethasone; ELISA, enzyme-linked immunosorbent assay; PND, postnatal day.

DISCUSSION

We report that prenatal exposure to DEX triggered a remodeling of microglial cell processes in the PFC in a gender-specific and long-lasting manner. Sexual dimorphism was translated into a gender-selective resistance to a therapeutic intervention, since chronic treatment with a selective A_{2A}R antagonist normalized microglial processes and anxiety behavior in DEX-treated males, but not in females.

We have previously characterized the model of prenatal DEX as a trigger of anxious behavior in critical phases of brain development.[4,40] In those previous studies, we used male rats and detailed the critical role of the PFC in mounting anxiety responses (see also refs 28,29,43), although other limbic structures such as the amygdala, bed nucleus of stria terminalis and hippocampus were also involved.[4,40] The present characterization of the gender-dependent effect of prenatal DEX on microglial cells and the gender-dependent resistance to a drug that selectively

blocks A_{2A}R constitutes one step ahead. Since there is increasing evidence supporting a role of glial cells in the etiology of neuropsychiatric disorders (reviewed in refs 26,44) and microglia undergo morphological alterations in the PFC upon chronic stress,[23] we now performed a detailed morphometric analysis of microglia in the PFC of DEX pups and young adult rats.

We report that prenatal DEX significantly altered the morphology of microglial cells, namely the fine structure of microglia processes, that play a critical role on the formation and homeostasis of synaptic contacts.[20,45,46] Prenatal DEX promoted a hyper-ramified state in males, which has been described as characteristic of stress-related conditions.[23,24] Notably, these alterations were selectively observed in males, since DEX-treated females displayed microglia with less and shorter processes. This probably results from the different morphology of microglia in the brains of males and females, typified by a higher basal complexity of microglial cells in females (higher number and longer processes) than in males (reviewed in refs 39,47). Microglia morphological differences between genders, which are driven by steroid hormones,[47] become apparent early in brain development, are dependent on the brain region analyzed[39] and impact on dendritic spine density,[48] gene expression of immune factors[39] and behavior.[48,49]

Recent studies have implicated a role of A_{2A}R in the control of stress-induced mood-related alterations, such as anxiety or depression.[34,35,37,50,51] Additionally, we and others have unravelled the ability of A_{2A}R to control microglia dynamics,[25,32] proliferation[30,31] and participation in neuroinflammatory responses.[52,53] This led us to test the impact of DEX on the density of A_{2A}R and the therapeutic potential of blocking A_{2A}R.

The therapeutic administration of a selective A_{2A}R antagonist (SCH58261) in a regimen described to ameliorate anxiety in rodents,[37] showed that A_{2A}R blockade did not change prenatal DEX-induced atrophy of microglia in adult females, while it decreased microglia hyper-ramification observed in males prenatally exposed to DEX. Coincidently, A_{2A}R blockade did not exert an anxiolytic effect in females, while ameliorating anxiety in males.

We performed *in vitro* studies using a microglia cell line, and observed that the exposure to DEX alters the density of GR and, subsequently, the density of A_{2A}R (Supplementary Figure 6), which control microglia morphology.[25] On the basis of these *in vitro* results, we propose that morphological alterations of microglia may be triggered by DEX-induced changes in A_{2A}R density. This hypothesis is also based on preliminary data obtained using the same *in vitro* model in an attempt to analyze microglia morphology in the presence of DEX and its regulation by A_{2A}R: DEX tended to increase the ratio ramified:ameboid microglia in the culture and this effect was prevented by blocking A_{2A}R (data not shown). In a complementary set of experiments, using our animal model, we observed that prenatal DEX caused opposite tendencies for alterations of A_{2A}R in the PFC of adult males and females: decreased A_{2A}R levels in females (Supplementary Figure 7A), paralleled by an atrophy of these cells, and higher levels of A_{2A}R in males (Supplementary Figure 7B), paralleled by the hyper-ramification of microglia. The observed decrease of A_{2A}R density in the PFC of females prenatally exposed to DEX is one possible explanation for the lack of efficacy of A_{2A}R blockade as anxiolytic and as a normalizer of microglia morphology in females. Considering these results, we anticipate a gender-specific regulation of adenosine A_{2A}R under the control of DEX. This would be in line with the recent observations by Mitrović *et al*.,[54] showing that steroid sexual hormones regulate one of the enzymes responsible for the formation of adenosine, the main ligand of A_{2A}R.

Considering that the sensor ability of microglial cells is highly dependent on the dynamics of cellular processes, basal and DEX-induced differences in microglia morphology between genders would anticipate functional consequences associated with sexual dimorphism. Intriguingly, DEX-induced gender-specific changes in A_{2A}R density in the PFC paralleled gender-specific alterations of microglia, but both males and females prenatally exposed to DEX displayed anxious-like behavior. The behavioral impact of microglia morphological differences between genders was only observed when we pharmacologically blocked A_{2A}R. These prompt the need to test if microglial cells from males and females react differently to stimuli known to interfere with A_{2A}R density in the brain, such as chronic stress[37] and if a correlation exists with the behavior. In particular, it would be important to clarify if DEX-induced dimorphic morphological differences in this model of chronic anxiety are associated with differential susceptibility to other psychiatric disorders, namely depression.

CONCLUSIONS

The major finding emerging from our study is that the exposure to the GC analog DEX during early development impacts on microglia differentiation in a gender-specific manner. There were gender-specific differences in microglia morphology in young adult animals, which displayed an anxious phenotype. We identified A_{2A}R as a possible candidate mediating the genesis of anxiety and showed that its pharmacological manipulation was effective in reverting anxiety in males, but not in females. Many psychiatric diseases have a gender difference in etiology and clinical presentation, and these disorders are often associated with alterations of the immune system. Considering the critical role of microglia in brain development and immunity, it is surprising that very few studies address gender differences in microglial cells in different phases of neurodevelopment.

Conceptually, this work approaches three relevant questions: the long-term effect of the prenatal exposure to GCs, the developmental genesis of a differential susceptibility to psychiatric conditions and drug efficacy between genders.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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