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FRANCISCO GANDRA FERRER ANTUNES

# *Prenatal Immunomodulation, Microglia and Behavioral Changes*

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Trabalho realizado sob a orientação de:

CATARINA GOMES

ANABELA MOTA PINTO

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# Prenatal Immunomodulation, Microglia and Behavioral Changes

Trabalho realizado por:

FRANCISCO GANDRA FERRER ANTUNES[1]

Sob a orientação de:

CATARINA ALEXANDRA DOS REIS VALE GOMES[1,2]

ANABELA MOTA PINTO[1,2]

1. Faculty of Medicine, University of Coimbra, Portugal

2. Coimbra Institute for Clinical and Biomedical Research (iCBR), Faculty of Medicine, University of Coimbra, Portugal

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**Resumo**

Alterações imuno-inflamatórias estão associadas à ocorrência de patologia do espectro ansioso. Em modelos animais, a exposição a níveis suprafisiológicos de glucocorticoides (GC) em fases importantes do neurodesenvolvimento resulta no aparecimento de padrões de ansiedade crónica. Neste quadro, as células imunes do sistema nervoso central são elementos essenciais, estando descritas alterações morfológicas das células da microglia no córtex pré-frontal medial (mPFC) e no hipocampo dorsal (dHIP), duas regiões cerebrais implicadas no comportamento do tipo ansioso. Este processo de remodelação está relacionado com alterações de comportamento animal.
O objetivo deste trabalho foi alargar o conhecimento de um modelo de ansiedade crónica baseado na exposição a GC no final da gestação, com o intuito de melhor caracterizar o impacto a longo prazo no perfil inflamatório sistémico, e estudar os efeitos na microglia numa região envolvida na resposta do medo, o Núcleo do Leito da Estria Terminal (BNST), averiguando se a sua morfologia está alterada e comparando estas alterações com as observadas em outras regiões envolvidas no comportamento ansioso, tendo em conta diferenças de sexo.

Administrou-se dexametasona (DEX) a ratos Wistar gestantes (no final da gravidez), sendo a sua descendência avaliada na idade adulta (90 dias). O marcador pró-inflamatório Interleucina-1beta (IL-1β) foi quantificado no soro dos animais e no sobrenadante de uma cultura celular de microglia (N9) exposta a DEX. Foram realizadas reconstruções tridimensionais de células da microglia do BNST para estudar a sua morfologia. Os indivíduos em estudo foram divididos de acordo com o sexo e a exposição ao fármaco.

A exposição a DEX resultou numa diminuição da IL-1β sérica em fêmeas, e uma tendência para redução nos machos, embora sem significado estatístico. A morfologia da microglia do BNST em condições fisiológicas apresentou um dimorfismo de sexo, sendo os processos microgliais mais longos no caso das fêmeas. Em resposta à DEX observou-se um aumento dos processos distais da microglia dos machos, efeito não observado no caso das fêmeas, em cujas células da microglia a DEX não induziu alterações nas condições do estudo desenvolvido.

A alteração dos níveis de IL-1 β observadas corroboram a existência de uma adaptação inflamatória sistémica a longo prazo à exposição pré-natal a GC. Quanto aos novos dados referentes à morfologia da microglia do BNST, vêm complementar o conhecimento das outras regiões envolvidas na ansiedade crónica, sugerindo que a microglia do BNST é afetada por

esta exposição de uma forma dependente do sexo, tal como observado no mPFC ou no dHIP. À semelhança do que foi feito nestas regiões, estudos comportamentais futuros (testes padronizados para avaliação do medo e envolvendo a ativação da BNST) serão importantes para esclarecer se alterações morfológicas da microglia se fazem acompanhar de alterações do medo entre sexos.

Este estudo não clínico reforça o envolvimento do sistema imune em geral e das células da microglia em particular na fisiopatologia da ansiedade e dos seus sintomas. Para além disso, reforça a importância de identificar e controlar os efeitos a longo prazo da exposição a GC durante o neurodesenvolvimento.

**Palavras-Chave:** Microglia, Transtornos de Ansiedade, Inflamação, Glucocorticoides, Núcleos Septais, Interleucina-1beta

**Abstract**

Immunoinflammatory changes are related to the occurrence of pathology of the anxious spectrum.
In animal models, exposure to supraphysiologic levels of glucocorticoids (GC) in key steps of the neurodevelopment leads to chronic anxiety. In the development of this condition, immune cells of the central nervous system are crucial elements, there being reports of changes of morphology of microglia cells in the medial prefrontal cortex (mPFC) and the dorsal Hippocampus (dHip), brain regions involved in anxious behavior.
We aim to widen the knowledge of this model of chronic anxiety based in the exposure to GC during pregnancy, to better portray the long-term impact of prenatal exposure to GC on the systemic inflammatory environment and study the effect it has upon the microglia of a region involved in the fear response, the bed nucleus of stria terminalis (BNST) , checking whether its morphology is altered and comparing these changes with the ones observed in other regions concerned in anxious behavior, taking to account sex differences.

Pregnant Wistar rats (in the final stages of pregnancy) were administered with dexamethasone (DEX) or saline, and the offspring was evaluated in adulthood (90 days of age). The pro-inflammatory marker Interleukin-1beta (IL-1β) was quantified in the rats' serum and in the supernatant of a microglia cell line (N9) exposed to DEX. Three-dimensional models of microglia from BNST were reconstructed, allowing precise morphological studies. Individuals were divided by gender and exposure.

Exposure to DEX resulted in a significative reduction of serum IL-1β in females, but the apparent reduction in males was not found significative. Microglia morphology from the BNST, in physiological conditions, was different between sexes as females presented longer processes. In response to DEX males' microglia showed a slight increase in distal processes, effect not observed in females, whose microglia didn't respond to DEX in the present study conditions.

These results regarding IL-1β are consistent with a long-term systemic inflammatory adaptation after prenatal exposure to GC. The new data regarding BNST microglia morphology complements what was known about the brain regions involved in chronic anxiety, suggesting that BNST microglia is affected by this exposure in a sex-dependent manner, as was seen in the mPFC and dHIP. As was done for these regions, future behavioral studies (standardized tests for fear evaluation and involving BNST activation) will be important to clarify if the

microglia morphologic changes are accompanied with changes regarding the fear response between sexes.

This non-clinical study strengthens the role of the immune system, in general, and of microglia cells, in particular, in the pathophysiology of anxiety and its symptoms. It also reinforces the importance to acknowledge and control the long-term effects of exposure to GC during neurodevelopment.

**Keywords**: Microglia, Anxiety Disorders, Inflammation, Glucocorticoids, Septal Nuclei, Interleukin-1beta

## Introduction

Inflammation is a crucial element for an organism's homeostasis, acting both in the autoregulatory processes and in its adaptation to the surrounding environment.
A growing body of evidence supports the existence of a relationship between the inflammatory status and the development of psychiatric pathology and altered behavior [1-3]. Namely, a number of studies support the theory that immunoinflammatory alterations are involved in the genesis of anxious spectrum pathology [4-7], reporting a pro-inflammatory state with increased inflammatory biomarkers (Interleukin-1β (IL-1β), Interleukin-6, TNF-α, Interferon-γ and C reactive protein) [4,5], as well as hypothalamic-pituitary-adrenal axis and autonomic nervous system dysregulation [4].

Exposure to supraphysiological levels of glucocorticoids (GC), either endogenous or exogenous, during key moments in the neurodevelopment is considered a risk factor for behavioral changes, namely the development of chronic anxiety [8-11]. Prenatal exposure to high levels of exogenous GC has been tested in animal models. This exposure resulted in a chronically altered behavior with features that qualified it as a chronic anxiety disorder, with alterations in standardized tests targeting anxious-like behavior (Elevated Plus Maze) [12,13,14], depressive-like behavior (Forced Swimming test) [12,13,15] and recognition memory (Novel Object Recognition) [13,15]. In this animal model of chronic anxiety, neuronal changes have been reported, including altered neuronal morphology [16-20], number [16,18-20] and survival [16,18-21], abnormal spine density [16,17] and impaired neuronal migration [22], in several brain regions, including the medial prefrontal cortex (mPFC) [23], the dorsal hippocampus (dHIP) [21,20], the Amygdala [16,23] and the bed nucleus of the stria terminalis (BNST) [16], key brain regions for the pathogenesis of anxious pathology and the regulation of fear responses [16,24,25].

The pathophysiological process behind this syndrome is not, as of now, completely understood. Initial approaches focused solely on the direct effect GC had on neurons, disrupting their function and architecture during the neurodevelopment and resulting in the described behavioral changes. Despite this being a simple explanation, it failed to completely explain the magnitude of the changes and their long-term persistence.
On the other hand, attending to the immunomodulating effects of GC, a novel line of studies is pursuing dysregulations of the brain's inflammasome that may further explain the mechanism behind this presentation. In this model, microglia cells are a key element.

Microglia are cells of the reticuloendothelial system in charge of the regulation of the physiological and pathological inflammatory responses in the central nervous system.
Regarding the physiological role of microglia, it is important to highlight that, since the early stages of neurodevelopment, these cells are involved in the formation of a functional synaptic architecture by controlling, partly through inflammatory mediators [26-30], the elimination [26,28-32,34], maturation [26,27,29,30,33-35] and creation of synapses [26-28,32-35].
Microglia morphology is closely linked to its function, and when responding to frankly pathological setting, an “activated state” morphology is observed, but when further studying this cell subtle changes in the morphology of the “resting state” microglia were found to disrupt their physiological role as architects of a sane synaptic network [36].
Furthermore, being an active component of the innate immune system, microglia are susceptible to a wide range of immunomodulators throughout life, but more markedly during early neurodevelopment. These cells, possessing receptors for GC, are directly affected by these hormones [12,37,38].

The immunomodulatory effect of GC, during this critical period of neurodevelopment, whether by direct effect or indirectly, or by altering the inflammatory environment, could differentially influence microglia of brain regions associated with anxious states, generating the observed pattern.

Based in this model, previous studies concerning the same animal model revealed that microglia is also affected by the prenatal exposure to GC. These studies found alterations in microglial morphology in the mPFC [12] and in the dHIP [13] that were present from early stages of life and were maintained in adulthood. Furthermore, in the mPFC these changes occurred differently in males and females, resulting from dimorphic basal morphology and opposite responses to the pharmacological stimuli between sexes [12].

A priming effect could help explain the persistence of cellular and behavioral modifications seen in this model. According to this theory, the effect of GC upon microglia leads not only to an acute neuroinflammatory response, but also to an altered response of these cells to future inflammatory stimuli, having a long-term impact in the neuroinflammatory environment with a bias towards a pro-inflammatory response. [39,40]

We now investigated the long-term effect of the prenatal exposure to the synthetic GC Dexamethasone (DEX):
- In the systemic inflammatory status, by quantifying serum levels of the cytosine IL-1β, a pro-inflammatory cytokine believed to be involved in the pathogenesis of chronic anxiety [4,7].

- On microglia morphology in the BNST, using a three-dimensional reconstruction method with ensuing morphometric analysis, as studying this region will complement previous studies of other brain regions that were likely involved in the genesis of chronic anxiety found in this model.

## Materials and methods

### Animal Model

Animals were housed under standard laboratory conditions with a light/dark cycle (12/12h), at room temperature (RT) and with *ad libitum* access to food and water. The procedures involving animals were performed in agreement with the EU guidelines for the use of experimental animals (EU Directive 2010/63/EU). All efforts were made to minimize animal suffering and to reduce the number of animals used.
Pregnant Wistar Han rats were administered either DEX (Sigma) (1mg/kg/day) or saline, subcutaneously, on the $18^{th}$ and $19^{th}$ day of gestation, a protocol of administration previously described as an inducer of chronic anxiety [12].

### N9 Murine Microglia Cell Line

Microglia cells were kept in Roswell Park Memorial Institute medium with added 5% heat inactivated Fetal Bovine Serum, 1% penicilin-streptomicin, 23.8 mM sodium bicarbonate and 30mM glucose, with a 7.2 pH level. The cells were kept at 37ºC in a humidified atmosphere with 5% $CO_2$ and 95% $O_2$.
The cells were exposed to DEX in different concentrations (0.1, 1 and 10 μM) for a period of 24 hours. The supernatant was then collected to analyze.

### IL-1β Quantification

Venous blood was sampled from the tail vein of adult rats (postnatal day (PND) 90) at 8h pm. Il-1β levels were measured using an IL-1β ELISA kit (Peprotech, USA), following the manufacturer provided protocol.

Using the same method IL-1β levels were measured in the supernatant of the N9 microglia cell lines.

Microglial Morphometric Analysis

The adult rats (PND90) were anaesthetized with intraperitoneal injection of 90 mg/kg Ketamine (Nimatek) and 10 mg/kg Xylazine (Ronpum 2%) and perfused with phosphate buffer saline (PBSPBS;137 mM NaCl, 2.7 mM KCl, 10 mM NaH2PO4.2H2O, 1.8 mM KH2PO4 in miliQ water, pH = 7.4) and 4% paraformaldehyde (PFA) solution in PBS 1x to clear vascularization and start fixation. After dissection the brains were kept at 4% PFA overnight for fixation and then were transferred to a 30% sucrose (2 days) solution and were stored at -80ºC. The frozen brains were sliced in 50 µm slices in the cryostat (Leica CM3050S, Germany) and were individually transferred into 24 wells plates filled with cryopreservation solution (50 mM NaH2PO4.H2O [Merck], 50 mM K2HPO4 [Sigma], 30% sucrose [Sigma], 30% ethylene glycol [Sigma], diluted in MilliQ H2O, pH 7.2) and stored at -20ºC.

For immunodetection of microglia, the brains sections were washed three times with PBS during 10 minutes intervals, in mild agitation, and were then blocked and permeabilized by incubation in a 5% BSA and 0.1% Triton X-100 in MilliQ $H_2O$ solution for 2 hours at room temperature, in mild agitation.
Samples were incubated with the primary antibody (rabbit anti-Iba-1, 1:1000 [WAKO]) for 48 hours at 4ºC and mild agitation. After new washing cycle, the brain slices were incubated with the secondary antibody (donkey anti- rabbit, 1:1000 [Invitrogen]) for a 2 hours period and were washed again. Finally, the sections were incubated with DAPI (1:5000) for 10 minutes and were then washed. Negative controls with no primary antibody incubation were included to validate the secondary antibody.
The brain sections were mounted in gelatinized microscope blades using DAKO glycergel, dried overnight and sealed.
Images of 15 randomly chosen microglia cells from the BNST from each animal were acquired by confocal microscopy (LSM 710 META connected via ZEN software, using a 63x objective lens).

The Z-stack images were then imported to the Neurolucida software (MBF Bioscience, Vermont, USA) and were manually reconstructed along the acquired planes, producing a three-dimensional reconstruction of each cell. Criteria for reconstruction was to consider every

microglial process ramification, regardless of its length in order to create a trustworthy reconstruction.
The morphometric data (branched structure analysis) of the reconstructed cells was obtained using the Neurolucida Explorer software extension, and the number and length of processes per branch order (division order counted from the cell body) was analyzed.

## Statistical Analysis

Data were analyzed using Graphpad Prism software.
All values are presented as means with the standard error of the mean interval displayed.
Normalization tests were performed to assess whether to use parametric or non-parametric tests.
Comparison between independent means was carried out using student's t-test.
Differences were considered significative at $p<0.05$

**Results**

Inflammatory profile

Prenatal exposure to DEX reduces IL-1β serum levels in adult female rats

We observed that exposed females presented decreased levels of the proinflammatory cytosine IL-1β when compared with control females (Figure 1 a), whereas in males we found no significant differences between DEX-exposed animals and controls (Figure 1 b).

IL-1β secretion by N9 murine microglia cell lines was decreased in cells exposed to DEX when compared to cells left in saline (Figure 1 c).

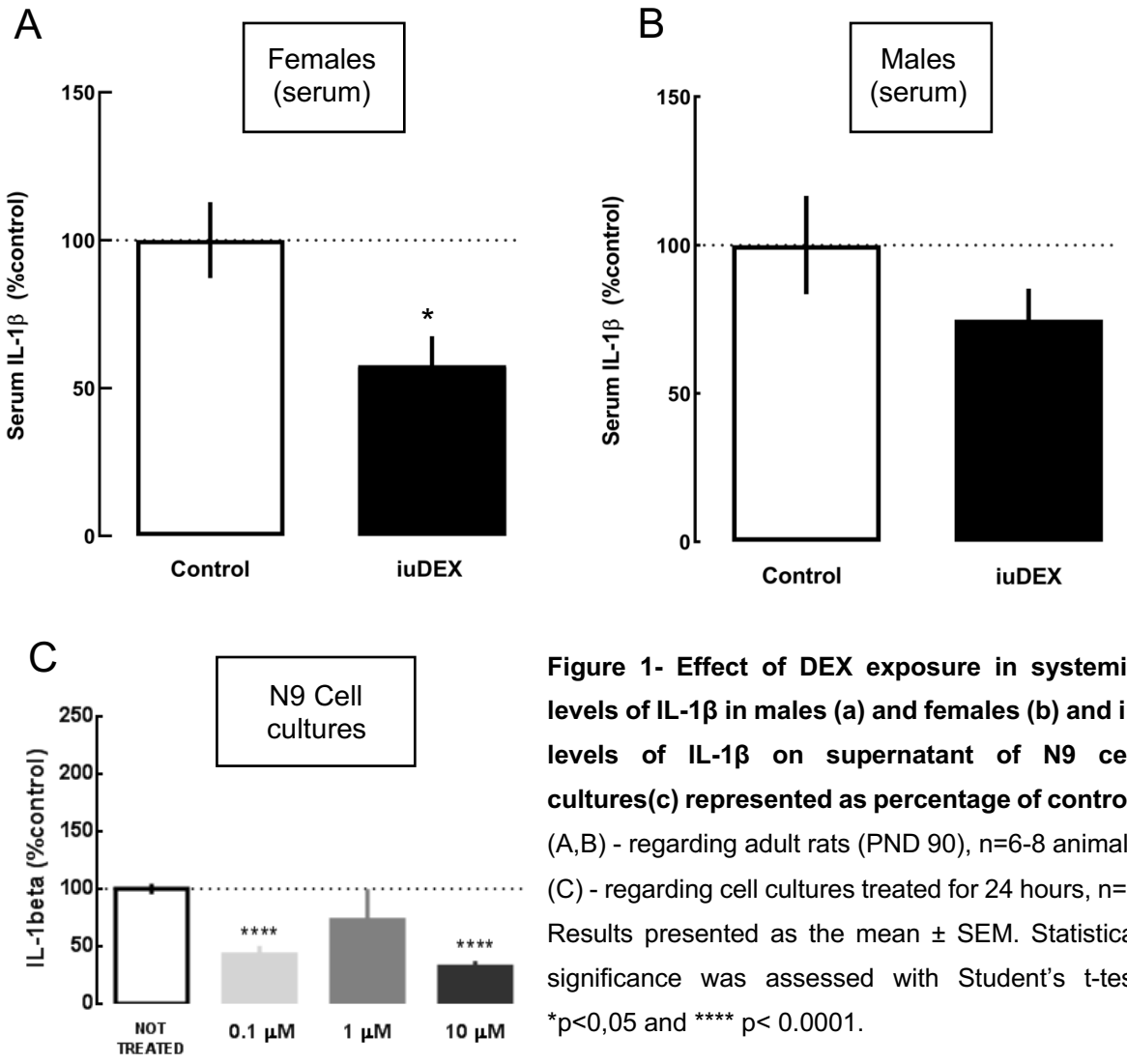


**Figure 1- Effect of DEX exposure in systemic levels of IL-1β in males (a) and females (b) and in levels of IL-1β on supernatant of N9 cell cultures(c) represented as percentage of control.** (A,B) - regarding adult rats (PND 90), n=6-8 animals (C) - regarding cell cultures treated for 24 hours, n=6 Results presented as the mean ± SEM. Statistical significance was assessed with Student's t-test *p<0,05 and **** p< 0.0001.

Microglia morphology

There is a physiologic sex dimorphism in microglia morphology in the BNST

Quantification of morphologic features of microglia was performed in the BNST of male and female non-exposed rats at PND 90 (adults), to assess sex inequalities in physiologic settings. We found the length of the processes to be significantly superior in females compared to the males (Figure 2 c) in some branch orders, with no differences found in the number of processes (Figure2 b).

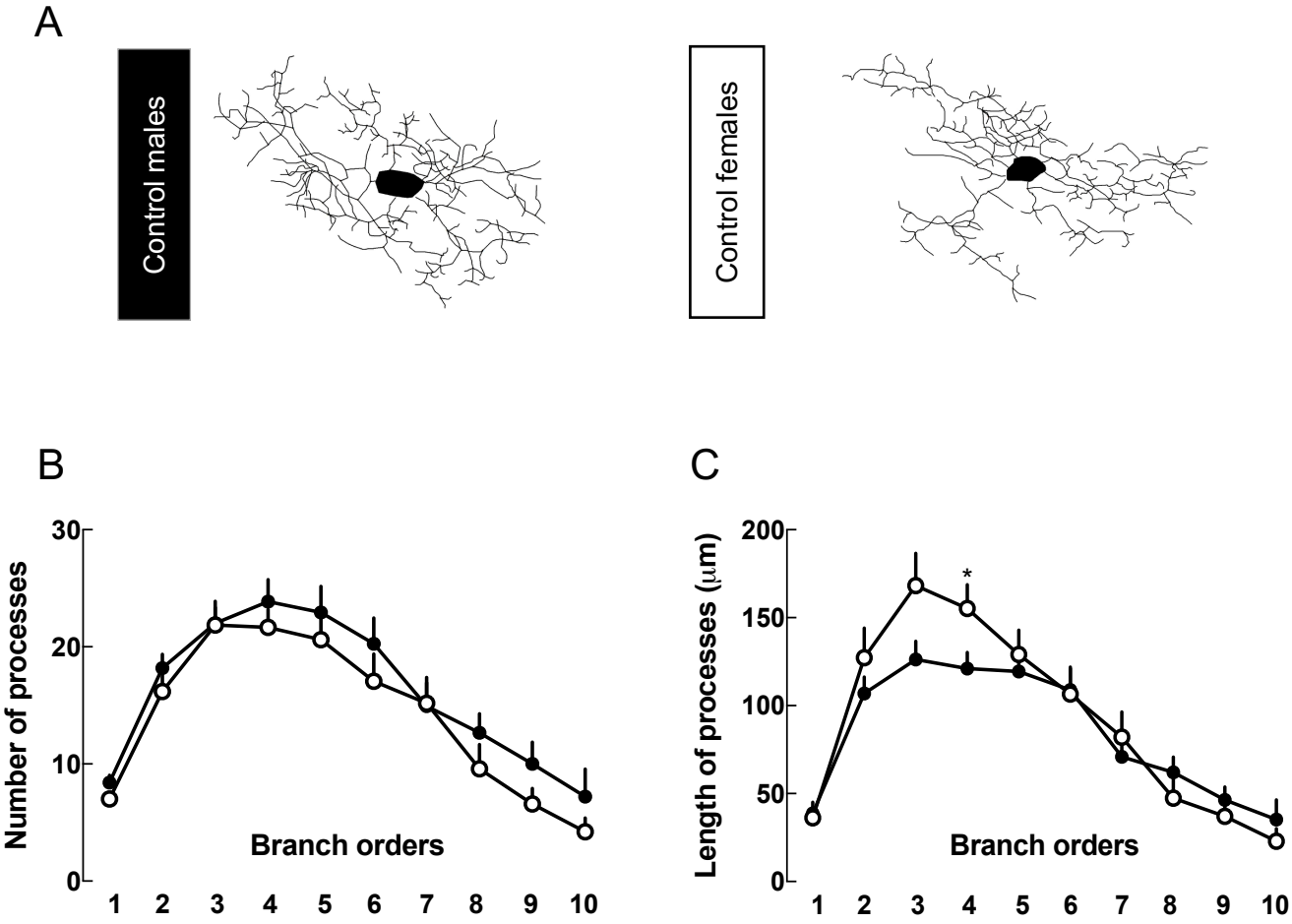


**Figure 2: Female microglia present longer processes than male microglia in the BNST at adulthood.** Results regarding adulthood (PND 90) females and males (n=6) are presented as the mean ± SEM. Microglia morphology was assessed by tridimensional manual reconstruction in Neurolucida software (A) and morphometric data was acquired in Neurolucida Explorer regarding the number (B) and length (C) of processes. Statistical significance was assessed with Student's t-test *p<0,05.

Prenatal exposure to DEX did not induce long-term alterations of microglia in the BNST of adult female rats

We then compared microglia morphology of adult females prenatally exposed to DEX with the unexposed controls in the same region, to assess drug-induced modifications in the morphology of microglia in the BNST.

We found no significative differences regarding the length of the processes (Figure 3 c) and the number of ramifications (Figure 3 b).

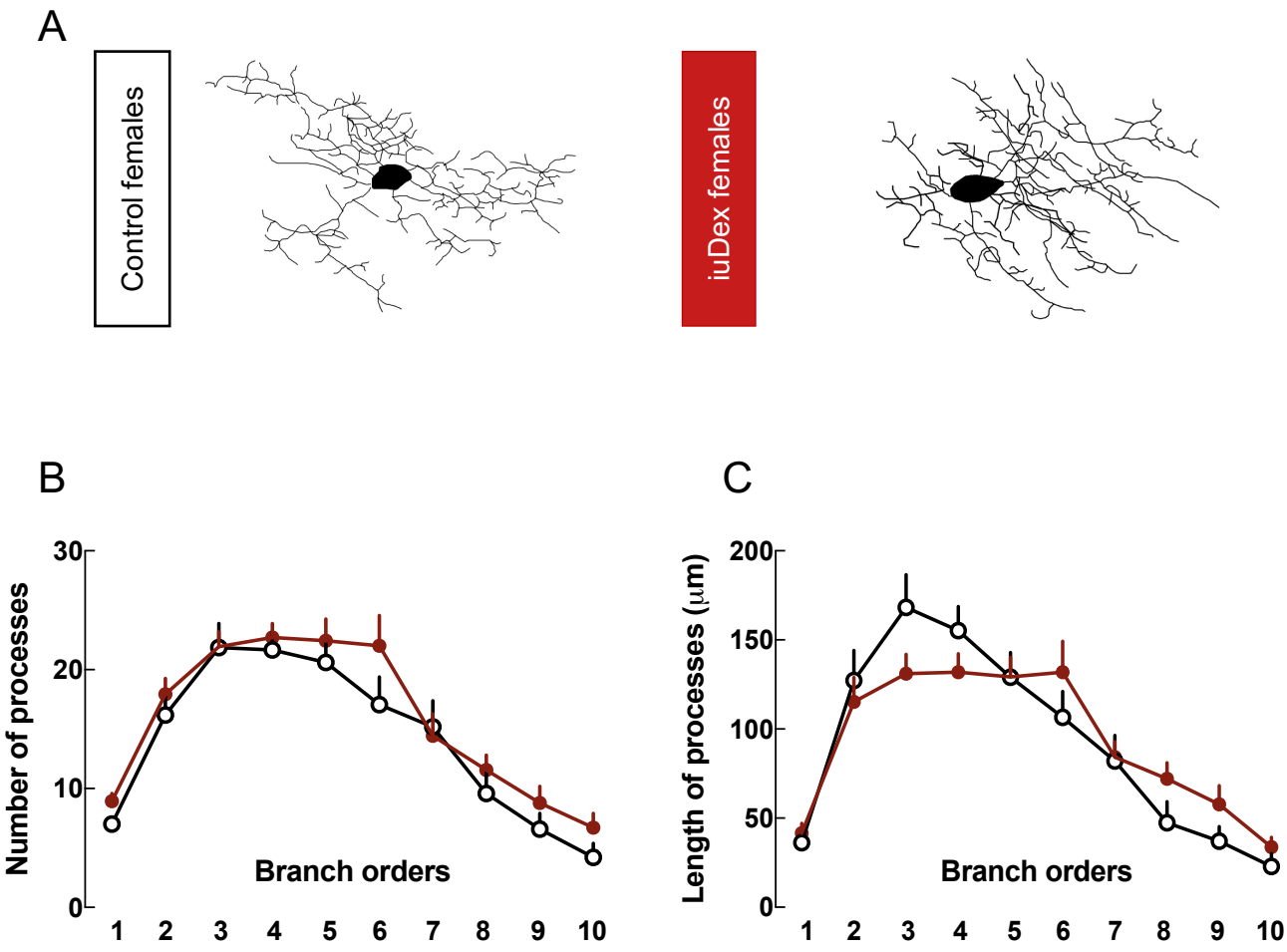


**Figure 3: iuDex did not affect the number and length of BNST microglia in adult female rats.** Results regarding iuDEX adulthood females (PND 90) CTRL (n=6) are presented as the mean ± SEM. Microglia morphology was assessed by tridimensional manual reconstruction in Neurolucida software (A) and morphometric data was acquired in Neurolucida Explorer regarding the number (B) and length (C) of processes.

Prenatal exposure to DEX induced long-term alterations of microglia in the BNST of adult male rats

In a parallel fashion to what we did with females, we compared microglial morphology of adult males prenatally exposed and unexposed to DEX, in the BNST, with the same purpose. Exposed males expressed an increase of both the number (Figure 4 b) and of the length (Figure 4 c) of distal ramifications.

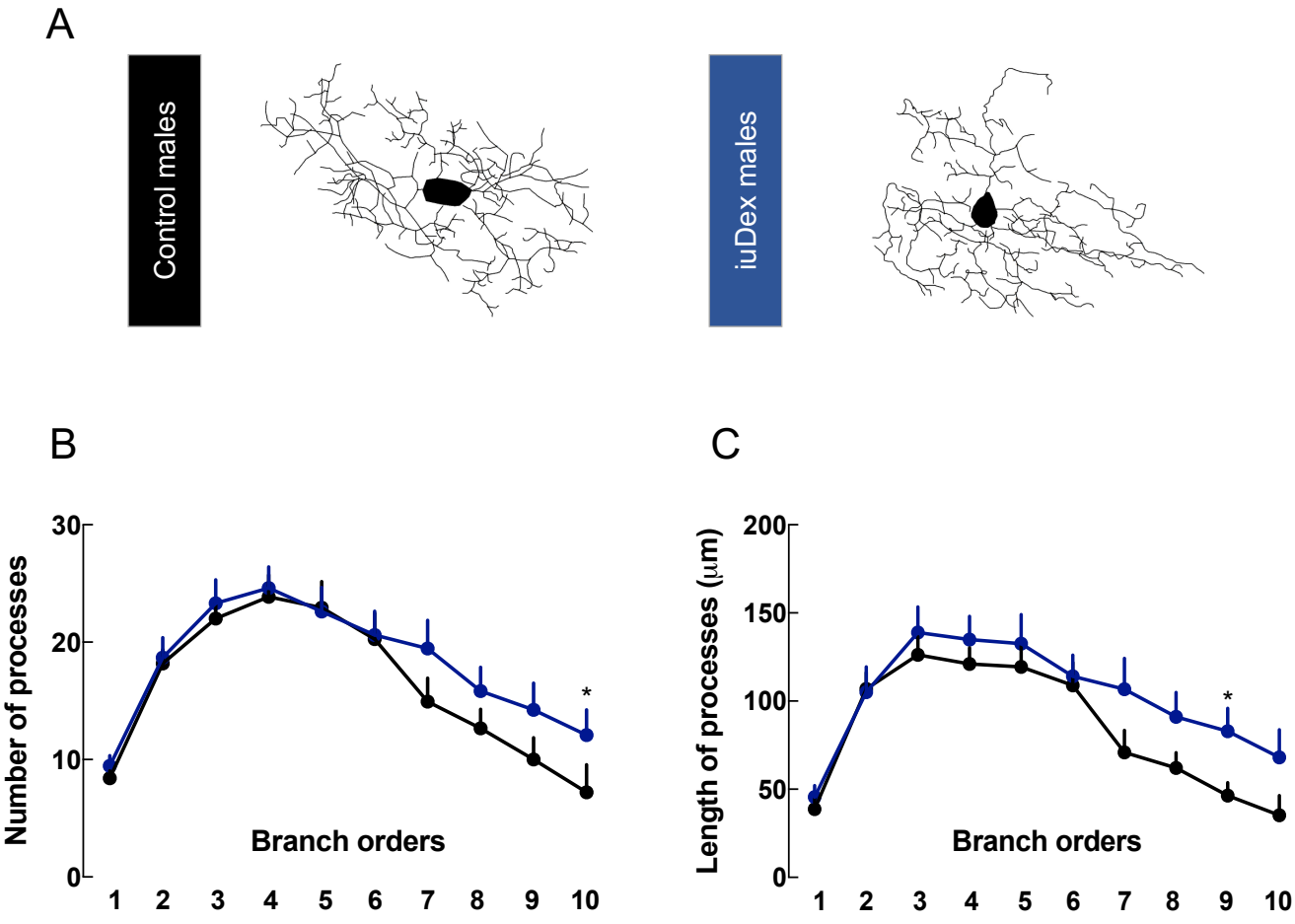


**Figure 4: iuDex increased the number and the length of BNST microglia distal processes in adult male rats.** Results regarding iuDEX adulthood females (PND 90) CTRL (n=6) are presented as the mean ± SEM. Microglia morphology was assessed by tridimensional manual reconstruction in Neurolucida software (A) and morphometric data was acquired in Neurolucida Explorer regarding the number (B) and length (C) of processes. Statistical significance was assessed with Student's t-test *p<0,05 comparing iuDEX with CTRL group.

Prenatal exposure to DEX has opposite effects according to sex, leading to the cancellation of the physiological sex difference

Finally, we compared microglia morphology in the BNST of males and females prenatally exposed to DEX, to search whether the physiologic differences between genders were maintained after pharmacological manipulation.
We found exposed males' microglia tend to have, distally, longer (Figure 5 c) and more numerous (Figure 5 b) processes when compared to exposed females' microglia.

These findings are a consequence of the previously exposed data: the increase in the number of microglia processes in exposed male rats compared to male controls combined with the non-significative reduction of length of microglial processes in exposed females compared to the control females.

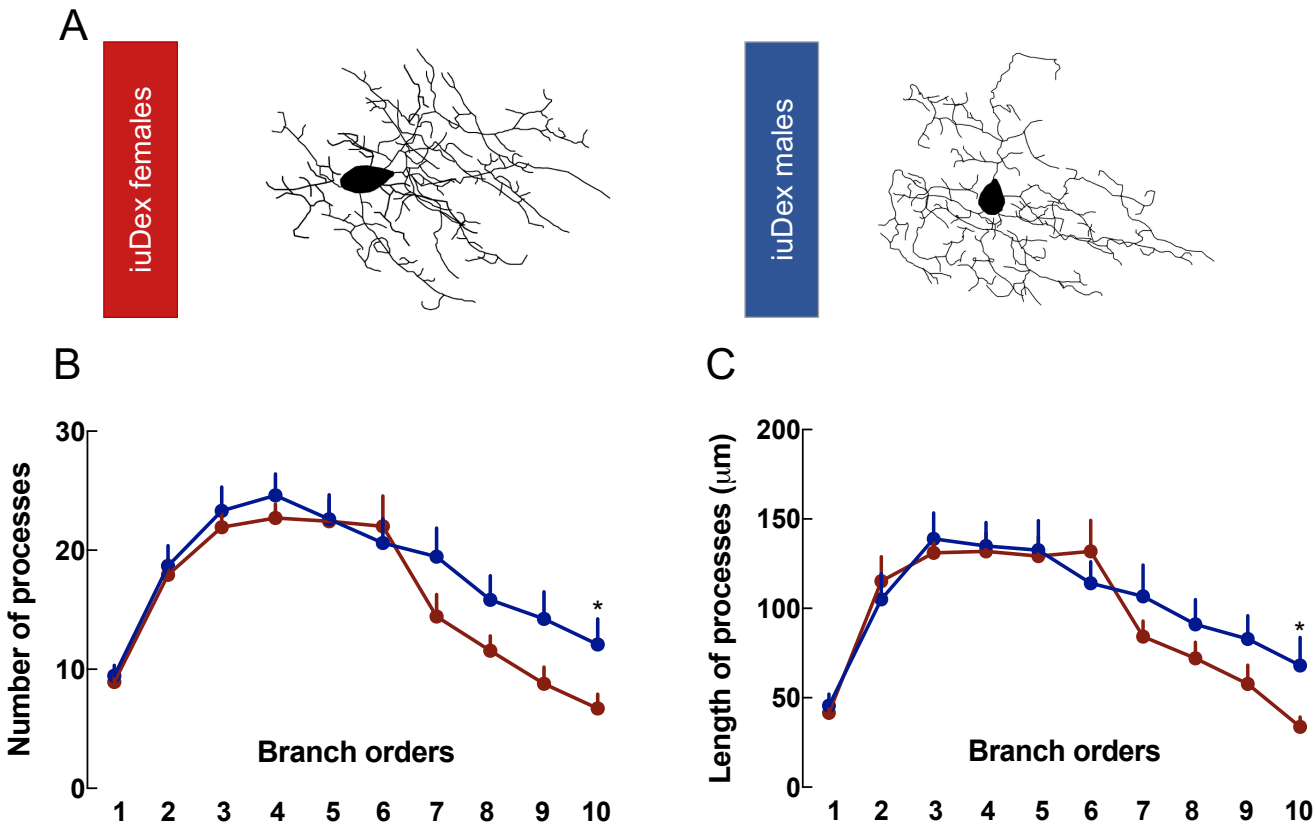


**Figure 5: iuDex altered the physiological difference in the length of BNST microglia in adult female and male rats.** Results regarding iuDEX adulthood (PND 90) females and males (n=6) are presented as the mean ± SEM. Microglia morphology was assessed by tridimensional manual reconstruction in Neurolucida software (A) and morphometric data was acquired in Neurolucida Explorer regarding the number (B) and length (C) of processes.

## Discussion

We report a long-term impact in the systemic inflammatory environment in response to prenatal exposure to GC. These results complement findings from previous studies using the same animal model, concerning corticosterone levels, which were significantly decreased in exposed females with an insignificant decrease in the male subjects when exposed [12]. These new data reinforce the idea of long-term systemic inflammatory modifications in response to prenatal immunomodulation. It also amplifies the suggestion of a sex-bias in these inflammatory changes, with a larger impact on females. A broadening of the testing series might help clarifying the existence of eventual changes in males, as well as reassert the presence of the suggested sex-bias.
On the other hand, the coexistence of a decrease in both corticosterone and IL-1β, apparently paradoxical [41], doesn't unequivocally means a bias towards an anti-inflammatory status, and in this sense, taking in consideration the levels of other cytokines [42] would allow us to further understand the effects in this complex endocrinal-inflammatory system and take more founded conclusions.

Our complementary study of the response of IL-1β secretion by N9 murine microglial cell line after DEX exposure, despite revealing the ability of microglial cells to respond to direct stimulation by the drug through the secretion of inflammatory cytokines, should not lead us to assume that microglia is the main responsible for the systemic alterations found.
In our view, it indicates that, having this decreased secretion mimicked what was found in systemic levels, taking in account that these cells are a constituent of the reticuloendothelial system, the globality of this system appears to be affected by the early exposure to GC, generating a chronic dysregulation of this system, with a reduction in the levels of pro-inflammatory markers.

Our group previously described [12,13] that, in response to DEX, rat microglia of the mPFC in females expressed a less complex morphology, as opposed to male, where hyper-ramification was observed, resulting in a narrowing of the gap found between unexposed individuals of the two sexes. In the dHIP we found that microglia responded with hyper-ramification in females, opposite to what was found in the mPFC.
We now report results consistent with a long-term remodeling of microglia morphology in the BNST with different expression between sexes. Even though this conclusion would be an overstatement if we were to analyze the new data by itself, when we compare it to the findings concerning the mPFC, we see that it appears to sketch a similar response albeit not as

expressive (Table 1). A bigger pool of samples could possibly overcome the brittleness of the reported results and provide a more conclusive insight.

**Table 1: Summary of the effects of iuDEX on Microglia morphology according to brain region**

| | mPFC | dHIP | BNST |
|---|---|---|---|
| Males | Increased Branching | NA | Slight Increase in distal Branching |
| Females | Decreased Branching | Increased Branching | NS |

NA = Data not available; NS = No significative differences found

When we compile all the information from the present and previous studies we can, now with more certainty, suggest that, in the animal model of chronic anxiety, there is a microglial remodeling in response to GC, that there are regional differences in said remodeling (as was previously reported), and that the remodeling has marked inter-sex heterogeneity. These conclusions support the importance to pursue the study of microglial response to GC in other brain regions concerned in the development of anxious-spectrum pathology as well as study aspects other than morphology, namely a more direct study of its function, to better integrate the knowledge we now have in an explanatory model to the pathogenesis of anxiety.

We believe this study reinforces the importance to acknowledge and control the long-term effects of exposure to GC during neurodevelopment in the clinical approach of situations where this exposure is considered necessary, such as prematurity and other less common situations. The arise of chronic neuropsychiatric pathology, namely chronic anxiety, in this setting reveals the need to rethink the therapeutic options to deal with this pathological entity.

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