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RESEARCH ARTICLE

Region-specific control of microglia by adenosine A_{2A} receptors: uncoupling anxiety and associated cognitive deficits in female rats

Joana Mendes Duarte[1,2] | Rita Gaspar[1,2] | Liliana Caetano[1,2,3] | Patrícia Patrício[4,5] | Carina Soares-Cunha[4,5] | António Mateus-Pinheiro[4,5] | Nuno Dinis Alves[4,5] | Ana Rita Santos[4,5] | Samira G Ferreira[3] | Vanessa Sardinha[4,5] | João Filipe Oliveira[4,5] | Carlos Fontes-Ribeiro[6] | Nuno Sousa[4,5] | Rodrigo A. Cunha[2,3] | António F. Ambrósio[1,2] | Luísa Pinto[4,5] | Ana João Rodrigues[4,5] | Catarina A. Gomes[1,2,6]

[1]Coimbra Institute for Clinical and Biomedical Research (iCBR), Faculty of Medicine, University of Coimbra, Portugal

[2]Center for Innovation in Biomedicine and Biotechnology (CIBB), University of Coimbra, Portugal

[3]CNC - Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

[4]Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal

[5]ICVS/3B's –PT Government Associate Laboratory, Braga/Guimarães, Portugal

[6]Institute of Pharmacology and Experimental Therapeutics, Faculty of Medicine, University of Coimbra, Coimbra, Portugal

Correspondence: Catarina Gomes, Institute of Pharmacology and Experimental Therapeutics, Faculty of Medicine, University of Coimbra Azinhaga de Santa Comba – Celas, Coimbra 3000-354, Portugal.
Email: catarina.gomes@fmed.uc.pt

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Abstract

Epidemiologic studies have provided compelling evidence that prenatal stress, through excessive maternal glucocorticoids exposure, is associated with psychiatric disorders later in life. We have recently reported that anxiety associated with prenatal exposure to dexamethasone (DEX, a synthetic glucocorticoid) correlates with a gender-specific remodeling of microglia in the medial prefrontal cortex (mPFC), a core brain region in anxiety-related disorders. Gender differences in microglia morphology, the higher prevalence of anxiety in women and the negative impact of anxiety in cognition, led us to specifically evaluate cognitive behavior and associated circuits (namely mPFC-dorsal hippocampus, dHIP), as well as microglia morphology in female rats prenatally exposed to dexamethasone (*in utero* DEX, iuDEX). We report that iuDEX impaired recognition memory and deteriorated neuronal synchronization between mPFC and dHIP. These functional deficits are paralleled by microglia hyper-ramification in the dHIP and decreased ramification in the mPFC, showing a heterogeneous remodeling of microglia morphology, both postnatally and at adulthood in different brain regions, that differently affect mood and cognition. The chronic blockade of adenosine A_{2A} receptors ($A_{2A}R$), which are core regulators of microglia morphology and physiology, ameliorated the cognitive deficits, but not the anxiety-like behavior. Notably, $A_{2A}R$ blockade rectified both microglia morphology in the dHIP and the lack of mPFC-dHIP synchronization, further heralding their role in cognitive function.

KEYWORDS

A_{2A} receptors, anxiety, cognition, gender dimorphism, microglia morphology

1 | INTRODUCTION

Exposure to high levels of glucocorticoids (GCs) during neurodevelopment is relatively common, occurring in maternal stress situations and GC-based therapies during pregnancy. During brain development,

high levels of GC have been associated with psychiatric disorders and behavior alterations, later in life (Drozdowicz & Bostwick, 2014). The impact of GC on brain wiring and function, affecting neuronal migration (Fukumoto et al., 2009), neuronal morphology (Leao et al., 2007; Oliveira et al., 2012; Sousa & Almeida 2002; Sousa et al., 1998), and spine density (Oliveira et al., 2012; Rodrigues et al., 2012; Tanokashira et al., 2012) in several brain regions, has been causally associated with their long-term consequences in terms of behavior (Li et al., 2014; Oliveira et al., 2006; Oliveira et al., 2012; Rodrigues et al., 2012) and predisposition to brain disorders (Drozdowicz & Bostwick, 2014).

Besides neurons, GC also affect microglia during development (Caetano et al., 2017). These immune cells colonize the brain early in development (Ginhoux et al., 2010; Ginhoux et al., 2013) and indirectly sculpt neuronal circuits by controlling synapse formation (Cristovao et al., 2014; Lim et al., 2013; Miyamoto et al., 2016; Parkhurst et al., 2013), maturation (Ji et al., 2013) and elimination (Paolicelli et al., 2011; Schafer et al., 2012). Microglia express functional glucocorticoid receptors (GRs) (Sierra et al., 2008) and their morphology is altered by the prenatal exposure to GC, an effect that persists until adulthood (Caetano et al., 2017). In a developmental model of anxiety (prenatal exposure to GC), microglia undergo a complex process of morphologic plasticity in the medial prefrontal cortex (mPFC), that is different between males and females (Caetano et al., 2017). This dimorphic effect is likely related with the function of adenosine A_{2A} receptors ($A_{2A}R$), which modulate microglia function and morphology (Caetano et al., 2017; Orr et al., 2009; Gyoneva et al., 2009; Gyoneva et al., 2014). In fact, $A_{2A}R$ blockade is anxiolytic in males (Caetano et al., 2017; Kaster et al., 2015; Wei et al., 2014) and corrects morphologic changes in microglia (Caetano et al., 2017), whereas $A_{2A}R$ blockade in females fails to counteract both the anxious-like phenotype and the morphologic changes in microglia (Caetano et al., 2017).

Since patients with anxiety disorders commonly display cognitive impairment (Kendler et al., 2017; Tanokashira et al., 2012) and $A_{2A}R$ blockade prevents cognitive deficits (Batalha et al., 2013; Cunha et al., 2008; Dall'Igna et al., 2007; Kaster et al., 2015; Li et al., 2015b; Prediger et al., 2005; Viana da Silva et al., 2016), we now investigated if $A_{2A}R$ blockade recovers cognition in females with anxiety resistant to $A_{2A}R$ antagonism. We further assessed if $A_{2A}R$ in the dorsal hippocampus (dHIP) and in the mPFC differently control microglia morphologic remodeling in these two brain regions, which process anxiety and cognitive performance.

2 | MATERIALS AND METHODS

2.1 | Animal handling and pharmacological treatment

Animals were handled according to the European Community guidelines on animal care and experimentation (2010/63/EU). The experimental protocols were approved by the Ethical committees of ICVS (Life and Health Sciences Research Institute, SECVS protocol no. 107/2015) and CNC (Center for Neuroscience and Cell Biology, Orbea 78/2013). All animals were housed under standard laboratory conditions (22 °C, light/dark cycle of 12 hr; food and water *ad libitum*). Pregnant Wistar rats were administered with DEX (sc, subcutaneous, 1mg/kg) or vehicle on gestation Days 18 and 19, as previously described in Caetano et al. (2017). Females from the offspring were treated during 21 consecutive days between postnatal day (PND) 67 and PND90 with saline + DMSO (vehicle-treated animals) or with the selective $A_{2A}R$ antagonist, SCH58261 (ip, intraperitoneal, 0.1 mg/kg/day), a dose displaying anxiolytic effects in adult male rodents subjected to stress protocols (Caetano et al., 2017; Kaster et al., 2015).

2.2 | Behavior evaluation

2.2.1 | Open field test

The locomotor behavior was evaluated in the Open field (OF) test. Each rat at PND98 was placed in the center of the arena (white floor and transparent acrylic walls, 43.2 × 43.2 cm; Med Associates Inc., St. Albans, VT) under bright white light. The locomotor activity was monitored using a two 16-beam infrared system for 5 min. Velocity and total distance travelled were analyzed using the Activity Monitor software (Med Associates, St Albans, VT).

2.2.2 | Forced swimming test

Depressive-like behavior was evaluated in the forced swimming test (FST). The test was conducted 24 h after a pre-test session (10 min), by placing the rats in glass cylinders (50 cm) filled with water (25 °C) for 5 min. This test was performed at PND92–93. Trials were video recorded and the time spent immobile and latency to immobility were analyzed blindly.

2.2.3 | Novelty suppressed feeding

Anxiety-like behavior was assessed using the novelty suppressed feeding (NSF) test. Following previously published protocols (Patricio et al., 2015), rats at PND91 were food-deprived (18 hr) before being placed in a novel arena for 10 min, where a single food pellet was centrally placed. The latency to eat was measured, being an indicator of anxiety-like behavior. Rats were then transferred to their home cage and the amount of food consumed during 10 min was measured, as an indicator of appetite drive.

2.2.4 | Elevated plus maze test

Anxiety-like behavior was additionally assessed by the elevated plus maze (EPM) test. Rats at PND 90 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 room illuminated with bright white light during 5 min. The time spent in the open arms and the number of total entries were measured with an infrared photo beam system and analyzed with a specific software (MedPCIV, MedAssociates, Georgia, VT). The level of anxiety-like behavior was measured by the ratio of time spent in the open arms/total time and the number of entries into each arm of the maze.

2.2.5 | Sweet drive test

Rats were allowed to freely explore the sweet drive test (SDT) arena, previously described in Mateus-Pinheiro et al. (2014), where regular

(Mucedola 4RF21-GLP) or sweet pellets (Cheerios, Nestlé) were accessible, for 10 min. This test was performed in tree trials at PND 82, 85, and 89. The preference for sweet pellets was calculated by the formula: sweet pellets consumed (g)/ total pellets consumed (g) (Mateus-Pinheiro et al., 2014). The decreased preference for sweet pellets was used as an indicator of anhedonic behavior.

2.2.6 | Novel object recognition test

Novel object recognition (NOR) test evaluates the ability to distinguish between a familiar and a novel object, a readout for recognition memory. This test was performed at PND94–97. Briefly, rats were placed in the arena in the presence of two identical objects in shape, color, and size, during 10 min; 2 h later, one of the objects was replaced by a different object in color and shape. Trials were video recorded and the time spent in the novel and familiar objects was measured. The recognition index was measured by the quotient: time exploring the novel object/(time exploring the familiar object + time exploring the novel object).

2.3 | Corticosterone determination

Blood samples from adult female rats were collected (puncture of the tail vein) at 8:00 a.m. (diurnal nadir) and 8:00 p.m. (diurnal zenith) in the day preceding the sacrifice and processed to isolate serum, where corticosterone (CORT) levels were measured using the CORT ELISA Kit (Abcam, Cambridge, UK), according to the manufacturer's instructions.

2.4 | Immunohistochemistry and tridimensional morphometric analysis of microglia

Rats were deeply anesthetized with an ip injection of sodium pentobarbital (60 mg/kg) and transcardially perfused with heparinized saline and 4% paraformaldehyde (PFA). Brains were fixed in 4% PFA overnight and transferred to 30% sucrose solution in phosphate buffered saline (PBS, 37 mM NaCl, 2.1 mM KCl, 1.8 mM KH2PO4 and 10 mM Na2HPO4.2H2O, pH 7.4) overnight at 4 °C. After fixation, brains were cryopreserved at −80 °C and sectioned (50 μm) in a cryostat. For immunodetection of microglia, free-floating coronal sections containing the dHIP (stereotactic coordinates of interaural 5.20 mm and bregma −3.80; Paxinos & Watson, 1998) were incubated in a permeabilization and blocking solution of 5% BSA (bovine serum albumin) and 0.1% Triton X-100 in PBS (2 hr at room temperature, RT). Incubation with the primary antibody (rabbit antiIba-1, 1:1,000, WAKO, Osaka, Japan) was performed for 48 hr in the blocking solution at 4 °C. After washing, sections were incubated with the secondary antibody (donkey antirabbit, 1:1,000, Invitrogen, Waltham, MA) for 2 hr at RT and with DAPI (1:5,000) for 10 min at RT. Sections were mounted on gelatinized slices using glycergel (Dako mounting medium). Images of 10 random microglial cells from each animal were acquired in the dorsal dentate gyrus (DG) of the hippocampus with a laser scanning confocal microscope LSM 710 META connected to ZEN Black software (Zeiss Microscopy, Oberkochen, Germany), using a 63 × objective lens (Plan-Apochromat 63×/1.40 Oil DIC M27).

Tridimensional (3D) reconstruction of microglial cells was obtained by manual reconstruction using the Neurolucida software (MBF Bioscience, Williston, VT). Morphometric data (quantification of the number and the length of cellular processes) were extracted using Neurolucida explorer.

2.5 | In vivo electrophysiology

In vivo analysis of neural activity was performed as previously described, with minor changes (Mateus-Pinheiro et al., 2017; Oliveira et al., 2013). Briefly, rats were anesthetized with 4% sevofluorane (SevoFlo, Abbott, USA) and placed in a stereotaxic frame (KOPF, USA) once they were deeply anesthetized. Concentric platinum/iridium electrodes (400 μm shaft diameter, Science Products, Germany) were implanted in the prelimbic area of the mPFC (3.3 mm anterior to bregma, 0.8-mm lateral, and 4.0 mm below bregma) and in the CA1 of dHIP (3.8-mm anterior to bregma, 2-mm lateral and 2-mm below bregma) (Figure 2a) (Paxinos & Watson, 1998). Local field potential (LFP) signals were amplified, filtered (0.1–300 Hz, LP511 Grass Amplifier, Astro-Med, Germany), acquired (Micro 1401 mkII, CED, UK) and recorded by a dedicated software (Signal Software, CED, UK).

Coherence was calculated as a measure of phase and amplitude synchronization between mPFC-dHIP. This analysis was performed on the 2-channel 100 s long LFP signals and was based on multi-taper Fourier analysis and calculated by custom-written MATLAB scripts, using the MATLAB toolbox Chronux. Coherence was calculated to reach 1s long segments and their mean was assessed for all frequencies: delta (<4 Hz), theta (4–12 Hz), beta (12–20 Hz), and low gamma (20–40 Hz).

2.6 | Data and statistics

Data are means ± *SEM*. Means were compared using the Student's *t* test, when comparing two conditions or one-way analysis of variance (ANOVA), followed by a Turkey's *post hoc* test, when comparing more than two conditions. The level of significance was set at $p < .05$.

3 | RESULTS

3.1 | Prenatal exposure to DEX leads to deficits in recognition memory in adult females

Females prenatally exposed to DEX (iuDEX, Figure 1a), as compared with vehicle-treated females (VEH), display a lower recognition index (VEH: 0.47 ± 0.06, $n = 9$; iuDEX: 0.21 ± 0.03, $n = 10$; $p < .001$; Figure 1b). Confirming previous reports, iuDEX females also present anxiety-like behavior, evaluated by two independent tests, the EPM (VEH: 0.35 ± 0.03, $n = 6$; iuDEX: 0.20 ± 0.04, $n = 7$; $p < 0.05$; Figure 1c) and the NSF (Supporting Information Figure S1A). Moreover, iuDEX females presented altered serum levels of CORT at 8 a.m. (iuDEX: 78.9 ± 5.90, $n = 7$, $p < .001$; Figure 1d). Also in line with previous results, iuDEX females did not display helplessness behavior, as assessed by the FST (Supporting Information Figure S1B) or anhedonic behavior, as evaluated by the SDT (Supporting Information Figure S1c). Moreover, iuDEX did not alter body weight (Supporting Information Figure S1d) at adulthood, nor the distance travelled or the velocity in the OF test, indicating that iuDEX does not affect global locomotor function (Supporting Information Figure S1e).

3.2 | iuDEX decreases spectral coherence between the dHIP and mPFC

Hippocampal–prefrontal connectivity is related to anxiety, spatial learning and memory-related tasks (Fell et al., 2011), which requires an adequate synchronization between these regions (Figure 2a), measured as coherence. When compared with the control animals (VEH), iuDEX leads to a significant decrease of dHIP and mPFC coherence in different frequency bands, namely: delta (<4 Hz;VEH: 0.71 ± 0.05, n = 9; iuDEX: 0.47 ± 0.08, n = 7; p < .05), theta (4–12 Hz;VEH: 0.79 ± 0.04, n = 9; iuDEX: 0.44 ± 0.07, n = 5; p < .001), beta (12–20 Hz;VEH: 0.80 ± 0.03, n = 8; iuDEX: 0.58 ± 0.04, n = 5; p < .01) and low gamma (20–40 Hz;VEH: 0.76 ± 0.03, n = 9; iuDEX: 0.60 ± 0.06, n = 6; p < 0.05) (Figure 2 b,c). In contrast, the levels of neuronal activity in the mPFC (Supporting Information Figure 2a,b) and in the dHIP (Supporting Information Figure 2c,d) were not altered by iuDEX.

3.3 | iuDEX induces short-term and long-lasting alterations in microglia morphology in the dHIP

We next evaluated the impact of iuDEX on microglia morphology in the dHIP of PND 7 and of adult female rats (PND 99), focusing in the DG, a region associated with cognition (Amaral et al., 2007; Saab et al., 2009) and particularly involved in NOR performance(Jessberger et al., 2009). As previously found by our group in the mPFC

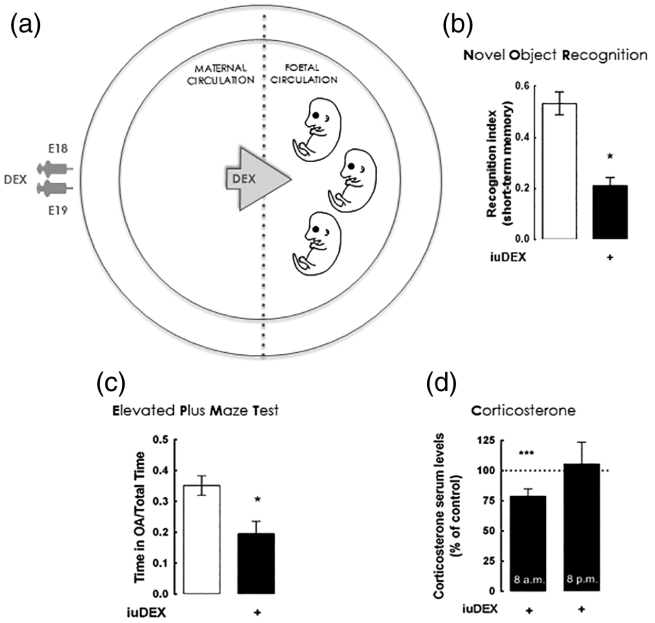

FIGURE 1 Effect of prenatal exposure to DEX (iuDEX) on cognition, anxiety and HPA axis. (a) Schematic view of the animal model and pharmacological treatment. Pregnant Wistar rats received DEX (1 mg/kg/day sc) on Days 18 and 19 of gestation. (b) The recognition index (time spent in the novel object *per* total time spent in the novel and familiar objects) was calculated to evaluate cognitive deficits, using the NOR test at PND94-97. (c) Time spent in the open arms *per* total time of the EPM test, performed to evaluate anxiety-related behavior at PND90. (d) Diurnal and nocturnal quiescent levels of CORT were measured by ELISA, to evaluate the endogenous CORT levels. Results are presented as the mean ± *SEM* (n = 6–10 animals); *p < .05 and ***p < .001, comparing with vehicle-treated animals, calculated using an unpaired Student's t test [Color figure can be viewed at wileyonlinelibrary.com]

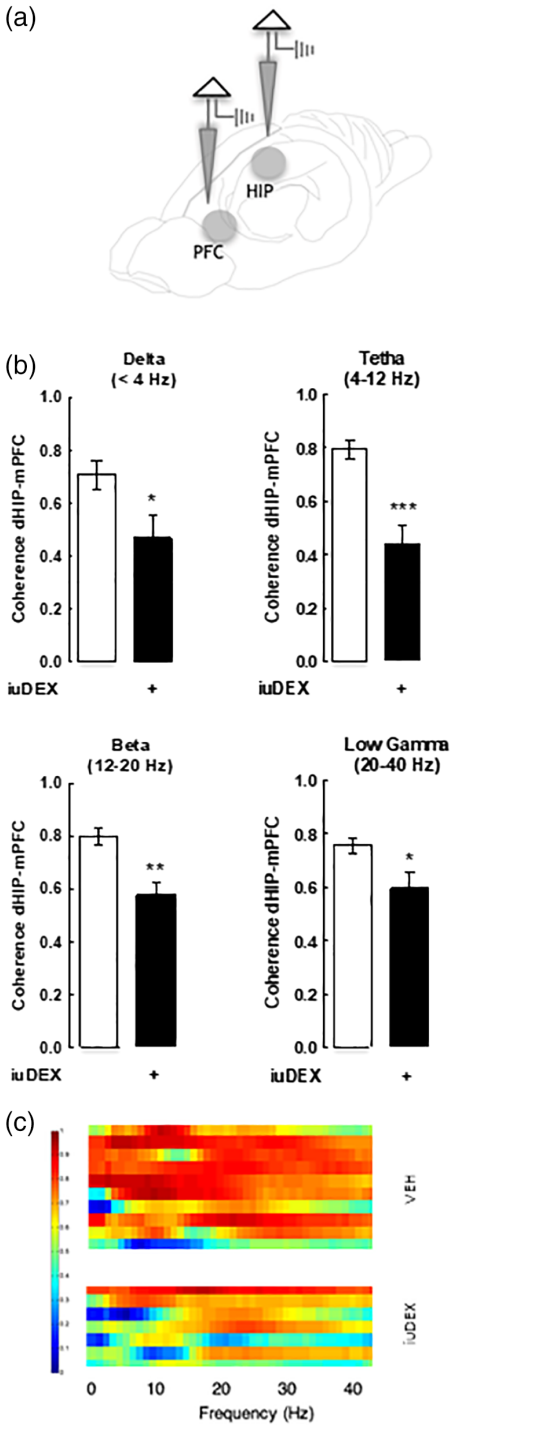

FIGURE 2 Prenatal exposure to DEX (iuDEX) decreases the coherence between the mPFC and the dHIP. Pregnant Wistar rats were administered with DEX (1 mg/kg/day, sc) at Days 18 and 19 of gestation. *In vivo* electrophysiology was performed to evaluate the coherence between mPFC and dHIP. (a) Scheme of the *in vivo* electrophysiological recordings in dHIP and mPFC. (b) Group comparison of the coherence values between mPFC and dHIP for delta (<4 Hz), theta (4–12 Hz), beta (12–20 Hz), and low gamma (20–40 Hz) frequency bands. Results are presented as the mean ± *SEM* (n = 5–10 animals); *p < .05, **p < .01, and ***p < .001, comparing with vehicle-treated animals, calculated using an unpaired Student's t test. (c) Spectrograms of mPFC-dHIP coherence. Each horizontal line represents the spectrogram of an individual rat [Color figure can be viewed at wileyonlinelibrary.com]

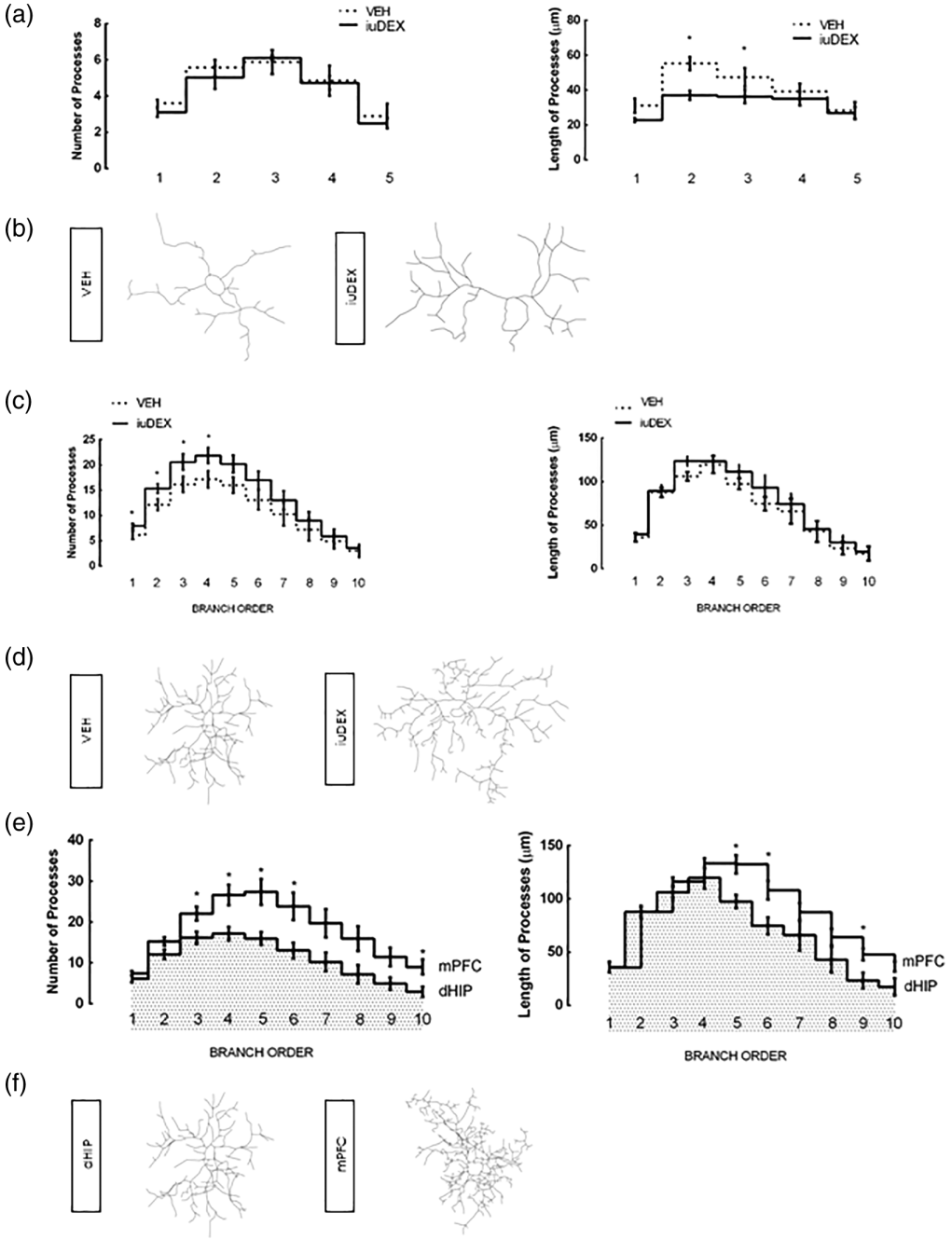

FIGURE 3 Effect of prenatal exposure to DEX (iuDEX) on the number and length of dHIP microglial processes. Pregnant Wistar rats received DEX (1 mg/kg/day, sc) at ED 18 and ED 19. Microglial cells of female brains were immunostained with Iba-1 at PND 7 and PND 99 and 3D reconstructions were performed using Neurolucida software. (a) Using the morphometric data extracted from the Neurolucida software, the number and length of microglial processes in the dHIP were assessed and compared between iuDEX-or vehicle-treated animals at PND 7. (b) Representative isolated manual reconstruction (skeleton) of microglial cells from the dHIP at PND 7 of females. (c) Number and length of microglial processes resulting from the morphometric analysis of reconstructed cells from dHIP, compared between iuDEX- or vehicle-treated animals at PND 99. (d) Representative isolated manual reconstruction (skeleton) of microglial cells from the dHIP at PND 99 of females. (e) Comparison of the number and length of microglial processes from mPFC and dHIP of control animals according to the respective branch order. (f) Representative isolated manual reconstruction (skeleton) of microglial cells from the dHIP and mPFC at PND 99 of females. Results are presented as the mean ± *SEM* (n = 3–6 animals); *p < .05, comparing with vehicle-treated animals, calculated using a using an unpaired Student's t test

(Caetano et al., 2017), iuDEX induced a decrease in the total length of cellular ramifications, without affecting their number at PND 7 (Figure 3a,b, Supporting Information Figure 3a and raw data and statistics in Supporting Information Table S1). Microglia morphologic alterations are still observed at PND 99. At this time point, an increase of the number of microglia ramifications was observed compared with control animals (Figure 3c,d, Supporting Information Figure 3b and raw data and statistics in Supporting Information Table S2), but the total length of ramifications is not different from the control (Figure 3c,d, Supporting Information

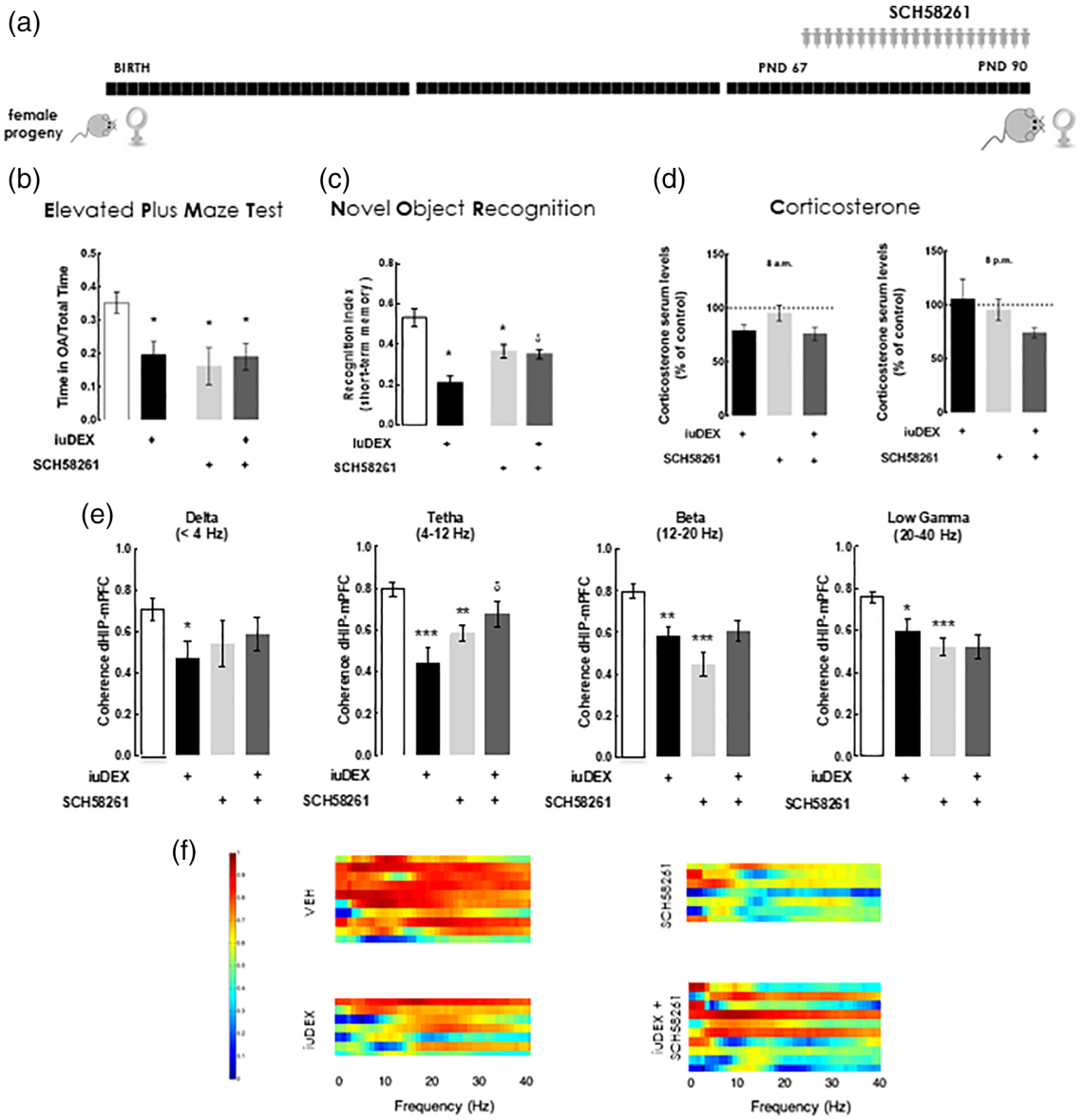

FIGURE 4 Effect of chronic blockade of $A_{2A}R$ on the behavior and mPFC-dHIP coherence effects induced by prenatal exposure to DEX (iuDEX). (a) Schematic drawing of the animal model and pharmacologic treatment: Animals from the offspring of vehicle-treated- or DEX-treated pregnant rats were chronically treated with SCH58261 (0.1 mg/kg/day, ip) or vehicle for 21 consecutive days between PND67-90. (b) Time spent in open arms *per* total time of the EPM test, performed to evaluate anxiety-related behavior at PND90. (c) The recognition index (time spent in the novel object *per* total time spent in the novel and familiar objects) was calculated to evaluate cognitive deficits, using the NOR test at PND94–97. (d) Diurnal and nocturnal quiescent levels of CORT were measured by ELISA, to evaluate the endogenous serum CORT levels. (e) Group comparison of the coherence values between mPFC and dHIP for delta (<4 Hz), theta (4–12 Hz), beta (12–20 Hz), and low gamma (20–40 Hz) frequency bands. (f) Spectrograms of mPFC-dHIP coherence. Each horizontal line represents the spectrogram of an individual rat. Results are presented as the mean ± SEM (n = 5-10 animals); $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$, comparing with vehicle-treated animals, $^{\$}p < .05$, comparing with iuDEX, calculated using a one-way ANOVA followed by a Turkey's multiple comparisons test [Color figure can be viewed at wileyonlinelibrary.com]

Figure S3b and raw data and statistics in Supporting Information Table S3).

3.4 | The morphologic complexity of microglia is different between the dHIP and mPFC

The effect of iuDEX observed in the dHIP contrasts with its effect in the mPFC, where we previously reported a decrease in the number of microglia ramifications (Caetano et al., 2017). Thus, we analyzed and compared microglia morphology under physiological conditions in both regions of adult female rats. Microglial cells in the mPFC exhibited a more complex morphology, with higher number of ramifications, which are longer, compared with microglial cells from the dHIP (Figure 3e,f, Supporting Information Figure S3c and raw data and statistics in Supporting Information Table S4).

3.5 | $A_{2A}R$ blockade rescues iuDEX-induced changes

Confirming our previous report (Caetano et al., 2017), the chronic blockade of $A_{2A}R$ with the selective $A_{2A}R$ antagonist, SCH58261, administered for 21 days (0.1 mg/kg/day) before PND 90 (Figure 4a), exerted, *per se*, an anxiogenic effect (SCH58261: 0.16 ± 0.06, $n = 7$;

VEH: 0.35 ± 0.03, n = 6; p < .05; Figure 4b) and was not able to ameliorate iuDEX-induced anxiety-like behavior in females (for the EPM, iuDEX+SCH58261: 0.19 ± 0.04, n = 10; iuDEX: 0.20 ± 0.04, n = 7; p > .05; Figure 4b; for the NSF, see Supporting Information Figure S1a). SCH58261 administered to control or iuDEX adult females did not interfere with the performance of the animals in tests for helplessness behavior (Supporting Information Figure S1b) or anhedonia (Supporting Information Figure S1c). Body weight and locomotor activity were also not affected by the treatment with SCH58261, alone or in combination with iuDEX (Supporting Information Figure S1d,e). Regarding serum levels of CORT, we again confirm our previous data (Caetano et al., 2017): SCH58261 per se did not alter these levels and was not able to normalize DEX-induced alterations (Figure 4d).

In contrast, the chronic treatment of adult females with the selective A_{2A}R antagonist, SCH58261, improved cognition in iuDEX females (iuDEX + SCH58261: 0.35 ± 0.02, n = 7; iuDEX: 0.21 ± 0.03, n = 10; p < .05; Figure 4c). However, iuDEX females treated with SCH58261 still presented a cognitive impairment in recognition memory, as compared with control animals (iuDEX + SCH58261: 0.35 ± 0.02, n = 7;VEH: 0.53 ± 0.04, n = 7; p < .05; Figure 4c). This might be due to the surprising observation that, in contrast to the well established absence of effect on learning and memory tasks of A_{2A}R antagonists in male rats (Cognato et al., 2010; Cunha et al., 2008; Kaster et al., 2015), the treatment with the A_{2A}R antagonist, *per se*, deteriorated memory performance in female rats (SCH58261: 0.37 ± 0.03, n = 6; VEH: 0.53 ± 0.04, n = 7; p < .05; Figure 4c).

In accordance with this effect of SCH58261 on memory performance, the chronic blockade of A_{2A}R in adult females also decreased the coherence between the dHIP and the mPFC in most of the frequency ranges analyzed: delta (<4 Hz; VEH: 0.71 ± 0.05, n = 9; SCH58261: 0.54 ± 0.11, n = 7; p > .05), theta (4–12 Hz; VEH: 0.79 ± 0.04, n = 9; SCH58261: 0.58 ± 0.04, n = 7; p < .01), beta (12–20 Hz; VEH: 0.80 ± 0.03, n = 8; SCH58261: 0.44 ± 0.06, n = 7; p < .001) and low gamma (20–40 Hz; VEH: 0.76 ± 0.03, n = 9; SCH58261: 0.52 ± 0.04, n = 7; p < .001) (Figure 4e,f), but most importantly, SCH58261 normalized the iuDEX-induced decrease in mPFC-dHIP coherence, an effect observed only for the theta frequency range: delta (<4 Hz; iuDEX+SCH58261: 0.59 ± 0.08, n = 10; p > .05 as compared with iuDEX), theta (4–12 Hz; iuDEX+SCH58261: 0.67 ± 0.06, n = 8; p < 0.05 as compared with iuDEX), beta (12–20 Hz; VEH: iuDEX+SCH58261: 0.61 ± 0.05, n = 10; p > .05, as compared with iuDEX) and low gamma (20–40 Hz; iuDEX +SCH58261: 0.52 ± 0.06, n = 10; p > .05 as compared with iuDEX)

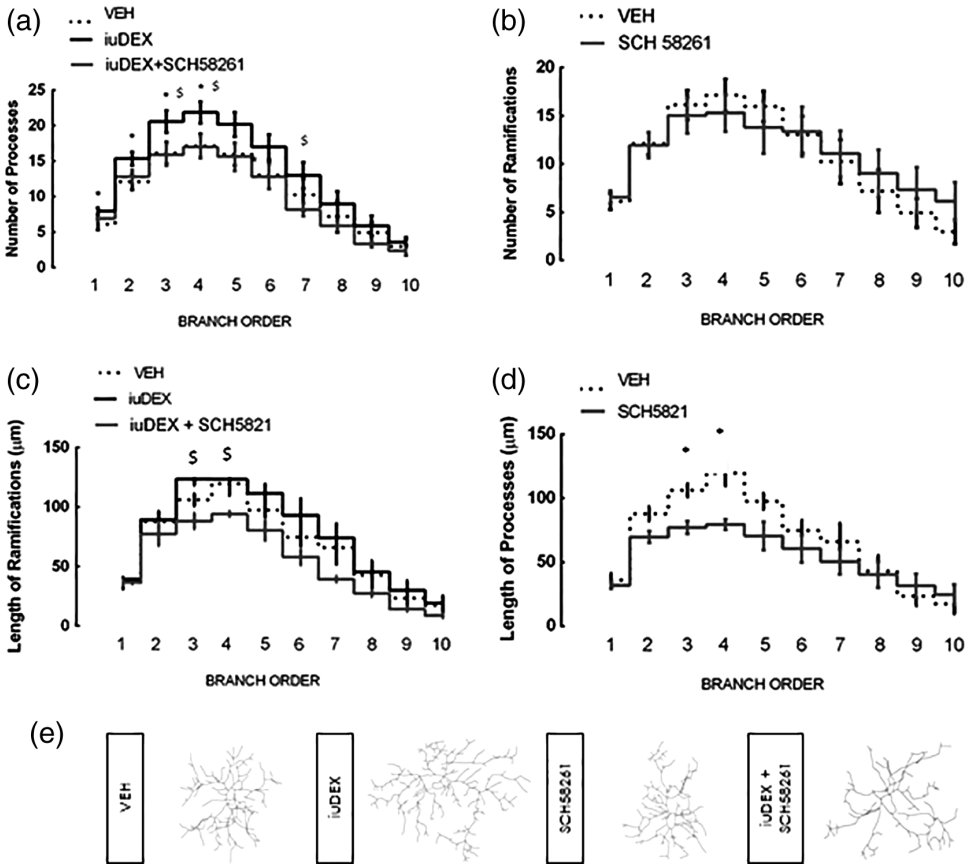

FIGURE 5 Effect of A_{2A}R chronic blockade on the morphologic alterations induced by prenatal exposure to DEX (iuDEX) on the number and length of dHIP microglia processes. Microglial cells of females at PND 99 were stained with Iba-1 and 3D reconstructions were performed using Neurolucida software. Using the morphometric data extracted from the Neurolucida software, the number (a, b) and length (c, d) of microglial processes in the dHIP was assessed and compared between treatments according to the respective branch order. (e) Representative isolated manual reconstruction of microglial cells. Results are presented as the mean ± *SEM* (n = 3–4 animals); *p < .05, comparing with vehicle-treated animals, $^{\$}p$ < .05, comparing with iuDEX, calculated using a one-way ANOVA followed by a Turkey's multiple comparisons test

(Figure 4e,f). Regarding microglia morphology, in contrast to what happens in the mPFC, where A_{2A}R blockade was unable to normalize iuDEX-induced changes in females (Caetano et al., 2017), we now observed in the dHIP that iuDEX adult females treated with SCH58261 show a significant reduction of the number of ramifications compared with iuDEX females, which recovers to control levels (Figure 5a,e, Supporting Information Figure S3b; Supporting Information Table S2). Furthermore, the length of processes was also diminished in iuDEX adult females treated with SCH58261compared with iuDEX females (Figure 5c,e, Supporting Information Figure S3b, Supporting Information Table S3). Importantly, SCH58261 alone reduced the length of some microglial processes in the dHIP, compared with control animals (Figure 5d, Supporting Information Figure S3b, Supporting Information Table S3), although not affecting the number of processes (Figure S5b, Supporting Information Figure S3b, Supporting Information Table S2). Interestingly, SCH58261 also decreased the number of microglia ramifications in the mPFC, although it also decreased the total length of these ramifications (Caetano et al., 2017).

4 | DISCUSSION

In animal models, the prenatal exposure to high levels of steroids, namely GC (as occurs in maternal stress or during gestations requiring GC therapy to prevent respiratory complications associated with premature delivery), impairs brain development and results in abnormal behavior in adult offspring (Fukumoto et al., 2009; Leao et al., 2007). We previously reported (Caetano et al., 2017) that the prenatal exposure to DEX, a synthetic GC used in obstetrics, was associated with an anxious phenotype, as already described by others (Oliveira et al., 2006; Oliveira et al., 2012). The behavioral impact of iuDEX is accompanied by the morphologic adaptation of microglia in the mPFC, a process of remodelling that is different between females and males. In the case of females, microglia adopt a less complex morphologic phenotype, while microglia from males acquire a hyper-ramified phenotype. We proposed that microglia morphologic plasticity is important to the anxious-like phenotype, considering that the chronic treatment of males with an experimental anxiolytic that is also able to modulate microglia morphology (a selective antagonist of adenosine A_{2A}Rs) ameliorates anxiety in parallel with the normalization of microglia morphology, whereas in females, the drug was unable to correct microglia morphology or to promote its anxiolytic effect (Caetano et al., 2017). In order to better explore and, eventually, take advantage of the therapeutic potential of drugs that regulate microglia, we decided to study a new group of females prenatally exposed to DEX and resilient to the anxiolytic effect of the A_{2A}R antagonist, considering that anxiety resilient to pharmacotherapy may be associated with important cognitive deficits. The main goal of the present work was to confirm cognitive deterioration of anxious females resilient to the anxiolytic effect of the A_{2A}R antagonist and to clarify if the drug, which is already known as a cognitive enhancer (Dall'Igna et al., 2007; Kaster et al., 2015; Li et al., 2015b; Machado et al., 2017), although unable to ameliorate anxiety, would be able to treat cognitive deficits and if this effect was paralled by microglia morphologic remodelling in the dHIP, critically involved in cognition.

We now report that female rats prenatally exposed to DEX present memory impairments at adulthood together with a disruption of the neuronal synchronization between the mPFC and the dHIP and morphologic remodeling of microglia (hyper-ramification) in the dHIP. Notably, we describe, for the first time, an opposite regulation of microglia morphology in different brain areas, namely in the dHIP (this work) and in the mPFC (Caetano et al., 2017). Contrasting patterns of cellular plasticity across the brain have already been described for neurons in response to stress conditions (for a review, see Chattarji et al., 2015); however, the regional morphologic adaptation of microglia to stress mediators, namely GC, was not yet described. Regarding this particular issue, a process of microglia atrophy in the hippocampus would easily reconcile with the already described neuronal atrophy in this brain area in stress conditions (for a review, see Chattarji et al., 2015). Although out of the scope of this work, it would be important to further explore the opposite plastic phenomena that we observed in the dHIP and in the mPFC of females upon *in utero* DEX exposure (Caetano et al., 2017): PFC, as well as the hippocampus, controls stress responses by negative feedback (Cerqueira et al., 2007; McEwen et al., 2013); thus, we would expect similar structural modifications triggered by prenatal exposure to DEX. We speculate (and the topic deserves further investigation) that the lost of synchronization between these regions, as now described (as assessed by *in vivo* electrophysiology), may contribute to the unexpected differences in the regulation of microglia morphology triggered by stress modulators, in the case, prenatal DEX. The detailed morphometric analysis here performed shows that, besides regional differences in microglia remodeling associated with the model, the morphologic phenotype of these cells in the mPFC is different when compared with the dHIP in basal, physiologic conditions. Physiologic differences in microglia morphology in the mPFC and in the dHip may also help explain the opposite effect of prenatal DEX in microglia morphology. These data are in line with previous studies reporting regional differences in microglia (Lawson et al., 1990; Schwarz et al., 2012), although most studies use rudimentary approaches rather than 3D reconstitutions to study microglia morphology. This regional heterogeneity of microglia is further heralded by distinct transcriptional identities of microglia in the cortex and in the hippocampus (Grabert et al., 2016). However, further studies are required to categorize region-specific functionalities of microglia to determine how these differences influence microglia modulation for instance by purines (such as adenosine), and microglia responses to insults.

We also observe that the chronic blockade of A_{2A}R in iuDEX adult females normalize microglia morphology in the dHIP and rescues cognitive deficits, as well as the lack of coherence between the dHIP and the mPFC. These results confirm the already described ability of A_{2A}R to control and rescue memory deterioration, now shown to be associated with a microglia morphologic adaptation in a context of anxiety. The observation that iuDEX-induced cognitive deficits in the NOR test, which involves the hippocampal formation (Broadbent et al., 2004), correlates with long-lasting changes in microglia morphology in this brain area, points towards an impact of microglia in cognition that is in line with previous studies showing that microglia are crucial

cellular elements during development (Tay et al., 2017; Wu et al., 2015). For instance, the transient depletion of these cells during development leads to long-term deficits in synaptic transmission, functional brain connectivity, synaptic plasticity and cognitive deficits (Paolicelli et al., 2011; Rogers et al., 2011; Torres et al., 2016; Zhan et al., 2014). Taking into consideration the role of microglia in synapse formation and pruning (Cristovao et al., 2014; Ji et al., 2013; Lim et al., 2013; Miyamoto et al., 2016; Paolicelli et al., 2011; Parkhurst et al., 2013; Schafer et al., 2012), we anticipated that iuDEX-induced microglia morphologic changes in the dHIP, already observed in the first post-natal week, would correlate with electrophysiologic changes and cognition. Indeed, although several studies demonstrate the impact of glia, namely astrocytes, in the synchronization of oscillations between the dHIP and the mPFC, whith impact on cognitive performance (Fellin et al., 2009; Lee et al., 2014; Sardinha et al., 2017), the compromise of microglia had not been previously studied as a morphologic correlate for deficits in the synchronization between mPFC and dHIP. The putative causal relation between long-lasting alterations of microglia morphology in the dHIP, mPFC-dHIP synchronization and cognitive deficits is further supported by our observation that the treatment of adult iuDEX females with a selective $A_{2A}R$ antagonist reverted both the cognitive deficits, the decrease in coherence between these brain regions, as well as microglia hyper-ramification in the dHIP.

Several studies have previously shown that $A_{2A}R$ blockade prevents memory deficits associated with chronic stress or depression (Batalha et al., 2013; Cunha et al., 2006; Kaster et al., 2015; Machado et al., 2017). These studies focused on the relation between synaptic dysfunction and memory deterioration and have shown that neuronal $A_{2A}R$ were both necessary and sufficient to trigger memory deficits (Li et al., 2015a; Pagnussat et al., 2015; Viana da Silva et al., 2016). Although the blockade of $A_{2A}R$ is globally accepted as a cognitive enhancer and anxiolytic, in animal models of disease, the majority of the studies were performed in males or the gender was not specified. This topic is of particular relevance, considering that mood disorders have a gender-specific susceptibility and that we previously described that the pharmacological manipulation of $A_{2A}R$ in our animal model of chronic anxiety (caused by prenatal exposure to DEX), differentially impact on anxious behavior of males and females (Caetano et al., 2017). Altogether, these data raise new questions about the physiological role of $A_{2A}R$ in behavioral differences between males and females and about the role of these receptors in brain regions critically implicated in anxiety and cognition.

We and others have described the ability of $A_{2A}R$ to modulate microglia morphology (Caetano et al., 2017; George et al., 2015; Gomes et al., 2013; Gyoneva et al., 2014; Orr et al., 2009; Santiago et al., 2014) and function (George et al., 2015; Gomes et al., 2013; Gyoneva et al., 2014; Gyoneva et al., 2009; Santiago et al., 2014). The data now presented make it clear that there is also a marked heterogeneity of $A_{2A}R$-mediated microglia modulation according to the brain region considered. In fact, in our previous work (Caetano et al., 2017), we observed that the selective $A_{2A}R$ antagonism was unable to correct microglia atrophy in the mPFC of females, but we now observe that the same antagonist reverts microglia hyper-ramification in the dHIP. In parallel, $A_{2A}R$ blockade reverts memory impairment (present results), but not anxiety (Caetano et al., 2017) in these iuDEX females. These results suggest that the functional uncoupling between anxiety and cognition in iuDEX females that were treated with a selective $A_{2A}R$ antagonist may be, at least partially, explained by a differential regulation of microglia morphology by $A_{2A}R$ in different brain regions. The reasons underlying this region-specific response are still unexplored, but may be due to regional differences of $A_{2A}R$ function in the brain (Li et al., 2015a; Shen et al., 2013; Wei et al., 2014) or to differences in microglia function in different brain regions.

In conclusion, the data obtained show that iuDEX, in conditions mimicking the clinical use of GC in the early periods of brain development, induces alterations in microglia morphology in a region-specific manner with impact in behavior. Microglia morphology remodeling in the dHIP correlates with cognitive deficits observed in our animal model and a treatment with a selective $A_{2A}R$ receptor antagonist was able to revert behavioral changes, electrophysiological abnormalities and the alterations of microglia morphology in the dHIP. These observations reinforce the link between microglia function and the control of mood and memory, and emphasize the importance of gender and brain region in the screening or design of novel therapeutic strategies targeting microglia homeostasis to manage brain disorders.

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SUPPORTING INFORMATION

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