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STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration. I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, 30 Outubro 2015

Full name: Susana Isabel Gonçalves Martins

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Don't be trapped by dogma — which is living with the results of other people's thinking.

Don't let the noise of others' opinions drown out your own inner voice.

Steve Jobs

ABSTRACT

The neuroimmunology field is at an exciting stage due to a set of revolutionary discoveries challenging the now old-fashioned dogma of the brain being "protected" from the peripheral immune system action. Immune components such as T lymphocytes and the cytokines they produce, once regarded as detrimental to the brain, are now considered integrant parts of the healthy nervous system since their regulated actions control immune surveillance but also modulate higher brain functions. The cytokine interferon-gamma (IFNγ), produced mainly by T lymphocytes, is a potent pro-inflammatory molecule, whose levels are altered in many neuropsychiatric and neurodegenerative diseases. Though studies assessing the effects of this cytokine, when administered into the brain, have shown that it affects different cellular and synaptic mechanisms underlying behavioural dimensions, it is still unclear whether this is a collateral damage of the inflammatory response or if IFNγ indeed plays a role in the modulation of non-pathological brain function. As so, we sought to explore the role of this cytokine in the modulation of brain function in physiological conditions and also after exposure to chronic stress – a paradigm known to trigger the development of psychiatric complications and also accelerate neurodegenerative processes.

In the first part of the thesis (2nd Chapter) we demonstrate that, in a healthy brain, the absence of IFNγ enhances dorsal hippocampus plasticity and associated cognitive function. At the structural level, an enlargement of the dorsal hippocampus volume contrasted with the absence of alterations observed in the ventral part, highlighting that the effects of this cytokine are more selective for cognitive behaviours. Moreover, the absence of this cytokine amplifies neuroplastic phenomena in the dorsal hippocampus, namely neurogenesis, size of neuronal dendritic arborisations and presynaptic functioning, most likely contributing for the enhanced cognitive performance.

On the 3rd chapter, we demonstrate that there are gender-differences on the behavioural phenotype of IFNγ KO mice, and discuss the possible association of estrogen and the IFNγ expression in the central nervous system.

In the following chapter (4th Chapter) we describe the optimisation of a chronic unpredictable stress (CUS) paradigm for use in C57BL/6 mice, a strain with higher resistance to stress. This mice model of stress-related disorders exhibits, beyond the stress-related neuroendocrine and behavioural alterations, mild changes in thymic cellular populations and relevant splenic myeloid cellular alterations, with an increased number of neutrophils as the most striking change.

At last (5th Chapter), we discuss the contributory role of IFNγ for the development of the immune maladaptive response to chronic stress. By submitting mice to the optimized CUS protocol, it was observed that mRNA levels of *Ifnγ* are elevated in the brain, specifically in the medial prefrontal and orbitofrontal cortices. Moreover, exposure to chronic stress leads to an increase of the adrenergic innervation of the spleen as to alterations on the percentage of neutrophils and monocytes/macrophages populations in the spleen. Importantly, the absence of this cytokine blunts the stress-related changes on these cell populations in the spleen.

The recognition of the proinflammatory cytokine – IFNγ, as a negative regulator of hippocampal plasticity and associated cognitive function, together with its contributory role for the stress-related immune dysfunction, suggests that this cytokine may articulate the complex network that underlies the inflammatory component of neuropsychiatric disorders.

Keywords: IFNγ, cognition; neuroimmunology; chronic stress; neutrophils

RESUMO

A área da neuroimunologia está a atravessar uma fase excitante devido a um conjunto de descobertas revolucionárias que desafiam o seu agora antiquado dogma que visiona o cérebro como um órgão "protegido" da ação do sistema imunitário periférico. Componentes imunitários, tal como os linfócitos T e respetivas citocinas que estes produzem, outrora vistos apenas como prejudiciais para o cérebro, são agora considerados partes integrantes do sistema nervoso, uma vez que a sua ação regulada controla a vigilância imunitária mas também a modulação de funções cerebrais superiores.

O interferão gama (IFNγ), uma citocina produzida principalmente por linfócitos T, é uma molécula pro-inflamatória cujos níveis estão alterados em diversas doenças neuropsiquiátricas e neurodegenerativas. Apesar de estudos demonstrarem que a administração desta citocina no cérebro afeta diferentes mecanismos celulares e sinápticos que estão na base de dimensões comportamentais, não é ainda claro se este efeito é um dano colateral da resposta inflamatória ou se o IFNγ tem de fato um papel na modulação da função do cérebro num contexto não patológico. Como tal, iremos aqui explorar o papel desta citocina na modulação de funções cerebrais em condições fisiológicas como também após exposição ao stress crónico – um paradigma que desencadeia o desenvolvimento de complicações psiquiátricas e acelera processos neurodegenerativos.

Na primeira parte da presente tese (2º Capítulo) nós demonstramos que a ausência de IFNγ no cérebro saudável leva a uma melhoria da plasticidade do hipocampo dorsal e função cognitiva associada. Ao nível estrutural, um aumento do volume do hipocampo dorsal contrasta com a ausência de alterações volumétricas na parte ventral, sublinhando assim que esta citocina afeta seletivamente o comportamento cognitivo. A ausência desta citocina leva também a uma amplificação dos fenómenos neuroplásticos do hipocampo dorsal, nomeadamente da neurogénese, o tamanho da arborização dendrítica neuronal e o funcionamento pré-sináptico, contribuindo, muito provavelmente, para a melhoria da performance cognitiva.

No 3º capítulo, nós demonstramos que existem diferenças entre géneros no fenótipo comportamental dos murganhos IFNγ KO, e discutimos a possível associação entre o estrogénio e a expressão de IFNγ no sistema nervoso central.

No capítulo seguinte (4º Capítulo) descrevemos a otimização de um paradigma de stress crónico imprevisível (CUS) para murganhos C57BL/6, uma estirpe que apresenta uma maior resistência ao stress. Este modelo de murganho de disfunção associada ao stress apresenta para além das alterações

neuroendócrinas e comportamentais relacionadas com o stress, alterações moderadas nas populações celulares do timo e importantes alterações celulares mielóides no baço, sendo o aumento de neutrófilos a alteração mais impressionante.

Por último (5º Capítulo), discutimos o papel contributório desta citocina para o desenvolvimento da resposta imunitária maladaptativa ao stress crónico. Após expor murganhos ao protocolo otimizado de CUS foi observado um aumento nos níveis de mRNA de *Ifnγ*, mais especificamente nos córtices pré-frontal medial e orbitofrontal. Para além disso, a exposição ao stress crónico leva a um aumento da inervação adrenérgica do baço assim como a alterações nas percentagens das populações de neutrófilos e monócitos/macrófagos no baço. A ausência de IFNγ preveniu as alterações induzidas pelo stress crónico nestas populações celulares do baço.

O reconhecimento da citocina pró-inflamatória – IFNγ, como um regulador negativo da plasticidade hippocampal e função cognitiva associada, juntamente com o seu papel contributório para a disfunção imunitária associada com o stress sugere que esta citocina poderá articular a rede complexa que está na base da componente inflamatória das doenças neuropsiquiátricas.

Palavras-chave: IFNγ, cognição; neuroimunologia; stress crónico; neutrófilos

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ABBREVIATIONS LIST:

A

Aβ amyloid beta

ACSF artificial CSF

APP amyloid precursor protein

AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA analysis of variance

ATP adenosine triphosphate

B

BBB blood-brain barrier

BCSFB blood- cerebrospinal fluid barrier

BCG *bacillus Calmette-Guérin*

BDNF brain-derived neurotrophic factor

BrDU bromodeoxyuridine

C

CA *Cornu Ammonis*

CCL chemokine (C-C- motif) ligand

CCR C-C chemokine receptor

CD- cluster of differentiation

cDNA complementary DNA

CMS chronic mild stress

CNS central nervous system

CP choroid plexus

CSF cerebrospinal fluid

CUS chronic unpredictable stress

CXCL C-X-C motif chemokine

D

DAPI 4',6- diamidino-2-phenylindole

dCLN deep cervical lymph nodes

dHip dorsal hippocampus

DG dentate gyrus

DMEM Dulbecco's modified eagle medium

DN double-negative

DNA deoxyribonucleic acid

DP double-positive

E

EA Eosine Azure

EGL external granule layer

ELISA enzyme-linked immunosorbent assay

EPM elevated plus maze

EPSP excitatory postsynaptic potentials

F

FCT portuguese science Foundation

FST forced swim test

G

GADPH glyceraldehyde 3-phosphate dehydrogenase

GABA γ-Aminobutyric acid

GAS gamma activated site

GADPH

GFAP glial fibrillary acidic protein

GNP granule neuronal precursor

GluR1 Glutamate receptor 1

H

HPA hypothalamic pituitary adrenal axis

HSC hematopoietic stem cells

I

ICVS Life and Health Sciences Research Institute

ICAM1 intercellular adhesion molecule

IDO indoleamine 2,3 dioxygenase

IL interleukin

IFNγ interferon gamma

IFNγR interferon gamma receptor

ISF interstitial fluid

J

Jak Janus Kinase

Jnk c-Jun N-terminal kinase

K

KO knock-out

L

LPS lipopolysacharide

LTP long-term potentiation

LTM long-term memory

M

mRNA messenger ribonucleic acid

MHC major histocompatibility complex

mPFC medial prefrontal cortex

MS multiple sclerosis

MSC mesenchymal stem cells

MWM Morris Water Maze

MPTP1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

N

NCAM neural cell adhesion molecule

NK natural killer cells

NMDA *N*-methyl-D-aspartic acid

NOR novel object recognition

NSC neural precursor cell

O

OCT optimal cutting temperature

OF open field

OFC orbitofrontal cortex

O-2A/OPC oligodendrocyte-type 2 astrocyte progenitor cell

P

P post-natal day

PCR polymerase chain reaction

PFA paraformaldehyde

PDGFR platelet-derived growth factor receptor

PFC prefrontal cortex

PI3K Phosphatidylinositol 3-kinase

PKR protein kinase R

PP paired-pulse

PSA-NCAM Polysialic acid-NCAM

$p27^{Kip1}$ cyclin-dependent kinase inhibitor 1B

Q

qPCR quantitative real-time polymerase chain-reaction

R

Rb retinoblastoma protein

Rit Ras-like protein in tissue

RNA ribonucleic acid

S

sACSF sucrose-based artificial CSF

SEM standard error of the mean

SCID severe combined immunodeficiency

SGZ subgranular zone

SHH sonic hedgehog

SNS sympathetic nervous system

SP single-positive

STAT Signal transducer and activator of transcription 1

SVZ subventricular zone

T

Th T-helper

TH tyrosine hydroxylase

TMB 3,3′,5,5′-tetramethylbenzidine

TNF tumor necrosis factor

TREK1 TWIK-related potassium channel1

TST tail suspension test

TWIK - Tandem of P domains in a Weak Inward rectifying K^+ channel

U

uCMS unpredictable chronic mild stress

V

vHip ventral hippocampus

W

WT wild-type

THESIS ORGANIZATION

The present thesis is divided into 7 chapters. The 1st Chapter is an overall introduction to the theme, while the 2nd to 5th chapters refers to the experimental work (in form of manuscripts), the 6th chapter is dedicated to an overall discussion and conclusions and finally, the 7th chapter, describes the future perspectives of the project. The 1st Chapter is a review article to be submitted. The 2nd Chapter is an accepted article in the journal *Translational Psychiatry*. The 3rd Chapter is a manuscript under preparation. The 4th Chapter is a published article in the journal *Frontiers in Psychiatry*. The 5th Chapter is a manuscript under preparation.

In the **1st Chapter**, the impact of IFNγ on brain functioning is revisited in light of new developments in the field of neuroimmunology. It is described, with special emphasis on IFNγ, the newly vision on how immune cells and cytokines residing on nearby compartments of the brain are important determinants for brain normal function and behaviour. Then, the specific effects of IFNγ at the level of the brain parenchyma are discussed, namely the impact on cell genesis, synaptic plasticity and neurodegeneration, and correlated with putative pathological implications.

On the **2nd Chapter**, a thorough behavioural characterization demonstrates that the absence of IFNγ selectively enhances cognitive behaviours, namely those engaging the hippocampus. It is shown that mice lacking IFNγ present enlarged volumes of different hippocampal sub-regions, an effect that is restricted only to the dorsal part – known for its major role on regulating learning and memory processes. At the level of the dorsal hippocampus it is demonstrated that the absence of this cytokine enhances adult neurogenesis, dendritic arborisation having also a modest impact on presynaptic functioning. In this manuscript, is shown for the first time, that the pro-inflammatory IFNγ is a negative regulator of hippocampal cognitive functioning, since its absence boosts different hippocampal phenomena that most likely are contributing for the enhanced cognitive phenotype observed in these animals.

In the **3rd Chapter** is demonstrated that the behaviour of IFNγ KO mice differs according to their gender. While female IFNγ KO mice exhibited an enhanced cognitive phenotype with no differences on anxiety, the male IFNγ KO mice presented anxious-like behaviour with no alterations on cognition. It is

shown that the administration of the sex hormone, estrogen, is able to modulate IFNγ production by glial cells.

The **4th Chapter** is composed by a published manuscript where it is discussed difficulties in using the widely used chronic unpredictable stress (CUS) paradigms in C57BL/6 mice for mimicking the maladaptive chronic stress response, since this strain presents a higher threshold of stress resistance. As so, an optimisation of the current protocol used in rats and other strains of mice is presented and compared with the former. It is thoroughly described chronic stress-alterations in biometric, endocrine, behavioural and immune parameters. With this improved CUS protocol it was possible to have a mice model of chronic stress in C57BL/6 background exhibiting body weight loss, overactivation of the HPA axis, anxiety and depressive-like behaviours but very mild cognitive alterations. Of note, from the immune alterations a special reference for the splenic myeloid cells, being a raise on neutrophils the most remarkable change.

On the **5th Chapter**, is discussed the role of IFNγ in the immune stress-response, using our recently optimised protocol. After exposure to chronic stress, there is an increase on the mRNA expression of *Ifnγ* in the prefrontal cortex. In the spleen, the chronic exposure to stress led to an increase on adrenergic innervation as to alterations of neutrophils and monocyte/macrophages populations. Importantly, mice lacking the expression of IFNγ did not display these cellular alterations in the spleen suggesting that this cytokine may be involved in the immune maladaptive response to chronic stress.

On the **6th Chapter**, are discussed the main achievements of each chapter integrated in the current literature, as also discussed eventual limitations of the work.

On the **7th Chapter** are stated the planned future directions of this work.

SECTION I – INTRODUCTION

1st CHAPTER

Brain interference: revisiting the role of IFNγ in the central nervous system

Monteiro. S., Roque, S., Correia-Neves, M., Cerqueira, J.J.

(manuscript to be submitted)

Brain interference: revisiting the role of IFNγ in the central nervous system

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Abstract

IFNγ is a pro-inflammatory cytokine first described as a secreted molecule capable of interfering with viral replication. Since then, this pleiotropic cytokine has been endorsed with many other important actions in the context of the immune response to invading pathogen (including those invading the brain) already extensively characterized. Yet, this hitherto immune molecule seems also to play a still-enigmatic role in neuropsychiatric disorders as well as in the regulation of normal brain function.

In this Review, we update the current knowledge of the immune action of this cytokine and integrate it with the growing body of evidence involving IFNγ on CNS cell genesis, plasticity and neurodegeneration and, discuss the putative pathological implications.

Introduction

If the history of cytokines was to be written, the discovery made by Alik Isaacs and Jean Lindenman in 1957 would make an excellent chapter. This remarkable breakthrough in the virology field first describes "interferon (IFN)"- a released substance that has the ability to interfere with subsequent viral infections allowing the invaded cell to survive (A. Isaacs & Lindenmann, 1957). Only a few years later was the substance "interferon" purified and three molecules isolated: the IFNα, β and the third one identified in 1965 by Wheelock – the IFNγ. These were exciting timings, but these scientists, at that time, were probably far from imagining that the pleiotropic cytokines they discovered had, besides their classical immune pro-inflammatory role in the immune response to infection and anti-tumoral defence, still intriguing roles in the modulation of brain function on health and disease.

IFNγ is a pro-inflammatory cytokine, belonging to the type II family of interferons. It is mainly produced by T lymphocytes, natural killer (NK) cells and NKT cells although other cells have been shown to also produce this cytokine. Its production has been mostly associated with the immune response to intracellular pathogens and is an important molecule both for the innate as for the adaptive immune systems. Its actions contemplate a vast array of immune responses, namely: the activation of macrophages (Mosser, 2003), the up-regulation of the major histocompatibility complex (MHC) expression (Giroux, Schmidt, & Descoteaux, 2003) and the modulation of the type of T helper (Th) response (Bradley, Dalton, & Croft, 1996; Smeltz, Chen, Ehrhardt, & Shevach, 2002).

In the healthy brain parenchyma T, NK and NKT cells are scarcely found, and for that reason the IFNγ impact on brain function was mainly discussed in pathological contexts as in infectious diseases of the central nervous system (CNS) or in diseases, such as Multiple Sclerosis (MS), cerebral trauma or stroke (Walter et al., 2011). Nonetheless, IFNγ is also involved in neuropsychiatric disorders, conditions for which the inflammatory component was not so evident and is starting to be unravelled.

Schizophrenic patients present lowered production of IFNγ that was correlated with acute exarcebation (Arolt, Rothermundt, Wandinger, & Kirchner, 2000) and polymorphisms of the *ifnγ* gene were also associated with the disease (H. J. Kim et al., 2012).

IFNγ serum levels have been shown to be increased in depression patients (Maes et al., 1994; Schmidt et al., 2014) and, in accordance, anti-depressants were shown to display immunomodulatory properties by reducing IFNγ levels while enhancing the anti-inflammatory cytokine interleukin (IL)-10 (Maes et al., 1999). Mice exposed to chronic mild stress (CMS), a paradigm that leads to the development of a constellation of symptoms resembling depression, exhibit increased serum levels of IL-2 and TNFα, which was blunted in stressed IFNγKO mice (Litteljohn et al., 2010). Moreover, the stress-induced

elevation on corticosterone serum levels, a central molecule in the development of depressive-like behaviour, was prevented in IFNγ knock-out (KO) mice (Litteljohn et al., 2010).

Conversely, peripheral infections can also trigger neurological/psychiatric complications. For instance, IFNγ mRNA levels in the hippocampus were increased after a respiratory infection, and more importantly, this was associated with a decreased performance in cognitive tasks (Elmore et al., 2014). Another example comes from the development of behavioural alterations after the infection with Bacillus Calmette-Guérin (BCG), a live attenuated form of the *Mycobacterium bovis* used as a vaccine. Interestingly, IFNγ was suggested to be required to the induction of BCG-related alterations on behaviour (O'Connor, Andre, et al., 2009).

Quite surprisingly, it also seems that IFNγ action could affect brain function even without an infection or an apparent inflammatory process. The first evidence that pointed out for this unexpected role of IFNγ on behaviour came from studies with mice with a target deletion of the *ifnγ* gene showing that the absence of this cytokine led to important alterations on emotional behaviours (Campos, Vaz, Saito, & Teixeira, 2014; Kustova, Sei, Morse, & Basile, 1998; Litteljohn et al., 2010), but more recent reports have showed that cognitive function can also be affected by this cytokine. Indeed, a recent report by Litteljohn et al. (Litteljohn, Nelson, & Hayley, 2014) showed that mice lacking IFNγ, when exposed to chronic stress, display a better cognitive performance than wild-type (WT) animals (Litteljohn et al., 2014). Of note, this seems to be also the case in humans. As an example, medical students with higher perception of stress before an exam display increased concentration of pro-inflammatory cytokines in the blood, including IFNγ, and lower levels of the anti-inflammatory cytokine IL-10 as well as IL-4 when compared with students with a low perceived stress (Maes et al., 1998).

Sure the above-mentioned reports represent substantial observations showing that IFNγ can impact brain function. Yet, the question remained: how could functioning of the brain, the so-called immune privileged organ, be affected by a cytokine that is produced by cells that seem to be absent from the brain parenchyma?

IFNγ-producing cells revisited

As stated earlier T, NK and NKT cells are undoubtedly the classical cell sources of IFNγ. However, in response to certain stimulus, it was demonstrated that other cell types have also the ability to produce IFNγ.

That IFNγ was able to activate macrophages was already an established concept, now the observation that macrophages could also produce its own IFNγ in response to IL-12 and/or IL-18 stimulation (Fig.1)

was a new-fangled finding (Munder, Mallo, Eichmann, & Modolell, 1998). Munder et al. demonstrated that while low amounts of IFNγ are produced in response to each of these interleukins separately, when administrated together they lead to a potent secretion of IFNγ, representing a novel cellular source of this cytokine (Munder et al., 1998). Moreover, the macrophage-derived IFNγ was able to act back on its own receptors and induce the production of more IFNγ in an autocrine way (Munder et al., 1998), a common form of signalling of the immune system.

Since the brain also has its own resident macrophages, the microglia, and since IFNγ can orchestrate microglia actions in a similar way to macrophages, the question of whether microglia could also produce IFNγ rapidly emerged. Wang et al. were the first to suggest that the macrophage ability to produce IFNγ was also transversal to microglia with the report of IFNγ-microglial production upon a *Toxoplasma gondii* infection (Fig.1) (X. Wang & Suzuki, 2007). Interestingly, Wang et al. further showed that this brain-derived IFNγ production was independent of T cells, since microglia from infected SCID mice (lacking T and B cells but not NK cells) also produced IFNγ (X. Wang & Suzuki, 2007), once more strongly suggesting that there are other cellular intermediates responsible for IFNγ production independently of its classical producing cells.

Since infection can be quite a unique situation, the question was reformulated to whether this microglial ability for IFNγ production was also true in the absence of an immune insult. The answer came in 2006 when Kawanocuchi et al. reproduced the Munder et al. findings in macrophages by demonstrating that stimulation of microglia with IL-12 and IL-18, or IL-12 alone also leads to IFNγ production (Fig.1) (Kawanokuchi et al., 2006). Curiously, the stimulation with IL-12 and IL-18 was also able to trigger IFNγ production by a subset of B cells. In Harris et al. [23] IFNγ production by B cells was detected after being stimulated with IL-12/IL-18 [23]. In addition, Bao et al. [22] show latter that this subset of B cells were able to mount an immune response by producing IFNγ to activate macrophages, in response to several immune challenges such as: *Listeria monocytogenes*, *Escherichia coli*, vesicular stomatitis virus and Toll-like receptor ligands [22].

Besides microglia, brain production of IFNγ can also be attributable to non-immune cell sources such as neurons and astrocytes (Fig. 1). Indeed, sensory neurons from cultured dorsal root ganglia express IFNγ, which was detected by immunohistochemistry. These sensory neurons not only express IFNγ as its respective cognate receptor - key components for the reported autocrine regulation of *ifnγ* gene expression in these cells (Neumann, Schmidt, Wilharm, Behrens, & Wekerle, 1997). In response to tumour necrosis factor (TNF)α (Xiao & Link, 1998) or in response to traumatic and metabolic injury (Lau & Yu, 2001) astrocytes represent other of the brain cellular sources of IFNγ (Fig.1). Wei et al.

demonstrated that IFNγ expression in the aged brain was mainly found in the epithelial cells choroid plexus (CP) and endothelial cells of the CP microvessels (Fig. 1) (Y. P. Wei et al., 2000), key structures on the interface of the brain parenchyma and the peripheral signals. However, further research should clarify to what extent the positive IFNγ immunolabelling in the CP is associated with IFNγ-producing T cells that have been shown to reside in the choroid plexus (Kunis et al., 2013).

Despite the addition of new cellular sources to the list of IFNγ-producing cells, the above-mentioned studies suggest that the production of this cytokine in the brain is still restricted to specific stimulus that most certainly are related to danger signals.

In clear contrast with the reduced number of cells capable of producing IFNγ, are the cells that express its receptor. Virtually all cells can express IFNγ receptor (IFNγR) making it tempting to think that an indirect signalling initiated by IFNγ at the periphery could represent an alternative mechanism underlying the IFNγ impact on brain function. This hypothesis is especially appealing in the absence of an evident immune insult, a scenario where the brain-barriers are well preserved. Hence, one attracting possibility would be that the classical IFNγ-producing cells might still play a role on brain function from neighbouring sites.

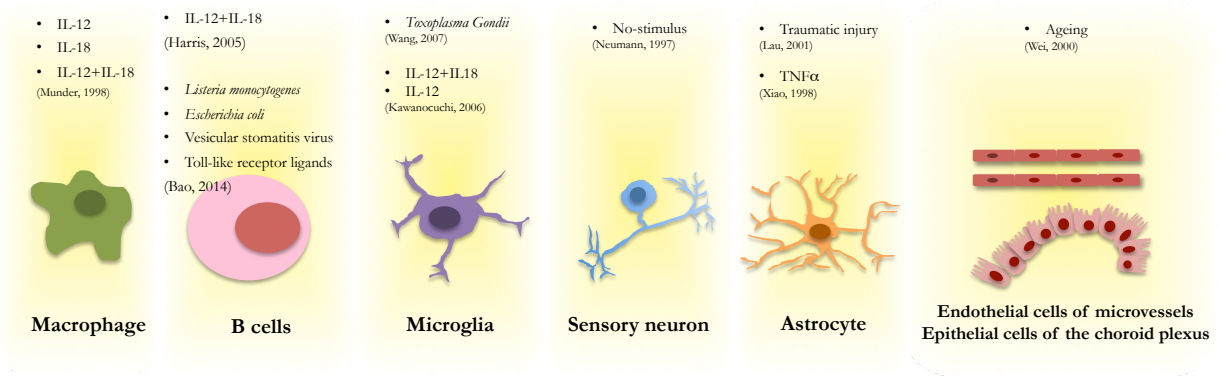

Fig. 1 – Non-classical cell sources of IFNγ. Studies demonstrate that, in response to specific stimulus, macrophages and B cells can produce its own IFNγ. In the CNS, microglia, sensory neurons, astrocytes, endothelial cells from microvessels and epithelial cells from the CP have been reported to have the ability to produce IFNγ in response to specific stimulus.

The concept of "immune privilege" revisited

The concept of "immune privilege" in respect to the brain was initially mainly associated with the presence of specialized barriers, such as the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB), that "protected" the brain from peripheral molecules that could represent insults for

the brain. At the initial periods the infiltration of peripheral immune cells into the CNS was regarded as a hallmark of pathology. However, in the last 20 years, this simplistic view has been overwhelmingly challenged being now accepted that while the classical immune activation is to some extent restricted at the brain parenchyma, immune cells do populate the CP, the cerebrospinal fluid (CSF) and meninges (Carrithers, Visintin, Viret, & Janeway, 2002), playing a crucial role on immune surveillance as in the support of classical brain function.

Produced by the CP the CSF is populated mainly by memory T cells. With more than 90% from the total CSF cells being memory T cells, less than 1% naïve T cells and a few B cells and monocytes, the CSF cellular composition differs largely from that of the blood (Kivisakk et al., 2003; Ransohoff & Engelhardt, 2012). It is believed that CSF memory T cells are particularly important for CNS immune surveillance, a concept extensively described in Ransohoff and Engelhardt (Ransohoff & Engelhardt, 2012). Briefly, memory T cells actively patrol the CNS by circulating in the CSF through the subarachnoid spaces where they can be re-activated upon encountering antigens presented by meningeal myeloid cells. This active immune surveillance is rapidly translatable into an efficient immune response in case of sensing pathogen or tissue damage (Hussain et al., 2014; Ransohoff & Engelhardt, 2012). The CP stroma also has its resident T cells although these differ from those of the CSF. Effector memory T cells represent more than 90% of the $CD4^+$ T cells in the CP stroma, expressing mainly the cytokines IL-4 or IFNγ in higher levels than those found in circulation and lymphoid organs (Baruch & Schwartz, 2013; Kunis et al., 2013) (Fig. 2).

Adding to their immune surveillance role, the T cells that populate the vicinity of the brain were shown to also support classical brain function. For instance, the ablation or reduction of T cells, using different genetically modified mice or pharmacological approaches, was shown to lead to impairment of cognitive function, and more importantly, those were reverted by the replenishment of normal levels/distribution of T cells (Brynskikh, Warren, Zhu, & Kipnis, 2008; Derecki et al., 2010; Kipnis, Cohen, Cardon, Ziv, & Schwartz, 2004; Wolf et al., 2009; Ziv et al., 2006). Meningeal T cells, for example, were reported to be of relevance for the maintenance of cognitive function, based on the observation that the demand from cognitive training results in an accumulation of IL-4 producing T cells in this compartment and consequently to a skewing of the meningeal myeloid cells to a M2 phenotype. Far more significant was the correlation of the meningeal M2 phenotype with improved spatial learning abilities (Derecki, Quinnies, & Kipnis, 2011) and increased brain-derived neurotrophic factor (BDNF) production by astrocytes which is further supported by the recognition that IL-4 supplementation is able to rescue the impaired cognitive function of IL-4 KO mice (Derecki et al., 2010; Radjavi, Smirnov, Derecki, & Kipnis,

2014) (Fig. 2). In other studies, T cells, specifically of the $CD4^+$ subset, were shown to support physiological hippocampal neurogenesis, since its depletion resulted in reduced neurogenesis together with worst performance in the Morris water maze, an effect not observed with $CD8^+$ T or B cells (Wolf et al., 2009; Ziv et al., 2006). Also involving T cells but this time in the CP, it was shown that enhancing the recruitment of these cells to the CP, improves the ability to cope with mental stress and rescues hippocampal BDNF to pre-stress levels (Lewitus & Schwartz, 2009).

It was suggested that antigen-specificity is a requirement for the T cells supportive role on cognitive function since transgenic mice with T cells that only recognize ovalbumin exhibit cognitive deficits that were reverted with adoptive transfer of $CD4^+$ T cells specific for a peptide present in the CNS (Radjavi, Smirnov, & Kipnis, 2014). In fact, it is known that brain specific proteins are actively drained into the deep cervical lymph nodes (dCLN) where they can be presented to local T cells (Fig. 2). In accordance the surgical removal of dCLN interrupts the flow of $CD4^+$ T cells into the meninges, and more importantly it results in the impairment of cognition (Radjavi, Smirnov, Derecki, et al., 2014).

Despite the reported advances, the question of how and where do these surveying and supportive immune cells enter and leave the CNS, is still to be elucidated.

Ransohoff et al. first explains three possible entry routes for peripheral T cells from the blood to the CNS: 1) the parenchymal perivascular space, 2) the subarachnoid space and 3) across the CP to the CSF, being this last one the most frequent in physiological conditions. The latter is characterised by a multi-step mechanism, in which the T cells at first travel from the blood to the CP stroma through the fenestrated endothelium of the CP vessels. In a second step, they mobilize from the stroma to the basolateral surface of the CP epithelial cell reaching the CSF and the subarachnoid space in a process orchestrated by adhesion molecules, chemokines and cytokines (Ransohoff, Kivisakk, & Kidd, 2003) (Fig. 2). Curiously, from these cytokines found in the CP, IFNγ was shown to have an important role as a regulator of peripheral T cells entry into the CSF across the CP. In response to IFNγ, the CP epithelial cells express the adhesion molecules ICAM-1 and immune cells chemoattractants like CXCL10 and CCL6, which will allow the T cells transmigration (Chang, Holtzman, & Chen, 2002) (Fig. 2). In accordance, mice with impaired IFNγ signalling (IFNγR KO) display reduced expression of molecules that regulate immune cell trafficking to the CP and therefore, reduced number of infiltrating $CD4^+$ T cells in the CSF (Kunis et al., 2013). Supporting the notion that the CP is crucial for the correct immune surveillance of the CNS is the study by Baruch et al. showing that danger signalling coming from the brain parenchyma is able to promote leukocyte recruitment across the CP (Baruch, Kertser, Porat, & Schwartz, 2015). Interestingly, a decreased IFNγ signalling at the CP is a common feature of ageing

(Baruch et al., 2014), but also of neurodegenerative disorders such as - Alzheimer's (Mesquita et al., 2015) and amyotrophic lateral sclerosis (Kunis, Baruch, Miller, & Schwartz, 2015), underlining the importance of the healthy immune surveillance capability to brain function.

Nevertheless, it is also conceivable that other sites for leukocyte entry to the CNS might be regulated by a similar mechanism as the one orchestrated by IFNγ at the CP. In support of this view Bittner et al. shows that treatment of both mouse and human endothelial and BBB cells with IFNγ together with TNFα also promoted leukocyte transmigration through a mechanism that was mediated by Tandem of P domains in a Weak Inward rectifying K^+ channel (TWIK)-related potassium channel-1 (TREK1) downregulation (Bittner et al., 2013), although is still unclear whether this T cell transmigration across the BBB may represent an alternative entry route for surveying/supportive T cells, or rather a mechanism underlying the pathophysiology of inflammation-mediated breakdown of the BBB.

At last, the very recent anatomical discovery of a brain lymphatic system definitely calls for a reappraisal of the immune cells and molecules circuits within the brain. This outstanding discovery ended with the long-held question on how did the surveying immune cells leave the CNS and also on how the CSF reach the dCLN. Louveau et al. and Aspelund et al. independently demonstrated that meningeal lymphatic vessels run along the sagittal venous sinus draining immune cells, small molecules and excess CSF from the CNS to the dCLN (Fig. 2). This represents a second stage of the CNS clearance pathway that is at first performed by the glymphatic system. In this system the CSF enters the brain parenchyma by following a para-arterial route exchanging solutes with brain interstitial fluid (ISF) - a process mediated by the astrocytic aquaporin 4 water channels (Iliff et al., 2012). The CSF carrying cleared soluble proteins, waste products and excess ISF is then drained into the lymphatic system. The lymphatic vessels leave the CNS through the foramina of the base of the skull alongside arteries, veins and cranial nerves into the dCLN (Aspelund et al., 2015; Louveau et al., 2015) (Fig. 2). These meningeal lymphatic vessels express specific markers of lymphatic vessels like those of other body parts and CCL21 – a chemoattractant that binds to CCR7 typically expressed in memory T cells.

The discovery of the brain lymphatic system is an exciting finding not only because it represents a route for specialized immune cells to transport brain antigens into the dCLN, a more classical form for peripheral T cell activation, but also because malfunction of this system results in the accumulation of waste products in the brain – a common feature of many neurodegenerative diseases.

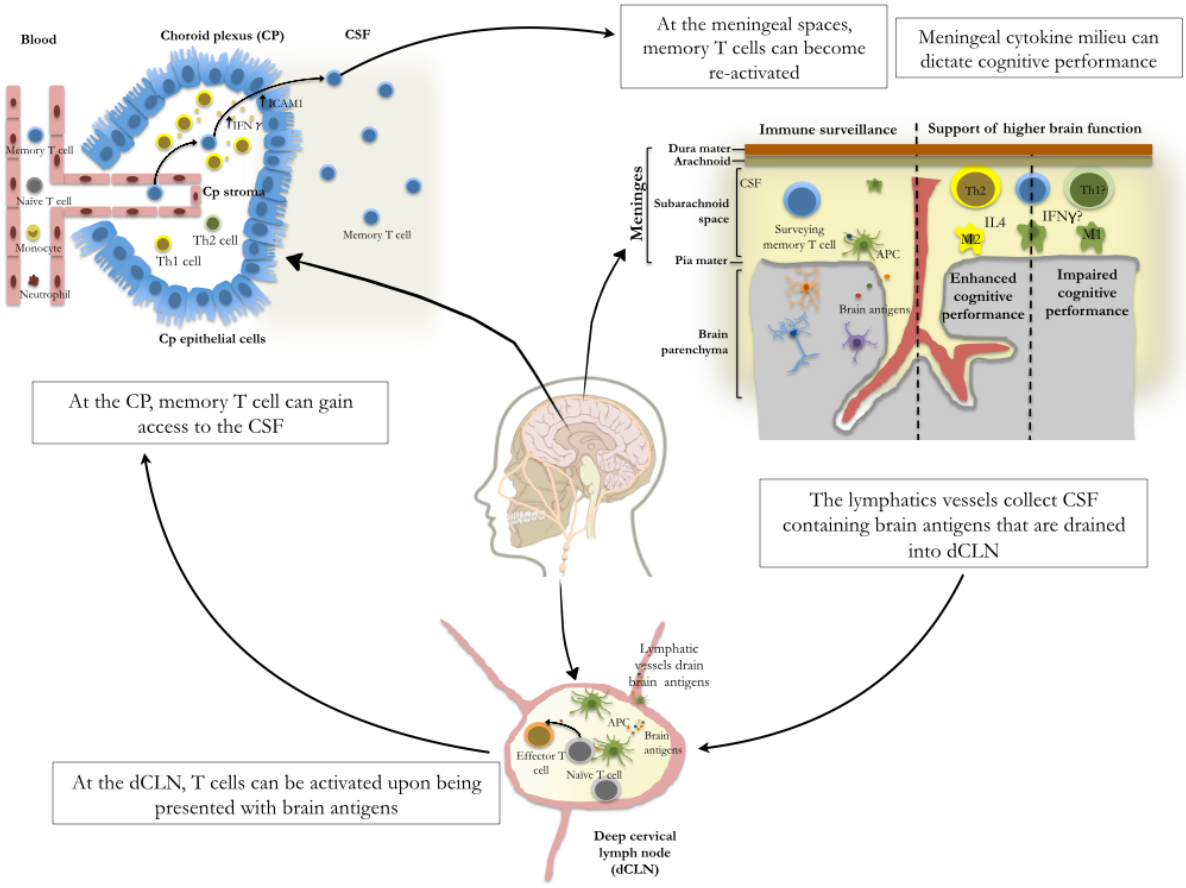

Fig. 2 – Neuroimmune modulation of the CNS. Brain lymphatic vessels transport CSF containing immune cells and also brain antigens draining it to the deep cervical lymph node (dCLN). Brain antigens can be presented to naïve T cells at the dCLN, that upon activation can mobilize in direction of the CNS. Depending on the levels of IFNγ in the choroid plexus (CP), transmigration of memory T cells from the blood into the CSF can occur in a multi-step mechanism. The CSF is rich in memory T cells that patrol the CNS by actively sensing the environment. At the meningeal spaces, they can encounter pathogens or danger signals presented by local myeloid cells and become rapidly re-activated. In addition, meningeal T cells are essential for higher brain function being the flow of T cells into the meninges but also their state of activation determinants of cognitive performance.

IFNγ cell signalling revisited

IFNγ classically acts by binding to its receptor - a multimeric complex composed by two chains, the cell-surface α-chain and a transmembranar β-chain. IFNγR α-chain binds IFNγ with high affinity causing the dimerization with β-chain. This dimerization induces the IFNγ signalling cascade activation through the Janus Kinase – signal transducer and activator of transcription (JAK-STAT) pathway. JAK-1 associates with the IFNγR α-chain while JAK-2 associates with β-chain. Activation of both JAKs 1 and 2 leads to the phosphorylation of (STAT)-1α, which in turn binds to gamma-interferon activation site (GAS), a specific DNA element present in several genes (Popko, Corbin, Baerwald, Dupree, & Garcia, 1997). In addition, IFNγ can also activate alternative signalling pathways such as CRK - a nuclear adaptor protein

for STAT5, or phosphatidylinositol 3-kinase (PI3K), important signaling cascades regulating cell cycle, proliferation and differentiation.

The multi-functionality of IFNγ can be partially attributed to the fact that more than 200 different genes possess the GAS element that together with the possibility of triggering different signaling cascades renders this cytokine capable of eliciting different cellular responses. Similarly, the activation of these same signaling pathways but now in the CNS can have a multiplicity of outcomes ("interferences") on brain function, as we will discuss below.

Interfering with cell genesis

The IFNγ anti-proliferative function is crucial for inhibiting viral replication, but likewise it may also interfere with physiological cell genesis (summarized in Table 1).

One example comes from the impact that IFNγ may have on haematopoiesis, an essential process in which new blood cells, including immune cells, are originated from hematopoietic stem cells (HSC). In de Bruin et al. it is demonstrated that IFNγ inhibits HSC proliferative capacity in the course of an infection through the phosphorylation of STAT5, an important transcription factor for many cell-cycle genes (de Bruin, Demirel, Hooibrink, Brandts, & Nolte, 2013). In a different study, IFNγ is now described as a promoter of long-term repopulating HSC proliferation (Baldridge, King, Boles, Weksberg, & Goodell, 2010). These findings, although with the divergent outcomes to be further elucidated, highlight the ability of this cytokine to affect normal cell genesis, and particularly proliferation.

IFNγ was also shown to inhibit the proliferation of both mouse and human mesenchymal stem cell (MSC), but perhaps more appealing for the scope of this review, is the report that it also modulates the neural differentiation potential of human MSCs. This effect is mediated through the activation of indoleamine 2,3-dioxigenase (IDO) and consequently of the kynurenine pathway (Croitoru-Lamoury et al., 2011).

Neural stem cells (NSCs) are self-renewing cells that can give rise to neurons, astrocytes and oligodendrocytes in response to certain environmental stimuli. They are particularly important for neurodevelopment but also exist in the adult brain where they are primarily located in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampal dentate gyrus. Control of NSCs self-renewal, proliferation and differentiation has been attributed to several different cytokines, and IFNγ is not an exception.

Both proliferating and differentiated NSCs express the IFNγR (Makela, Koivuniemi, Korhonen, & Lindholm, 2010; Walter et al., 2011) and *in vitro* stimulation with IFNγ was shown to inhibit

neurosphere formation from NSCs isolated both from the neonatal and adult SVZ (Li, Walker, Zhang, Mackay, & Bartlett, 2010; Makela et al., 2010). Also interesting is the observation that the IFNγ inhibition of neurospheres formation is a direct effect not dependent on other cellular intermediates since it was observed in a single-cell culturing system (Li et al., 2010). In an independent study, it was also reported the IFNγ–induced reduction on neurosphere formation, being an effect mediated by the cyclin-dependent kinase inhibitor p21 (Makela et al., 2010). In line with studies based on IFNγ administration, SVZ cells from IFNγ KO mice were shown to originate more and larger neurospheres and display increased self-renewal capacity when compared to WT (Li et al., 2010).

The same inhibitory effect of IFNγ on proliferation was also demonstrated in oligodendrocyte-type 2 astrocyte progenitor cells (O-2A/OPCs) – a type of progenitor cell that populates the CNS giving rise to oligodendrocytes and astrocytes. IFNγ reduced the platelet-derived growth factor receptor (PDGFR)-α and Ki-67 - important proliferation regulators. Once again, the reduced number of proliferating cells was not due to cell death but to an arrest of the cell cycle as shown by the upregulation of cyclin-dependent kinase inhibitor 1B ($p27^{Kip1}$) and retinoblastoma protein (Rb)(Tanner, Cherry, & Mayer-Proschel, 2011).

One interesting possibility would be that the reduction on NPC proliferation induced by IFNγ would represent rather an increased differentiation of these cells into neurons, an important step of neurogenesis. In accordance, Kim et al. showed an enhancement of differentiation promoted by IFNγ with the observation of increased expression levels of the neuronal marker βIII-tubulin - an effect mediated through c-Jun N-terminal Kinases (JNK) (S. J. Kim et al., 2007). Also supporting this idea is a recent *in vivo* study in which was demonstrated that IFNγ decreases the proliferation of $Nestin^{+}$ progenitor cells in the SVZ while enhancing the formation of neuroblasts, through STAT1 (Pereira, Medina, Baena, Planas, & Pozas, 2015). Indeed, the formation of neuroblasts in the SVZ is a known step of neurogenesis in this area that is followed by the migration of these new neurons through the rostral migratory stream to be integrated in the olfactory bulb. Surprisingly, in this study no alterations were observed in the olfactory bulb despite the observed increased differentiation of progenitors cells into neuroblasts (Pereira et al., 2015).

The increased differentiation along with reduction of NPCs proliferation, promoted by IFNγ treatment, was also reported in Walter et al., nevertheless a dysfunctional differentiation was also described leading to aberrant cells expressing both neuronal and glial markers (βIII-tubulin and glial fibrillary acidic protein (GFAP)) and displaying deregulated functional and molecular properties. In fact, IFNγ led to the activation of an abnormal downstream signalling, with an upregulation of sonic hedgehog (SHH)

pathway together with an overall downregulation of the pro-neurogenic factors: Pax6, Math1, Mash1, Neurogenin1 (Walter et al., 2011).

Actually, the SHH pathway was associated with another intriguing finding, that contrarily to what happens with postnatal or adult, embryonic neurospheres treated with this cytokine display increased proliferation (Li et al., 2010). The first evidence that IFNγ was a regulator of SHH, an instrumental signalling pathway for brain development, came from *in vitro* studies showing an increase of the SHH protein caused by IFNγ. Binding of STAT1 to the SHH promoter activates a cascade that culminates in the augmentation of granule neuron precursor cell (GNP) proliferation. In accordance, blockage of SHH blunted the proliferative action of IFNγ on GNPs (Sun, Tian, & Wang, 2010). In support of the *in vitro* findings, inducible-expression of IFNγ in the prenatal mouse brain leads to cerebellar dysplasia characterized by a persistent and abnormal proliferation of granule neurons in the external granule cell layer (EGL) of the cerebellum. This abnormal proliferation pattern was shown to disrupt granular cell migration from the EGL through the molecular layer into the internal granule cell layer of the cerebellum, an effect mediated by the expression of SHH by granule neurons (J. Wang, Lin, Popko, & Campbell, 2004). In transgenic models in which IFNγ is overexpressed in the CNS or specifically in astrocytes, by using either a transcriptional regulatory element of the myelin basic protein (MBP) gene (Corbin et al., 1996) or glial fibrillary acidic protein (GFAP) gene (LaFerla, Sugarman, Lane, & Leissring, 2000) cerebellar dysplasia and high hypomyelination in the cerebellum and hippocampus was also observed. In addition, these animals display severe ataxia and die before reaching sexual maturity (Corbin et al., 1996; LaFerla et al., 2000).

Conversely, in IFNγ KO mice neurogenesis was enhanced in the SVZ, an observation supported by the increased numbers of bromodeoxyuridine (BrdU)-positive cells together with a rise on newborn neurons in the olfactory bulb (Li et al., 2010). In clear contrast are the studies on the expression of IFNγ in Alzheimer's disease models, where limited production of this cytokine in the brain seems to actually support neurogenesis (Baron et al., 2008; Mastrangelo, Sudol, Narrow, & Bowers, 2009), an effect that may be possibly related with the impact of the immune function of this cytokine on the inflammatory component of the disease.

Other puzzling observation is the differential effect of IFNγ in the fate of NSCs. As opposed to neurons and oligodendrocytes, differentiation to astrocytes is actually enhanced by IFNγ treatment (Tanner et al., 2011). This differential effect on astrocytes is also supported by others studies such as in Li et al. in which isolated neurospheres from IFNγ KO mice display increased capability of differentiating into neurons and oligodendrocytes although giving rise to less differentiated astrocytes when compared to

WT. This study also showed that treatment of neurospheres with IFNγ mirrored these results, with enhanced differentiation of astrocytes and less of neurons and oligodendrocytes when compared to non-treated neurospheres (Li et al., 2010). It may be possible that this increase on astrocytes may be part of the IFNγ-immune response since several studies demonstrate that IFNγ can grant astrocytes the ability to act as non-professional antigen presenting cells through an upregulation of MHC expression (Jarosinski & Massa, 2002; Vardjan et al., 2012), although this issue needs to be further addressed.

Table 1 – summary of the studies describing the effect of IFNγ on cell genesis.

Measure	Experiment	Model	Outcome	Reference(s)
Proliferation	*In vitro*	Hematopoietic stem cells (HSC)	Inhibited	(de Bruin et al., 2013)
		Hematopoietic stem cells (HSC)	Enhanced	(Baldridge et al., 2010)
		Mesenchymal stem cells (MSC)	Inhibited	(Croitoru-Lamoury et al., 2011)
		Oligodendrocyte-type 2 astrocyte progenitor cells (O-2A/OPCs)	Inhibited	(Tanner et al., 2011)
		Embryonic neurospheres	Enhanced	(Li et al., 2010)
		Granule neuron precursor (GNP)	Enhanced	(Sun et al., 2010)
	In vivo	Neural stem cells (NSC) from the subventricular zone (SVZ)	Decreased	(Pereira et al., 2015)
		Mice with pre-natal inducible-brain expression of IFNγ	Increased but dysfunctional cerebellar proliferation	(J. Wang et al., 2004)
		Mice with inducible IFNγ expression in astrocytes	Cerebellar dysplasia; hypomyelination; severe ataxia; death before puberty	(Corbin et al., 1996; LaFerla et al., 2000)

Neurosphere formation	*In vitro*	NSC	Inhibited	(Li et al., 2010; Makela et al., 2010)
		NSC from IFNγ KO	Enhanced	(Li et al., 2010)
Differentiation	*In vitro*	MSC	Enhanced	(Croitoru-Lamoury et al., 2011)
		NSC?	Enhanced	(S. J. Kim et al., 2007)
		NSC	Increased but dysfunctional	(Walter et al., 2011)
	In vivo	SVZ	Increased	(Pereira et al., 2015)
Neurogenesis	*In vivo*	IFNγ KO mice	Increased	(Li et al., 2010)
		Alzheimer's disease model producing limiting amounts of IFNγ	Increased	(Baron et al., 2008; Mastrangelo et al., 2009)

Interfering with synaptic plasticity

The brain is a highly dynamic structure being able to reorganize its neural circuits forming new connections between neurons (synapses), and/or ending others in the course of learning a new task, in response to environmental stimuli or even in the recovery of a damaged area. Depending on their activity, synapses can be strengthened or weakened over time, a phenomena highly dependent on neurotransmission but also on dendritic remodelling.

Glutamatergic transmission

Glutamate is one of the most abundant neurotransmitters in the brain and an important excitatory molecule involved in synaptic plasticity believed to be central for learning and memory processes. However, excessive glutamate on the synaptic cleft and over glutamatergic receptor stimulation leads to excitotoxicity, which is characterised by high influx of calcium ions triggering an enzymatic cascade that culminates in neuronal damage by overstimulation. The excitoxicity is a common underlying mechanism of neurodegenerative diseases such Alzheimer's, Parkinson, Multiple Sclerosis, and others.

The involvement of IFNγ with excitatory activity comes from observations that many CNS-viral infections, in which the IFNγ levels typically peak, are accompanied by seizures – for which glutamatergic transmission is central. Infection of C57BL/6 mice with West Nile virus induced seizures that were absent in IFNγ KO mice although both strains presented similar levels of the virus in the brain. However, seizures were not prevented when chimeric C57BL/6 were reconstituted with IFNγ KO mice bone marrow which suggests that IFNγ may play a role in seizures-related circuitries during brain development rather than act directly in the adult brain. Nevertheless, the induction of seizures with the glutamate receptors agonists - N-methyl-D-aspartate (NMDA) or kainic acid was diminished/abolished in the chimeric mice reconstituted with IFNγ KO bone marrow. However, seizures were not prevented in these chimeric mice when induced by administration of Pentylenetetrazol, an antagonist of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) receptor, which probably indicates that GABAergic transmission is not involved in the IFNγ-inducted seizures (Getts et al., 2007).

The IFNγ involvement in excitatory activity in a pathogen-free brain context is demonstrated in Muller et al. report (Muller, Fontana, Zbinden, & Gahwiler, 1993), in which IFNγ added to hippocampal slices produced a clear excitatory effect on pyramidal neurons of CA3 and decreased evoked inhibitory postsynaptic potential amplitude. After around 50min of the initial administration of IFNγ, abnormal epileptic burst behaviour was observed in these pyramidal neurons. This delayed response of IFNγ on excitatory activity is also described in Vikman et al. (Vikman, Owe-Larsson, Brask, Kristensson, & Hill, 2001) that demonstrate that although no major alterations were observed after the acute administration of IFNγ, after 48h it was observed a spontaneous excitatory postsynaptic potential (EPSP) mediated through the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (Vikman et al., 2001). Still to understand is if this delayed response of IFNγ on excitatory activity is due to the activity of other molecular intermediates or if IFNγ exerts a direct effect.

Based on the excitatory potential of IFNγ in the glutamatergic system it is possible that some of the IFNγ-induced neuronal damage may be mediated through excitotoxicity. Indeed, treatment of cortical neurons with IFNγ led to a reduction of intracellular ATP and to the formation of dendritic beads – a classical presentation of dendritic damage caused by excitotoxicity. This dendritic beads formation might be mediated by the activation of the IFNγR and the AMPA/kainate receptors. This has been shown since the antagonists for each one of these receptors prevent dendritic bead formation but not MK-801, an NMDA receptor antagonist. Furthermore it was, shown by immunoprecipitation, that binding IFNγR to the AMPA receptor subunit GluR1 gives rise to a unique complex, which is phosphorylated at serine 845 triggering the JAK1, 2/Stat1 pathway. The activation of this pathway

culminates in the calcium influx, production of nitric oxide and dendritic bead formation, common features of excitotoxic damage (Mizuno et al., 2008).

In a different brain region, the suprachiasmatic nucleus, a 'biological clock' that synchronizes physiological daily rhythms and recently involved in neurodegenerative diseases, patch-clamp analyses show that IFNγ chronic treatment leads to decreased spiking activity as well as a reduction of AMPA receptors expression (Kwak et al., 2008). This decrease on synaptic plasticity was independent of GABAergic activity since the addition of the $GABA_A$ receptor antagonist – bicuculline, did not lead to alterations on spontaneous spiking frequency. More importantly, this changing in spiking patterns has a functional correlate since IFNγ treatment led to a decrease on circadian clock gene expression (Kwak et al., 2008).

The protein kinase RNA-activated - PKR, that senses double-strand viruses, stopping translation into proteins and therefore crucial for a protective immune response against viral defence, was shown to have a crucial role on cognitive function through IFNγ action (Cohen-Chalamish et al., 2009). In accordance with the above-mentioned studies, the suppression of PKR is accompanied by an increase on IFNγ and induces hippocampal and cortical hyperexcitability. Importantly this hyperexcitability was shown to be mediated through IFNγ decrease on GABAergic synaptic transmission. Importantly both the genetic as well as the pharmacological suppression of PKR led to an enhanced induction of the late LTP and enhance long-term memory (LTM) (Zhu et al., 2011). Moreover, the IFNγ-mediated reduction of GABAergic inhibition was also demonstrated in a separate study and this time in the spinal cord horn (Vikman, Duggan, & Siddall, 2007). Furthermore linking the GABAergic transmission to IFNγ is the report of Wei et al. (M. Wei et al., 2010) showing that agonists of benzodiazepines receptors (which are themselves components of GABA receptors) inhibit the production of this cytokine, which highlights the possibility of this receptor being involved in the development of anxious-like behaviour associated with IFNγ.

Long-term potentiation (LTP)

Also occurring at the excitatory synapses, LTP is a form of long-term plasticity that may occur for minutes or even hours and underlies learning and memory acquisition. Knowledge on IFNγ involvement on LTP comes from studies addressing the impact of neuroinflammation in the LTP decline seen with aging. Griffin et al. demonstrate that the decreased LTP response seen in aged animals is accompanied by the increased levels of hippocampal IL-1, IL-18 and IFNγ (Griffin et al., 2006). Later Maher et al.

demonstrates that intracerebroventricular administration of IFNγ abrogates the LTP response concurrently with an increase on hippocampal IL-1 levels (Maher, Clarke, Kelly, Nally, & Lynch, 2006).

Serotonergic signalling

The monoamine neurotransmitter serotonin is synthesized from tryptophan being important for the regulation of mood, appetite and sleep, and the restoration of its normal levels at the synaptic cleft has been considered of relevance for depression treatment. The association of the tryptophan metabolism with serotonin depletion as an underlying mechanism for depressive-like behaviour was first reported nearly 50 years ago (Lapin & Oxenkrug, 1969). IFNγ is capable of inducing IDO, a rate-limiting enzyme of tryptophan catabolism, and therefore shifting the availability of this aminoacid essential for the serotonin production into the kynurenine pathway, inducing the neurotoxic metabolites quinolic acid and 3-hydroxy-kinurenine. Moreover, IDO has been shown to be an important mediator of the depressive-like behaviour and impaired recognition memory (Heisler & O'Connor, 2015; H. Kim et al., 2012; O'Connor, Lawson, et al., 2009). In fact, the IFNγ-induced depletion of serotonin through the kynurenine pathway is suggested to explain the association of this cytokine with the pathophysiology of aging-related neuropsychiatric disorders and also, depression (Myint et al., 2013; Oxenkrug, 2011).

Dendritic remodelling

Dendrites are neuronal projections that receive synaptic inputs and are critical for transmitting information. Each dendritic tree can combine information from multiple synapses with different axons in a single neuron. The dendritic tree is highly plastic and can be rapidly shaped by different stimuli. IFNγ influences synaptic connectivity in the brain by selectively retracting dendrites. Indeed, IFNγ administration to cultured sympathetic and hippocampal neurons induced a reduction of 88% of the dendritic arbor and inhibited synapse formation without affecting axonal outgrowth or cell survival. This was an effect mediated by STAT1 since the expression of a dominant-negative STAT1 mutant blunted the dendritic retraction. Furthermore the exposure of the axonal part to IFNγ also led to STAT1 mediated dendritic retraction and synaptic loss (I. J. Kim, Beck, Lein, & Higgins, 2002). Additionally, the *Ras*-like protein in tissue (Rit)-p38 pathway was demonstrated to be involved in the IFNγ induced dendritic retraction since IFNγ administration caused a rapid Rit activation. Conversely the expression of a dominant negative Rit mutant inhibited the dendritic retraction observed after IFNγ administration (Andres, Shi, Bruun, Barnhart, & Lein, 2008).

Interfering with neurodegeneration

One of the mechanisms of the anti-microbial/anti-tumor response of macrophages is the production of nitric oxide upon IFNγ activation. Similarly, microglia when activated with IFNγ as well as other stimuli like lipopolysaccharide produces nitric oxide. However this microglial-mediated production of nitric oxide was shown to lead to a marked decrease on neuronal survival (Chao, Hu, Molitor, Shaskan, & Peterson, 1992). Analogously, astrocytes may also be mediators for neurodegeneration upon activation with this cytokine. Indeed, Lee et al. showed that conditioned media from human astrocytes treated with IFNγ, led to the loss of viability of a human neuroblastoma cell line, the SH-SY5Y cells. The astrocytes' neurodegenerative potential was shown to be mediated through the production of secreted substances such as: IL-6, glutamate, proteases, oxygen-free radicals and prostaglandin, since the blockage of each of these components from the glial conditioned media, reduced the loss of cell viability. Conversely, the addition of each of these secreted components individually contributed to an overall viability loss of SH-SY5Y cells comparable to that induced by the astrocytes' conditioned media (Lee, McGeer, & McGeer, 2013). Other study also demonstrated that astrocytes may be activated by IFNγ together with IL-1 β leading to the production of TNFα (Chung & Benveniste, 1990). The glial activation with the production of inflammatory mediators is a classical component of neuroinflammation, a chronic form of CNS inflammation leading to neuronal damage and loss. Not surprisingly, IFNγ, probably due to its ability to induce neuroinflammation, has been shown to be involved in some neurodegenerative diseases, as it will be discussed below.

IFNγ and Parkinson's disease

Parkinson's disease is a neurodegenerative disease characterized by dopaminergic cell loss in the substantia nigra and striatum leading primarily to motor deficits but also having an impact on emotional behaviours. The reports showing that Parkinson's patients and parkinsonian monkeys have increased levels of IFNγ in the blood (Barcia et al., 2012; Mount et al., 2007; Reale et al., 2009) prompted research on the possible involvement of this cytokine in Parkinson's pathophysiology. Barcia et al. demonstrates that IFNγ and TNFα act synergistically to induce the chronic glial activation observed in Parkinson's disease(Barcia et al., 2012), a mechanism known to induce neurodegeneration. Mount et al. show that conversely, mice deficient for IFNγ expression, display reduced substantia nigra and striatal cell loss after a challenge with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Additionally dopaminergic system components were also less affected in these IFNγ KO mice together with less microglial reactivity. Moreover, the deleterious effect of IFNγ on dopaminergic neurons seems to be

mediated by microglia, since only co-cultured neurons with microglia were vulnerable to the effect of IFNγ administration. Moreover, the rotenone-induced death of dopaminergic neurons was only observed when microglia was added to the culture and importantly, prevented when antibodies against IFNγ were added (Mount et al., 2007).

IFNγ and Alzheimer's disease

Alzheimer's disease is a neurodegenerative disorder characterized by an abnormal accumulation of extracellular amyloid-β (Aβ) deposits and hyperphosphorylation of tau protein leading to neurofibrillary tangles. Memory deficits that progress with age to dementia are typical clinical symptoms of the disease.

IFNγ involvement with this neurodegenerative disease comes especially for its role in Aβ pathology. Blasko et al. showed that the stimulation with a combination of IFNγ and TNFα can trigger the production of Aβ peptides in a human neuroblastoma cell line (Blasko, Marx, Steiner, Hartmann, & Grubeck-Loebenstein, 1999) and later reported the same effect but by astrocytes, since the co-stimulation of primary astrocytes or astrocytoma cells by IFNγ with TNFα, or IFNγ with IL-1β (but not with TNFα or IL-1β alone) triggered $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ production (Blasko et al., 2000). Moreover the *in vitro* pre-treatment of neuronal cells (cortical, cerebellar and SH-SY5Y neuroblastoma cells) with IFNγ sensitized these cells to amyloid-$\beta_{1\text{-}42}$ leading to neuronal death (Bate, Kempster, Last, & Williams, 2006). Furthermore the adoptive transfer of IFNγ producing Aβ-specific-CD4 Th1 cells into APP/PS1 mice, a model for Azheimer's disease, accelerated markers of the disease such as: increased microglial activation and Aβ deposition and more importantly were associated with cognitive decline, which did not happened with Th2 or Th17 cells (Browne et al., 2013).

Other pathway in which IFNγ may be involved Alzheimer's disease is through IL-12/IL23 signalling. IL-12 and IL-23 share a common sub-unit p40 that is increased in the cerebrospinal fluid from Alzheimer's disease patients. Genetic deletion of p40 subunit or the administration of p40 antibodies resulted in the amelioration of brain amyloid deposition and cognitive improvement in APP1 transgenic mice (Vom Berg et al., 2012).

More recently, the specific role of IFNγ in this disease started to be questioned after the study of Mastrangelo et al., suggesting that IFNγ could have contradictory roles in Alzheimer's pathology. While IFNγ overexpression in the brain during 10 months leads to an accumulation of intracellular Aβ, quite surprisingly, a reduction on phosphorylated tau levels was observed together with an increase on neurogenesis (Mastrangelo et al., 2009). In addition, Baron et al. also reported that enhanced

neurogenesis in the dentate gyrus induced by limited amounts of IFNγ improving spatial learning and memory. This effect was more pronounced in older mice both WT and in an Alzheimer's disease model (Baron et al., 2008).

IFNγ and Down syndrome

Although Down syndrome is primarily considered a developmental disorder characterised, among other symptoms, by intellectual disability and progressive memory loss, nearly all patients exhibit a neurodegenerative process resembling Alzheimer's disease at the age of 40 (Lockrow, Fortress, & Granholm, 2012). The extra chromosome in these patients codifies for the amyloid processing protein (APP) gene but possess also many IFNγ-related genes. It was demonstrated that the brain of trisomic mouse (Ts16), a mouse model for Down syndrome, significantly displays higher levels of IFNγ and Fas receptor (an apoptosis marker) immunoreactivity, and cultured Ts16 cortical neurons have higher concentration of caspase-1, altered calcium homeostasis and pH. The IFNγ addition to the cultured Ts16 cortical neurons led to neuronal apoptosis through caspase-1 activation (Hallam, Capps, Travelstead, Brewer, & Maroun, 2000). In accordance, treatment of cultured Ts16 cortical neurons with anti-IFNγ antibodies resulted in higher viability and lower apoptosis rates of these cells (Hallam & Maroun, 1998), highlighting the role of this cytokine in mediating apoptosis in this disease.

Concluding remarks

In the last decade, the neuroimmunology field has assisted to extraordinary challenges to its central dogma - the concept of the healthy brain being separated from the peripheral immune system by a set of barriers. Currently it is accepted that under physiological conditions these barriers are selectively permeable to peripheral immune cells that populate brain neighbouring sites, from where they play an essential role on supporting immune surveillance and higher brain function. Although, a significant amount of data has been dedicated to T cells and particularly to the T cell-derived IL-4 in this context, less is known about one of major cytokine of the opposite arm of T-cell response – the IFNγ.

In this review, we revisited the IFNγ action in the CNS and efforts were made in order to integrate it in light of this new framework of physiological neuroimmune modulation of the CNS (Fig. 2). A compilation of studies reveals here how this cytokine impacts important cellular and synaptic processes that are on the basis of different behavioural dimensions. Noteworthy, many of the behavioural effects of IFNγ are easily attributable to its immune function adjusted to a unique environment, which is that of the brain.

As so, it is logical that its anti-proliferative immune function may negatively interfere with active neurogenesis with implication in cognitive processing. Or for instance, its immune role as an activator of macrophages may parallel with the microglial activation ability that due to the excessive production of inflammatory metabolites culminates in neuronal damage, a common event in the pathophysiology of neurodegenerative diseases. Nonetheless, the impact of IFNγ in neurotransmission or in the dendritic retraction, mechanisms that are unique to the nervous system, make it tempting to regard this cytokine also as a neuromodulator rather than solely a classical immune cytokine.

Based on many of the studies discussed in this review it may be suggested that IFNγ plays an overall negative 'interference' in normal brain function. This may be a biased view since most of the studies describe a direct effect of IFNγ action in the brain parenchyma, not reflecting the complex network of neuroimmune interactions happening at the vicinity of the brain with important implications for brain function. An important example comes from the crucial role of IFNγ on enabling the regular entry of surveying/supportive immune cells across the CP, being the dysfunction of this transmigration deleterious for brain function.

Despite the efforts to compile a comprehensive and clear review of IFNγ action in the brain, data concerning this subject are in several instances still ambiguous and many times contradictory. Most likely, some of the apparent disparity between studies stems from differences in experimental conditions, namely being in vivo or in vitro, the amount of this molecule, the timing and the mode of administration, rather than simply its presence/absence. Indeed, *in vitro* studies showed that IFNγ could induce or inhibit a neuroprotective phenotype on astrocytes depending on the amount of cytokine administrated. While low amounts were shown to induce the release of neuroprotectants and enhance glutamate clearance capacity, these effects are inhibited at high concentrations (Garg, Kipnis, & Banerjee, 2009). Likewise, disease context, stage of pathogenesis, and cellular/cytokine milieu also dictate the disease resolving and/or contributory nature of this cytokine, as seen, for instance in Alzheimer's disease models, in which the expression of IFNγ has been shown to be involved in the worsening of Aβ pathology being however able to enhance neurogenesis. Curiously, the adoption of anti-inflammatory strategies by using the anti-inflammatory cytokine - IL-10 has been shown to worsen plaque burden by blocking microglial phagocytosis with consequent worsening of cognitive performance in an Alzheimer's disease mice model. In line with these results, the IL-10 deletion was shown to ameliorate the disease (Chakrabarty et al., 2015; Guillot-Sestier et al., 2015). The above-mentioned studies highlight the idea that rather than trying to end with inflammation, the re-establishment of proper immune response balance may hold the key for neurodegenerative diseases treatment.

The necessity for a proper balance on brain immunity for optimal brain function is emphasized by the differential and multiplicity of outcomes of IFNγ in the CNS presented here. In that sense, and since this cytokine is altered in many neuropsychiatric and neurological disorders, components of its signalling pathway may represent attractive candidates for therapeutic targets being necessary further research on this topic.

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SECTION II – EXPERIMENTAL WORK

2nd CHAPTER

Absence of IFNγ promotes hippocampal plasticity and enhances cognitive performance

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Absence of IFNγ promotes hippocampal plasticity and enhances cognitive performance

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Running title: Absence of IFNγ enhances cognitive performance

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Abstract

Cognitive functioning can be differentially modulated by components of the immune system. Interferon-γ (IFNγ) is a pro-inflammatory cytokine whose production is altered in many conditions displaying some degree of cognitive deficits, although its role in cognitive functioning is still unclear. Here we show that the absence of IFNγ selectively enhances cognitive behaviours in tasks in which the hippocampus is implicated. Moreover, the absence of IFNγ leads to volumetric and cell density changes that are restricted to the dorsal part of the hippocampus. In the dorsal hippocampus, the absence of this pro-inflammatory cytokine leads to an increase in the numbers of newly-born neurons in the subgranular zone (SGZ) of the dentate gyrus (DG), an adult neurogenic niche known to support learning and memory, and to an enlargement of the dendritic arborisation of DG granule and *Cornu Ammonis (*CA)1 pyramidal neurons. Moreover, it also modestly impacts synaptic plasticity, by decreasing the paired-pulse facilitation in the Schaffer collateral to CA1 pyramidal cell synapses.

Taken together our results provide evidence that IFNγ is a negative regulator of hippocampal functioning, since its absence positively impacts on dorsal hippocampus structure, cell density, neuronal morphology, and synaptic plasticity. Importantly, these neuroplastic changes are associated with improved performance in learning and memory tasks. Therefore, blockage of the IFNγ signalling may present as promising therapeutic targets for the treatment of inflammation-associated cognitive dysfunction.

Keywords: IFNγ, cognition, hippocampus, learning, memory

Introduction:

The modulation of cognitive functioning by immune mediators is receiving increasing attention, as a common inflammatory component is recognized in many neurodevelopmental and neuropsychiatric disorders. The association of cytokines with cognitive functioning is underpinned by studies showing the differential action of these molecules on learning and memory (Depino et al., 2004; Derecki et al., 2010; Donegan, Girotti, Weinberg, & Morilak, 2014; McAfoose, Koerner, & Baune, 2009), synaptic plasticity (Balschun et al., 2004; I. J. Kim et al., 2002; Maher et al., 2006; Zhu et al., 2011) and neurogenesis (Butovsky et al., 2006; Depino et al., 2004; Derecki et al., 2010; Donegan et al., 2014; Johansson, Price, & Modo, 2008; Kelic, Fagerstedt, Whittaker, & Kristensson, 1997; I. J. Kim et al., 2002; Li et al., 2010; McAfoose et al., 2009; Sun et al., 2010; Walter et al., 2011). One of the most relevant cytokines in these interaction is Interferon-γ (IFNγ), a pro-inflammatory mediator produced mainly by T, natural killer (NK) and NT cells, whose levels are altered with chronic stress exposure (Maes et al., 1998), ageing (Oxenkrug, 2011; Y. P. Wei et al., 2000) and in several neuropsychiatric (Maes et al., 1998; Oxenkrug, 2011) and neurodevelopmental disorders (Nateghi Rostami, Douraghi, Miramin Mohammadi, & Nikmanesh, 2012), all of which present, to some extent, with cognitive impairment. Several studies have demonstrated that IFNγ is able to impact brain function by affecting systems that are on the basis of cognitive behaviors. For instance, different studies have highlighted the important role of IFNγ on the regulation of neural precursors cells (Butovsky et al., 2006; Li et al., 2010; Sun et al., 2010; Walter et al., 2011). Indeed, it was shown that neural precursor cells (NPCs) express the IFNγ receptor and that IFNγ reduces NPCs viability and increases cell death through caspase-3 expression (Makela et al., 2010). Furthermore, it has been shown that mice lacking IFNγ display increased neurogenesis in the sub-ventricular zone (SVZ) (Li et al., 2010), an adult neurogenic niche. Controversially, there are also some studies showing that IFNγ can increase proliferation of NPCs (Sun et al., 2010), or enhance neurogenesis in an Alzheimer's disease mouse model (Baron et al., 2008). In fact, it is known that IFNγ can have opposing effects on inhibiting or promoting proliferation depending on the molecular pathways activated (Asao & Fu, 2000). For instance, it has been shown that the increase on NPCs proliferation mediated through the sonic hedgehog pathway is dysfunctional in these mice leading to the differentiation of aberrant cells (Walter et al., 2011).

IFNγ was also associated with disturbances of the serotonergic signaling, since this cytokine activates the rate-limiting enzyme for tryptophan - indoleamine-pyrrole 2,3-dioxygenase (IDO) a serotonin precursor. In fact, disturbances of tryptophan metabolism caused by IFNγ have been discussed in the context of depression (Myint et al., 2013), ageing and ageing-related neuropsychiatric disorders

(Oxenkrug, 2011). Moreover, kynurenine metabolites which result from IDO activity, such as 3-hydroxy-kynurenine and quinolinic acid, are known to be toxic to the brain (Myint et al., 2013; Wichers et al., 2005).

Another line of research has consistently shown the importance of T lymphocytes in supporting cognition (Brynskikh et al., 2008; Kipnis et al., 2004; Kipnis, Gadani, & Derecki, 2012a; Serre-Miranda et al., 2015; Wolf et al., 2009; Ziv et al., 2006). For instance, training in a cognitive task was shown to induce the accumulation of interleukin-4 (IL4) producing T cells in the meninges, and conversely, mice lacking this cytokine exhibit cognitive deficits together with a skewed pro-inflammatory meningeal phenotype (Derecki et al., 2010). These are very interesting findings considering that one of the known biological functions of IL4 is the suppression of Th1 differentiation – cells for which IFNγ is considered the main effector cytokine.

Despite the above-mentioned studies and others showing that IFNγ can impact different brain functions, the role of IFNγ as a modulator of cognitive performance in the healthy brain is far from being understood. Therefore, we set out to: 1) understand if IFNγ is indeed a regulator of cognitive performance; 2) study the impact of IFNγ on structural and electrophysiological correlates of cognitive functioning in the hippocampus.

Herein we show that the absence of endogenous IFNγ leads to increased neurogenesis and dendritic length in the dorsal hippocampus that correlates with enhanced performance in learning and memory tasks; in addition, our results provide evidence of a brain regional selectivity for endogenous IFNγ action with the dorsal, but not the ventral, part of the hippocampus exhibiting the most significant alterations caused by the absence of this cytokine.

Material and methods

Animals

3 to 5-month old female IFNγ knock-out (KO) mice on C57BL/6 background (Dalton et al., 1993) and littermate homozygous wild-type (WT) were housed (5 per cage) under standard laboratory conditions [12 h light/12 h night cycles (8 h/20 h)], 22-24 °C, relative humidity of 55% and with *ad libitum* access to water and food. Cages were enriched with paper rolls and soft paper for nesting. All procedures were carried out in accordance to EU directive 2010/63/EU and Portuguese national authority for animal experimentation, Direção Geral de Veterinária (ID:DGV9457), guidelines on animal care and experimentation. Littermate WT were used as controls in all experiments to minimize genetic variability (Holmdahl & Malissen, 2012) and were obtained from an initial crossing of IFNγ KO mice with C57BL/6 mice (Charles River Laboratories, Barcelona, Spain) and by crossing the heterozygous progeny between them. Mice were sacrificed at 5-month of age. To exclude the possible impact of hormonal variability on the results, female mice were only sacrificed whenever they were at proestrus phase.

Behavioural assessment

Behavioural experiments were performed during the mice active period (between 8 pm and 8 am). Before behavioural testing, mice were gently handled by the same experimenter for 2 weeks every other day. Prior to each behavioural assessment mice were transported to the testing room and left for habituation to room conditions during 1 h. All behavioural data analysis was performed with the experimenter blinded to the genotype.

Morris Water Maze (MWM)

In order to assess spatial reference memory, 3-month old mice were tested in a white circular pool (170 cm diameter) filled with water (24 - 25 °C) placed in a dimly lit room. Spatial cues were placed in the walls around the pool (square, stripes, triangle and a cross). The pool was divided in 4 imaginary quadrants and a hidden transparent platform was placed in one of the quadrants. Data was collected by a fixed camera placed in the ceiling and connected to a video-tracking system (Viewpoint, Champagne-au-Mont-d'or, France).

Mice had to learn the position of a hidden platform over a period of 4 days. In each day, mice were placed facing the wall of the pool at different quadrants (north, west, south and east), in a pseudorandom order that varied from day to day, as a starting point for each trial. Each trial was

completed whenever the mouse reached the platform or when 120 s elapsed. Latency to reach the platform (escape latency) was recorded for each trial during the 4 days. In the 5th day, the platform was removed and a single trial of 60 s was performed (probe trial). The percentage of time that each mouse swam in each quadrant was recorded to confirm the acquisition of platform location through reference memory. Behavioural flexibility was assessed in the reversal task that took place on the 5th day immediately after the probe trial; briefly, the platform was positioned in a new (opposite) quadrant and the animals has 3 consecutive 120 s trials to find it in the new position. Latency to reach the platform (escape latency) in the new location was recorded for each trial.

Novel Object Recognition (NOR)

Recognition memory was evaluated by submitting animals to the NOR test. Briefly, 3-month old mice were habituated to a black box for 1 h in 3 consecutive days. In the 4th day, two similar objects were symmetrically placed in one side of the box and animals were allowed to freely explore both objects for 10 min. Mice were then immediately returned to their home cages and the box and objects were cleaned with a 10% ethanol solution. The test session was carried out 1 h later. One of the objects was replaced by a novel one (similar size but different colour and shape); each mouse was then reintroduced into its original test box where it was allowed to freely explore both objects for 5 min. The exploration time in each object was recorded. Animals were considered to be exploring whenever the nose was facing the object. Recognition memory was assessed by the discrimination ratio: time exploring the novel object minus time exploring the old object over the total exploration time.

Elevated-plus maze (EPM)

3-month old mice were tested for anxious-like behaviour using the EPM test. Briefly, this test consists on placing each mouse in the hub of a plus-like apparatus elevated 72.4 cm from the floor, with two opposing open arms (50.8cm x 10.2 cm) and two opposing closed arms (50.8 cm x 10.2 cm x 40.6 cm) (ENV560; MedAssociates, Inc., St. Albans, VT, USA) and letting the animal freely explore it for 5 min. Time in the open arms and in the closed arms was used as a behavioural parameter of anxious-like behaviour.

Open field (OF)

3-month old mice were tested for locomotor activity and anxiety-like behaviour using the OF. Each mouse was left in the center of a square arena (43.2 cm x 43.2 cm), which the mouse was free to explore for 5 min. This arena was equipped with infrared beams for activity detection (MedAssociates,Inc., St. Albans, VT, USA). Data were collected using the activity monitor software (MedAssociates,Inc., St. Albans, VT, USA). Distance travelled was used as a measure of locomotor activity. The ratio between time spent in the center (10.8 cm x 10.8 cm) and periphery of the arena was used as a measure of general anxiety.

Determination of estrous cycle stage

Vaginal smears were performed by inserting a drop of sterile 0.9% saline solution in the vagina with the help of a 1 ml syringe, collecting the cell suspension by inserting a small plastic inoculation loop and performing a smear into a glass slide. Smears were air-dried, fixed in alcohol 96% for 5 min and stained using the Papanicolaou protocol. Briefly, smears were hydrated in tap water, stained with Harris haematoxylin for 1 min, rinsed in running tap water for 2 min, regressively stained by a single-dip in a alcohol-acid solution, rinsed in tap water for 2 min, dehydrated in alcohol 96% for 1 min, stained with orange G for 1 min, washed in alcohol 96% for 1 min, stained with Eosine Azure (EA) 50 for 1 min, dehydrated in a decreasing series of alcohol concentration and cleared with xylene. Slides were analysed under a light microscope and the proportion of cornified epithelial cells, nucleated epithelial cells and leucocytes was used for the determination of the estrous cycle phases (Byers, Wiles, Dunn, & Taft, 2012).

Hippocampal formation volumes

5-month old mice were anesthetized with 20% sodium pentobarbital (Eutasil, (100mg/Kg), Ceva, Algés, Portugal) and transcardially perfused with 0.9 % saline solution and 4% paraformaldehyde (PFA). After being removed brains were further fixed in 4% PFA solution with constant agitation for 24 h and kept in 4% PFA until tissue processing (approximately 4 wks). Brains were processed for stereology according to the method described previously (Keuker, Vollmann-Honsdorf, & Fuchs, 2001). Briefly, brains were embedded in glycolmethacrylate (Tecnovit, 7100; Heraeus Kulzer, Werheim, Germany) and 30 μm-thick sections were obtained using a microtome. Every other section was collected into non-coated glass slide, stained with Giemsa and coverslipped using entellan (Entellan New, Merck, Darmstadt, Germany). To minimize bias, each brain was coded to keep the experimenter blind to the genotype. The

hippocampal formation was analyzed according to its main anatomical divisions: dentate gyrus (DG), *Cornu Ammonis* (CA) 3 and CA1 (strata oriens, pyramidale and radiatum). The hippocampal formation analysis was further divided into dorsal and ventral parts. Volumes of the different hippocampal sub-regions were determined using the Cavalieri's principle (Gundersen et al., 1988). Briefly, every 4th section was used and it's cross sectional area was estimated by point counting. For this we randomly superimposed onto each area a test point grid in which the interpoint distance, at a tissue level, was 150 μm for the DG and 200 μm for CA3 and CA1 regions. The volume of the region of interest was calculated from the number of points that felt within its boundaries and the distance between the systematically sampled sections. Average neuronal cell density numbers was estimated using the optical fractionator method (West, Slomianka, & Gundersen, 1991). Briefly, as for volumes, every 4th section was selected and the boundaries of every hippocampal sub-region were defined as stated above. A grid of virtual three-dimensional boxes for granule cell layer of the DG (20 x 20 x 20) and for pyramidal cell layer of CA1 and CA3 regions (40 x 40 x 40) was superimposed onto each section and neurons were counted whenever their nucleus (identified by size, shape and a prominent nucleoli) came into focus within the counting box.

Volume and neuronal number estimations were performed using Newcast software (Visiopharm Horsholm, Denmark) and a camera attached to a motorized microscope (BX51, Olympus, Japan).

Immunofluorescence

5-month old mice were deeply anaesthetized with 20% sodium pentobarbital and were transcardially perfused with cold 4% PFA. Brains were removed and post-fixed in 4% PFA under constant agitation for 24 h, immersed in 30% sucrose solution, preserved with optimal cutting temperature (OCT) compound and snap-frozen. Serial coronal sections (20 μm) were cut in a cryostat (Leica CM1900, Leica Biosystems, Nussloch, Germany) and collected to slides for immunofluorescence. Sections were stained for proliferating cells with anti-Ki-67 polyclonal antibodies (1:300, AB9260, Millipore Darmstadt, Germany) followed by staining for neuroblasts with polysialic acid-NCAM antibodies (PSA-NCAM) (1:300, clone 2-2B, MAB5324, Millipore, Darmstadt, Germany). Finally, for nuclear staining all sections were incubated with 4',6- diamidino-2-phenylindole (DAPI) (1:1000, Sigma-Aldrich, St. Louis, MO, USA). For each animal, Ki-67 positive cells double stained with PSA-NCAM within the subgranular zone (SGZ) of the dorsal DG were counted using confocal microscopy (Olympus FluoViewTM FV1000, Hamburg, Germany). For assessing neurogenesis (differentiation) we counted the number of neuroblasts (double-positive cells) divided by the total number of proliferating cells (Ki-67^{+}). We performed this analysis in

the dorsal hippocampus subgranular zone, including at least 4 sections per animal, from the dorsal hippocampus region (bregma -1.58 and -2.06). To minimize bias, each slide was coded to keep the experimenter blind to the genotype.

Hippocampal neurons morphology

To assess the 3-dimensional (3D) dendritic morphology of hippocampal neurons, we used the Golgi-Cox method. Briefly, 5-month old mice, were anesthetized with 20% sodium pentobarbital and transcardially perfused with 0.9% saline solution. Brains were removed and immersed in Golgi-Cox solution for 21 days and then transferred to a 30% sucrose solution and cut on a vibratome. Coronal sections (200 μm thick) were collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Rochester, NY, USA), fixed in Kodak Rapid Fix, dehydrated, xylene cleared, mounted and coverslipped with entellan. All incubation steps were performed in a dark room. To minimize bias, each brain was coded to keep the experimenter blind to the genotype. 3D reconstruction of Golgi-impregnated neurons from the DG and CA1 were evaluated. 5 to 8 neurons were randomly selected for 3D reconstruction having the following criteria in consideration: 1) full Golgi-impregnation along the dendritic tree; 2) complete dendrites without truncated branches; and 3) relative isolation from neighboring impregnated neurons, astrocytes or blood vessels to avoid interference with the analysis. Slides containing the region of interest were randomly searched and the first 5 to 8 neurons fulfilling the criteria (maximum of 3 neurons per section) were selected. For each selected neuron, all branches of the dendritic tree were reconstructed at 600x (immersion oil) magnification using a motorized microscope (BX51 Olympus), with a camera attached (DXC390; Sony, Tokyo, Japan) and Neurolucida software (Micro Bright Field, Williston, VT, USA). A 3D analysis of the reconstructed neurons was performed using NeuroExplorer software (MicroBright Field, Williston, VT, USA). Dendritic morphology was examined by the total dendritic length and arrangement of dendritic material using a 3D version of Sholl analysis (Sholl, 1956) of intersections. The number of dendritic intersections with concentric spheres positioned at radial intervals of 10 μm from the soma was registered.

Electrophysiological recordings

Slice preparation

3-month old mice were sacrificed by decapitation after deeply anaesthesia with sodium pentobarbital (30 mg/kg). The brains were quickly removed and placed in ice-cold sucrose-based artificial cerebrospinal fluid (sACSF) containing the following solution: 2.5 mM KCl, 7 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 110 mM sucrose, 25 mM $NaHCO_3$, 7 mM glucose, bubbled with carbogen gas (95% O_2, 5% CO_2). After a hemisection of the brain along the sagittal plane, the dorsal hippocampus of the right hemisphere was partially dissected and glued vertically with the dorsal-most part facing up. Horizontal slices (300 μm) were prepared in sACSF using a tissue slicer (Leica VT 1200s; Leica Biosystems, Nussloch, Germany) and incubated for 20 min at 30 °C in standard artificial cerebrospinal fluid (ACSF) containing: 124 mM NaCl, 4.4 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM $NaHCO_3$, 10 mM glucose, bubbled with carbogen gas. Slices were stored in ACSF at room temperature for at least 30 min before recording, after which they were transferred to a submerged chamber, maintained at 31 °C and continuously perfused with ACSF at a rate of 5 mL/min. Two slices per animal were used for electrophysiological recordings.

Electrophysiological recording

Extracellular field recordings were made with a Multiclamp 700B amplifier (Molecular Devices, CA, USA) in bridge mode and digitized with the Digidata 1440 digitizer (Molecular Devices, Sunnyvale, CA, USA) using pCLAMP10 software (Molecular Devices, Sunnyvale, CA, USA). Signals were low-pass filtered at an effective corner frequency of 3 kHz and sampled at 10 kHz. For recording, boro silicate glass recording pipettes (3–5 M) were pulled using a micropipette puller (P-97, Sutter Instruments, Novato, CA, USA) and filled with saline solution (0.75 M NaCl). For stimulation of the Schaffer collaterals, a stimulus isolating unit (STG4002, Multichannel Systems, Reutlingen, Germany) and a custom made bipolar tungsten electrode (Science Product, Hofheim, Germany) were used. Both recording and stimulating electrodes were placed in the middle of CA1 stratum radiatum. The frequency of baseline stimulation was of 0.03 Hz and for input–output relation monitoring, a series of increasing stimulus intensities were applied (0.5–8.0 V). Stimulus strength was then adjusted to have approximately 40% of the maximum slope of the local field excitatory post-synaptic potential (fEPSP).

The paired-pulse (PP) ratio was assessed before long-term potentiation (LTP) induction by giving two close stimulus of varying inter-pulse intervals (25, 50, 100, and 300 ms) (Citri & Malenka, 2008). The ratio was calculated by dividing the slope of fEPSP2 by the slope of EPSP1. For LTP induction, fEPSP slopes were monitored for a period of at least 20 min. If synaptic transmission was stable, 3 bursts of 1 s duration, separated by 15 s, and with a burst frequency of 100 Hz, were delivered and followed by 80 min of baseline recording. All points of each individual curve were normalized to the average value of baseline. All stored traces were averages of 4 consecutive recordings. Final slopes were calculated offline using the LTP software (Anderson & Collingridge, 2001). To minimize bias, the experimenter who performed all electrophysiology experiments was blinded to the genotype.

Statistical analysis

Adequate sample size was determined a priori using G-Power software v3.1.9.2, based on results of a previous pilot experiment suggesting a η^2_p of 0.15 for the effect of genotype on the Morris Water Maze test and assuming a 90% power and 5% probability of type I errors. All values were calculated as means + standard error of the mean (SEM). Kolmogorov-Smirnov normality test was used to assess whether data presents a normal distribution. Equality of variances was tested with an F test. Data from the reference memory, reverse task, sholl analysis and electrophysiology were analysed using ANOVA repeated-measures and differences between groups compared with the post-hoc Bonferroni test. To quantify the strength of the differences, partial eta-square (η^2_p) was calculated as a measure of effect size (0.02 was considered a small, 0.13 a medium and 0.26 a large effect size). All other data was analyzed using two tail Student's T-test and Cohen *d* was calculated as a measure of effect size (0.2 was considered a small effect size, 0.5 a medium effect size, and 0.8 a large effect size). Differences were considered significant if $p<0.05$. Statistical analyses were performed with Graphpad Prism version 5.0b (La Jolla, San Diego, USA).

Results

Absence of IFNγ leads to an enhanced performance in cognitive tasks that engage the hippocampus

We tested cognitive behaviour of IFNγ KO mice and WT in the MWM using 2 different paradigms: the spatial reference memory task (Fig. 1A) to test learning that relies on hippocampal functioning (during 4 consecutive days), and the reversal task (in the 5th day) for testing behavioural flexibility (Fig. 1C), a prefrontal cortex (PFC) function(Cerqueira, Mailliet, Almeida, Jay, & Sousa, 2007). Both IFNγ KO and WT mice were able to successfully learn the spatial reference memory task (time: $F_{(3,141)}$=43.1; p<0.0001; η^2_p=0.48), but animals lacking IFNγ had a significantly better performance (genotype: $F_{(1,47)=}$10.47; p=0.002; η^2_p=0.13; $d_{(day4)}$=0.63; interaction: $F_{(3,141)}$=0.61; p=0.60) (Fig. 1A). Interestingly, this effect seemed to be specific for hippocampal dependent tasks since in the reversal task there were no differences between groups (Fig. 1C). To further clarify this specificity, we used the NOR test to evaluate non-spatial recognition memory, a different dimension of hippocampal functioning. In accordance with our previous results, IFNγ KO mice also exhibited a better performance than WT in this test ($t_{(22)}$=2.21; p=0.03; d=0.90) (Fig. 1B).

To clarify whether the impact of IFNγ absence is specific for cognitive-related tasks (more related with dorsal hippocampal functioning) or it also affects non-cognitive dimensions such as anxiety-like behaviour (to which the ventral hippocampus is more relevant) we tested the animals in the EPM and the OF (Fig. 1D and E) and observed no significant differences between the experimental groups. There were also no differences between groups in total distance travelled therefore validating behavioural tests that are dependent on an intact locomotor function (Fig. 1F).

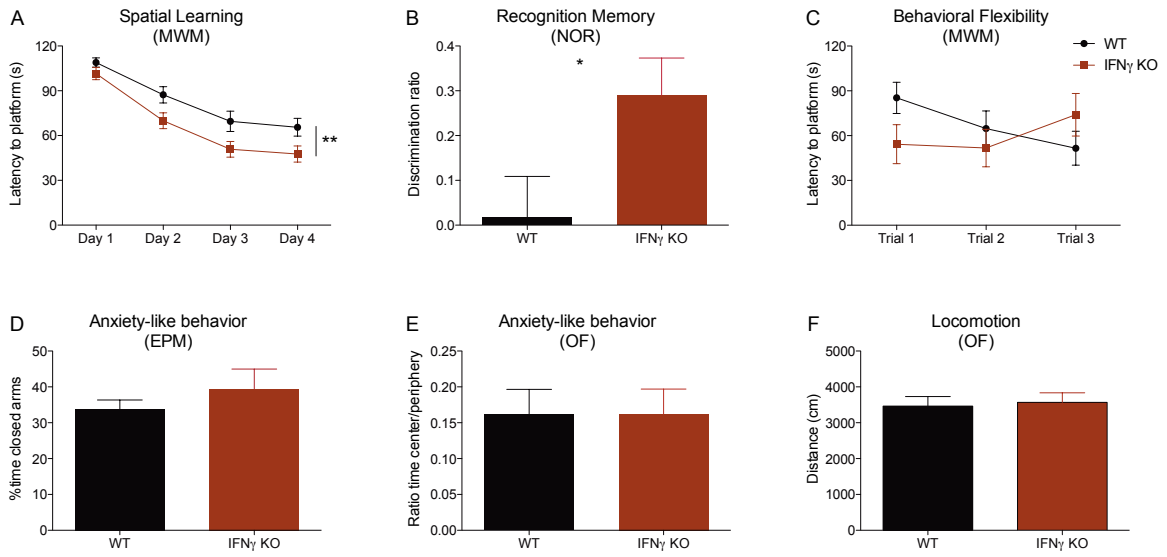

Fig. 1 – Spatial learning and recognition memory are enhanced in IFNγ KO mice. Hippocampal-dependent spatial learning was tested in the MWM (IFNγ KO n=23 and WT=26) (A). Hippocampal-dependent non-spatial recognition memory was assessed in the NOR task (IFNγ KO n=12 and WT=12) (B). Prefrontal cortex-dependent behavioural flexibility was assessed in the reversal task (IFNγ KO n=23 and WT=26) (C). Anxiety-like behaviour was assessed using the EPM (IFNγ KO n=7 and WT=10) (D) and OF (IFNγ KO n=7 and WT=10) (E). Motor activity was assessed in the OF (IFNγ KO n=7 and WT=10) (D). Error bars denote SEM; * P<0.05; ** P<0.01.

Absence of IFNγ induces structural changes in the dorsal hippocampus

Since the only observed behavioural differences were in cognitive tasks that are known to be dependent on hippocampal functioning (particularly the dorsal region), we next measured volumes of different sub-regions of the dorsal (Fig. 2A) and ventral (Fig. 2B) hippocampus using a stereological approach. Our results show that IFNγ absence results in an increased volume of the dorsal CA1 region as compared with WT ($t_{(8)}$=2.589; p=0.0322; d=1.64); of note there was also a tendency for increased volumes in the dorsal DG of IFNγ KO mice ($t_{(8)}$=1.695; p=0.1286.; d=1.0708), with a strong effect size, that did not, however, reach statistical significance (Fig. 2A).

Within the dorsal CA1 region, IFNγ KO mice exhibited enlarged *strata oriens* ($t_{(8)}$=2.763; p=0.02; d=1.75) and *radiatum* ($t_{(8)}$=2.608; p=0.03; d=1.65) of CA1 with no statistically significant differences on *stratum pyramidale* ($t_{(8)}$=1.891; p=0.0953; d=1.20) despite the strong effect size (Fig. 2C and D). Importantly, in line with the results of the behavioural testing, there were no volumetric differences in any of the subregions of the ventral hippocampus (Fig. 2B).

We also counted the number of neurons in the dorsal and ventral part of the hippocampus (Fig. 2E and F) using an unbiased stereological approach and observed an increased number of cells in the dorsal CA1 stratum pyramidale ($t_{(8)}$=2.700; p=0.0271; d=1.71) and a tendency for increased cell numbers in the dorsal DG granule cell layer (Fig 2E) from IFNγ KO mice (t(8)=1.973; p=0.0839; d=1.25). Curiously, no alterations were observed in the ventral region of the hippocampus (Fig 2F).

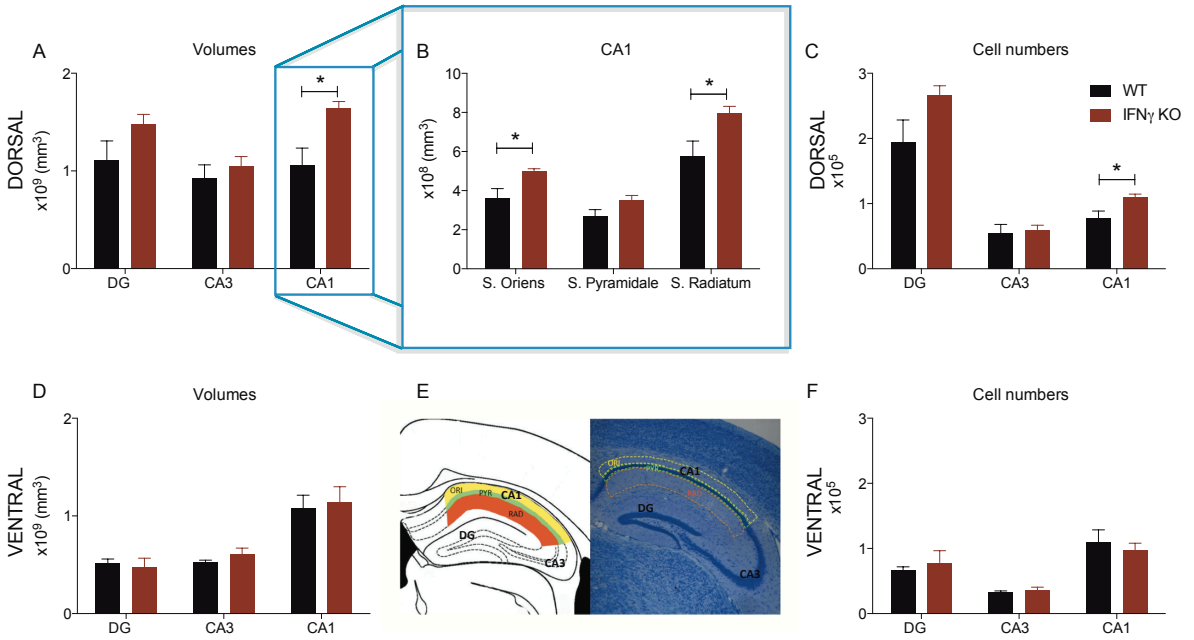

Fig. 2 – The structure of the dorsal but not the ventral hippocampus is altered in IFNγ KO mice. Stereological estimations of volumes of the dorsal DG, CA3 and CA1 (A); and of the ventral DG, CA3 and CA1 (D). Stereological estimations of volumes from CA1 strata oriens, pyramidale and radiatum (B). Outlining of the different subareas: DG, CA3 and CA1 - diagrams were adapted from the Paxinos mouse brain atlas (Paxinos, 2001) , corresponding brain slices were stained with Giemsa (E). Stereological estimations of cell numbers on the dorsal (C) and ventral (F) hippocampus (strata pyramidale for CA1 and CA3 and granule cell layer for DG). IFNγ KO n=5 and WT=5. Error bars denote SEM; *p<0.05.

Absence of IFNγ enhances neuroplastic phenomena in the dorsal hippocampus

Since our stereological and behavioural results pointed to a specific effect on the dorsal hippocampus, we focused our subsequent analyses in this brain region. Neurogenesis, a process characterized by the generation of newly-born neurons, is known to occur in the SGZ of the DG throughout life and is an important contributor for cognitive processes (reviewed by Kemmperman et al. 2004 (Kempermann, Wiskott, & Gage, 2004)). Since IFNγ is a known regulator of cell proliferation, we explored whether the effects observed above could also be related with altered hippocampal neurogenesis. In order to do so,

we counted in the SGZ the percentage of proliferating-cells ($Ki67^+$) also expressing the early neuronal marker PSA-NCAM (double-labeled cells) in the SGZ. In accordance with our hypothesis, IFNγ KO mice displayed enhanced neurogenesis compared to WT ($t_{(4)}$=3.239; p=0.03; d=2.64)(Fig 3A-C).

Another neuroplastic phenomena known to modulate cognitive performance is dendritic remodelling. We observed that dendrites from dorsal DG granule neurons of mice that lack IFNγ are longer (total length: $t_{(10)}$=3.888; p=0.003; d=2.24) (Fig. 3A) and more ramified than those of WT (Fig. 4A-C), particularly at a distance between 50-130 μm from the soma (Sholl analysis: genotype - $F_{(1,\ 10)}$=11.98; p=0.0061; η^2_p=0.39, genotype*distance to soma - $F_{(23,\ 230)}$=3.817; p<0.0001; η^2_p=0.28). While the total length of CA1 pyramidal neurons dendrites were not different between the two genotypes (Fig 4D, apical: $t_{(9)}$=1.418; p=0.1899; d=0.85; Fig. 4F, basal: $t_{(9)}$=2.001; p=0.0764; d=1.17), there was a clear tendency, with a strong effect size, for basal dendrites of CA1 pyramidal neurons of IFNγ KO animals to be larger; in fact they had significantly more branches, particularly at a distance between 70-90 μm near the soma (Sholl analysis: genotype – $F_{(1,\ 9)}$=3.553; p=0.0921; η^2_p=0.18, genotype*distance from soma - $F_{(23,\ 207)}$=2.406; p=0.0006; η^2_p=0.21) (Fig. 4G and H).

Together with structural plasticity, functional synaptic plasticity is the hallmark of learning and memory. In order to study the impact of the absence of IFNγ in functional synaptic plasticity we recorded extracellular field potentials on fresh slices of the CA1 region of the dorsal hippocampus. To address both pre and post-synaptic mechanisms we analysed three plasticity-related electrophysiological phenomena: the input-output response (global), LTP induction after thetanus stimulation (mainly post-synaptic) and paired-pulse facilitation (mainly pre-synaptic). While the first two phenomena were not different between IFNγ KO mice and WT (Fig. 5A and B), the EPSP ratio in the paired pulse facilitation paradigm was lower in IFNγ KO mice compared to WT ($F_{(1,\ 15)}$=5.104; p=0.039; η^2_p=0.48) (Fig. 5C), implying that IFNγ absence is associated to an increased pre-synaptic neurotransmitter release probability.

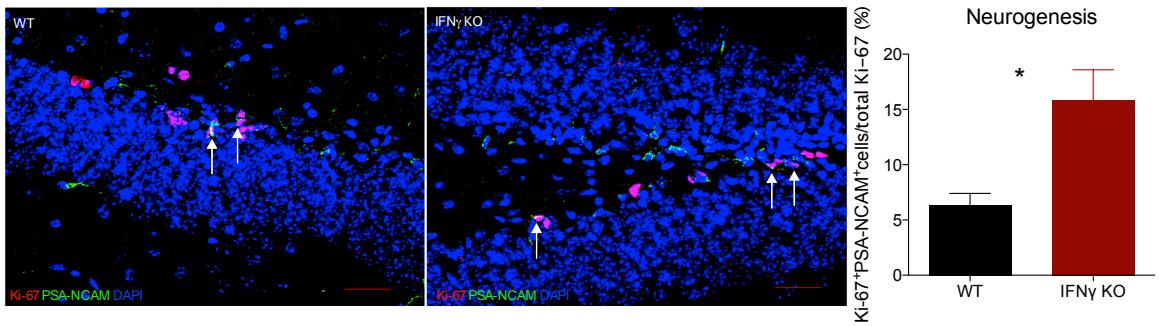

Fig. 3 – Neurogenesis is increased in the DG of the dorsal hippocampus of IFNγ KO mice. Photomicrograph depicts Ki-67 (red) and PSA-NCAM (green) double-staining in the DG SGZ from WT (A)

and IFNγ KO (B) mice. Graph displays the percentage of newly-born neurons (Ki-67+ cells that express PSA-NCAM+) by the total proliferating cells (Ki-67+) (for assessing neurogenesis (differentiation) in the DG SGZ of the dorsal hippocampus (C). IFN*γ* KO n=3 and WT=3. Scale bar=30μm. Error bar denotes SEM * P<0.05.

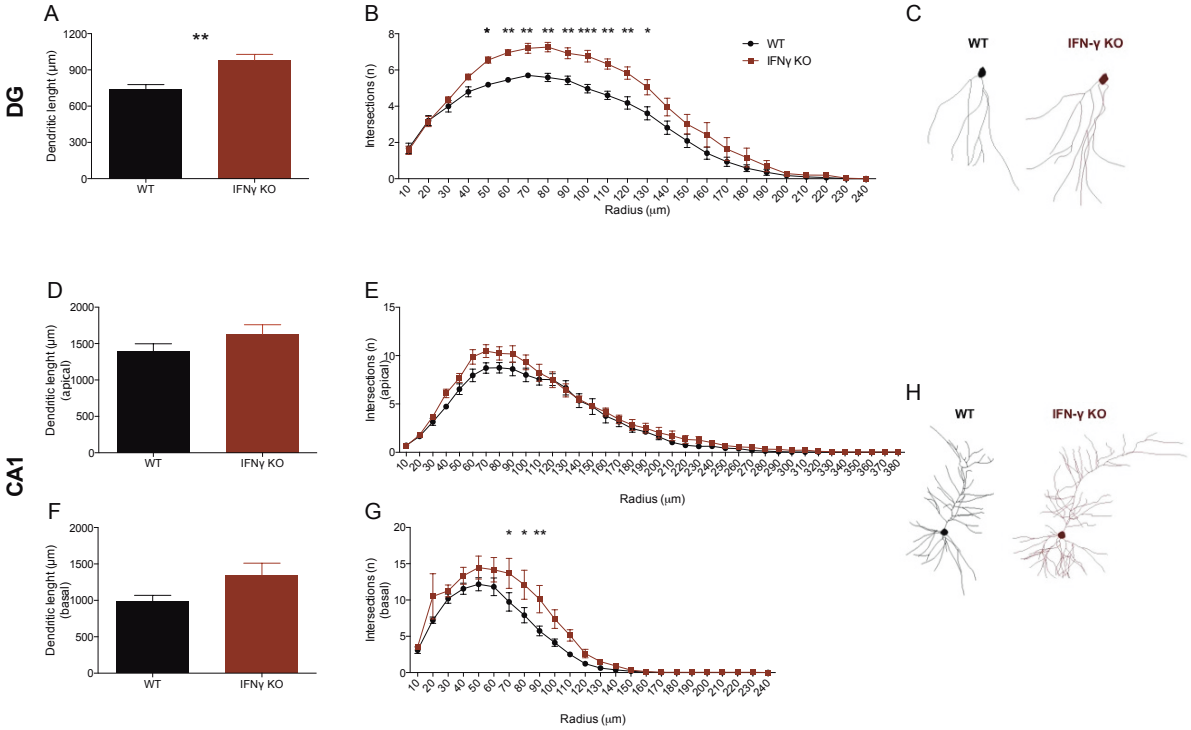

Fig. 4 – Neuronal dendritic trees are enlarged in IFN*γ* KO mice. Morphometric analysis of Golgi-stained hippocampal neurons of the: DG - total dendritic length (A), differential rearrangement of dendrites (B) and computer-assisted 3D reconstructions of a representative granule neuron (C); CA1 - total length of the apical (D) and basal dendrites (F), differential rearrangement of the apical (E) and basal (G) dendrites and computer-assisted 3D reconstructions of a representative pyramidal neuron (H). (IFN*γ* KO=5-6 and WT=6). Error bars denote SEM; *p<0.05;**p<0.01 and ***p<0.001.

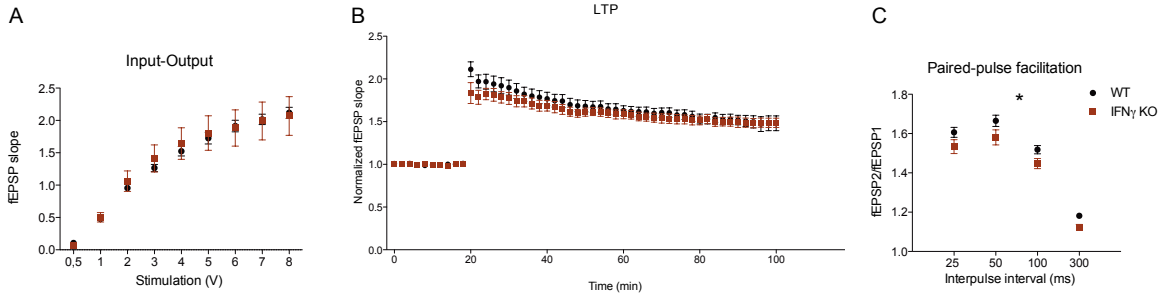

Fig. 5 - Pre-synaptic plasticity is altered in IFN*γ* KO mice. Electrophysiological analysis using dorsal hippocampal slices: input-output (A), long-term potentiation response (B) and paired-pulse facilitation (C) assays. (IFN*γ* KO=8 and WT=9). Error bars denote SEM; *p<0.05.

Discussion

Herein, we have identified the pro-inflammatory cytokine, IFNγ, as a regulator of cognitive performance. Moreover, we have also identified that its effects are regionally selective since most alterations caused by its absence were allocated to the dorsal, but not the ventral, hippocampus. We also demonstrated the impact of the absence of this cytokine on neuroplastic phenomena that are known to support cognitive functioning, namely an increase on the generation of adult newly-born neurons in the DG, enlarged dendritic arborisations and higher pre-synaptic vesicle release probability.

Of the several behavioural dimensions assessed, the absence of IFNγ selectively alters the performance on cognitive-related tasks and more specifically, on tasks engaging the hippocampus. Interestingly, the volumetric data, showing only alterations in the dorsal part of the hippocampus, support the cognitive-specificity already noted in the behavioural results, since the dorsal hippocampus is functionally distinct of its ventral counterpart, assuming cognitive-related functions while the latter is responsible for emotional processing (Fanselow & Dong, 2010).

The hippocampus plays a central role in learning and the acquisition of new memories. Integrity of these functions requires ongoing neurogenesis in the (hippocampal) subgranular zone of the adult brain such that its pharmacological arrest results in long-term emotional and cognitive changes (Mateus-Pinheiro et al., 2013; Shors et al., 2001). In accordance, here we demonstrate that IFNγ KO mice present higher numbers of adult newly-born neurons in the DG. Of note, a previous study reported a decrease on doublecortin-positive cells in IFNγ KO mice (Campos et al., 2014). Although these results may apparently go in a different direction from ours, we cannot exclude that we are targeting a different subset of immature neurons, since only actively proliferating neuroblasts were considered. Moreover, the reduction of doublecortin-positive cells in IFNγ KO mice may reflect a reduced immature neuronal stage, with more neurons being integrated, although this needs further investigation.

In line with our results, data by Li et al. (Li et al., 2010) also showed an increase of neurogenesis in IFNγ KO mice with increased number of proliferating cells in the SVZ and an increased percentage of newly-born neurons in the olfactory bulb (Li et al., 2010). However, the novel finding that these mice also present more newly-born neurons in the DG has a special relevance for cognitive function.

Of note, since IFNγ is a pro-inflammatory cytokine with an important function in the context of the immune response, it may be possible that IFNγ effect on neurogenesis in pathological contexts differs from that of the healthy brain. Indeed, in the paper by Baron et al. (Baron et al., 2008) is shown that in

an Alzheimer's disease model, genetically engineered for producing limited amounts of IFNγ, neurogenesis is enhanced. Here, the disease context, stage of pathogenesis, and cellular/cytokine milieu may also dictate the disease resolving and/or contributory nature of IFN γ.

One of the key components of neuronal plasticity is the complexity of the dendritic arbor. Learning, for instance, shapes the dendritic morphology of recently generated neurons in order to integrate them into neuronal circuits (Tronel et al., 2010). Previous studies have demonstrated that IFNγ by itself is capable of negatively shape neuronal morphology (Kelic et al., 1997; I. J. Kim et al., 2002). In fact it was shown that exposing neurons in a *in vitro* culture to IFNγ inhibited dendritic outgrowth, induced dendritic tree retraction and diminished the rate of synapse formation (I. J. Kim et al., 2002). Adding to these previous observations, our current data reveals that the DG granule neurons from IFNγ KO mice present larger dendritic trees, which together with the increased proliferation of newly-born neurons, contributes to the tendency for an increased volume observed in this hippocampal subregion. Likewise, the fact that the volume of strata oriens and radiatum from the CA1 region were also increased in IFNγ KO mice can be potentially attributed, at least in part, to the tendency for increased dendritic length of both the apical and basal dendritic tree in pyramidal neurons of this area. Apart from structural plasticity, functional plasticity at the synapse/circuit levels, as assessed by LTP and paired pulse protocols, is also critical for learning and memory. We here show that the absence of IFNγ has no effect on LTP induction, since mice lacking this cytokine are equally capable of inducing an efficient hippocampal LTP response when compared to WT animals. While this finding might seem in contradiction with the LTP impairment by acute IFNγ local administration (Maher et al., 2006), it might be because under basal conditions IFNγ has a very limited role in post-synaptic plasticity or simply reflect the development of adaptive mechanisms (as our model is a constitutional knockout). Yet, we have demonstrated that mice lacking IFNγ exhibit a decreased paired-pulse ratio without changes in the input/output curve. Importantly, although this is sometimes interpreted as decreased synaptic plasticity, it more probably reflects an increased pre-synaptic release probability (Ho et al., 2006). Of note, the co-existence of enhanced cognitive performance and decreased paired-pulse ratio observed in the present work is in line with preclinical models of Alzheimer's disease in which an increased paired-pulse ratio is one of the first signs of synaptic dysfunction and cognitive deficits (Larson, Lynch, Games, & Seubert, 1999).

Given the fact that our mouse line is a constitutional deletion model, it is plausible that the structural, morphological and functional alterations herein observed may have been induced during neurodevelopmental stages. As an example, IFNγ has been implicated in the cognitive deficit of Down

Syndrome, a neurodevelopmental disorder characterized by an extra copy chromosome 21 (chromosome 16 in mice), which codifies for many interferon regulated genes and IFN receptors (Hallam et al., 2000; Maroun, 1996), with patients displaying increased levels of this cytokine (Nateghi Rostami et al., 2012) and increased sensibility to IFN action.

Interestingly, although endogenous brain IFNγ is only very weakly expressed at basal conditions, here we show that its absence leads to important structural, morphological and functional alterations in the hippocampus that contribute to an enhanced cognitive performance. Therefore, our results suggest that in a pathological context, where IFNγ levels may be significantly altered, its role in mediating cognitive decline may emerge as particularly important to explore. Moreover, this cytokine and its downstream molecules may be promising therapeutic targets to prevent and/or treat cognitive decline associated with inflammation.

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Conflict of interest

The authors declare no conflict of interest.

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3rd CHAPTER

Gender differences on IFNγ KO mice behavioural phenotype: is estrogen involved?

Monteiro, S., Silva, N.A., S. Roque, Correia-Neves, M., Cerqueira, J.J.

Gender differences on IFNγ KO mice behavioural phenotype:

is estrogen involved?

Introduction

In the previous sub-chapter we demonstrated that IFNγ is a negative regulator of cognitive performance with no involvement on anxiety-related behaviour. However, our behavioural data seemed to be conflicting with three published reports (Campos et al., 2014; Kustova et al., 1998). Specifically, it was previously shown that the IFNγ KO mice exhibited an anxious-like phenotype (Campos et al., 2014; Kustova et al., 1998). Moreover, in the paper by Littlejohn et al. (Litteljohn et al., 2014), although an enhanced cognitive performance by IFNγ KO mice was demonstrated after chronic stress exposure, no basal differences between IFNγ KO and WT littermate controls were found. Since in our experiments we used female mice, and the above-mentioned studies were performed using males, we tested IFNγ KO male mice in the same paradigms and found relevant gender-differences.

The sex hormone estrogen has been shown to modulate different behaviours, such as cognition (Asthana et al., 2001; Bean, Ianov, & Foster, 2014; Hampson & Morley, 2013; J. Wei et al., 2014) and anxiety (Carrier et al., 2015; Pandaranandaka, Poonyachoti, & Kalandakanond-Thongsong, 2006). In addition, estrogen has been shown to have anti-inflammatory properties as demonstrated by the studies where this hormone blocked the LPS-induced inflammatory response in macrophages (Ghisletti, Meda, Maggi, & Vegeto, 2005) and also in microglia (Vegeto et al., 2001).

Importantly, estrogen was shown to be able to modulate the promoter of the *Ifnγ* gene (Fox, Bond, & Parslow, 1991), meaning that variations of this sex hormone most probably impact IFNγ expression. In line with this study, several reports have demonstrated that the administration of estrogen alters IFNγ production profile by splenocytes (Karpuzoglu-Sahin, Hissong, & Ansar Ahmed, 2001; Karpuzoglu-Sahin, Zhi-Jun, Lengi, Sriranganathan, & Ansar Ahmed, 2001; Nakaya, Tachibana, & Yamada, 2006), invariant natural killer T (iNKT) cells (Gourdy et al., 2005) and dendritic cells (Siracusa, Overstreet, Housseau, Scott, & Klein, 2008).

In the present sub-chapter we tested the working hypothesis if estrogen could also modulate the IFNγ production profile by cells of the central nervous system, similarly to what was already reported for peripheral cells,.

Material and methods

Animals

3 to 5-month old female and male IFNγ knock-out (KO) mice on C57BL/6 background (Dalton et al., 1993) and littermate homozygous wild-type (WT) were housed (5 mice per cage) under standard laboratory conditions [12 h light/12 h night cycles (8 h/20 h)], 22-24 °C, relative humidity of 55% and with *ad libitum* access to water and food. Cages were enriched with paper rolls and soft paper for nesting. All procedures were carried out in accordance to EU directive 2010/63/EU and Portuguese national authority for animal experimentation, Direção Geral de Veterinária (ID:DGV9457), guidelines on animal care and experimentation. Littermate WT were used as controls in all experiments to minimize genetic variability (Holmdahl & Malissen, 2012) and were obtained from an initial crossing of IFNγ KO mice with C57BL/6 mice (Charles River Laboratories, Barcelona, Spain) and by crossing the heterozygous progeny between them. Mice were sacrificed at 5-months of age for molecular analysis. To study the effect of endogenous estrogen on IFNγ expression levels, female mice were only sacrificed whenever they were at proestrus phase since estrogen levels are known to peak at this stage.

Behavioural analysis

Behavioural experiments were performed during the mice active period (between 8 pm and 8 am). Before behavioural testing, mice were gently handled by the same experimenter for 2 weeks every other day. Prior to each behavioural assessment mice were transported to the testing room and left for habituation to room conditions during 1 h. All behavioural data analysis was performed with the experimenter blinded to the genotype.

Morris Water Maze (MWM)

In order to assess spatial reference memory, 3-month old mice were tested in a white circular pool (170 cm diameter) filled with water (24 - 25 °C) placed in a dimly lit room. Spatial cues were placed in the walls around the pool (square, stripes, triangle and a cross). The pool was divided in 4 imaginary quadrants and a hidden transparent platform was placed in one of the quadrants. Data was collected by a fixed camera placed in the ceiling and connected to a video-tracking system (Viewpoint, Champagne-au-Mont-d'or, France).

Mice had to learn the position of a hidden platform over a period of 4 days. In each day, mice were placed facing the wall of the pool at different quadrants (north, west, south and east), in a

pseudorandom order that varied from day to day, as a starting point for each trial. Each trial was completed whenever the mouse reached the platform or when 120 s elapsed. Latency to reach the platform (escape latency) was recorded for each trial during the 4 days.

Elevated-plus maze (EPM)

3-month old mice were tested for anxious-like behaviour using the EPM test. Briefly, this test consists on placing each mouse in the hub of a plus-like apparatus elevated 72.4 cm from the floor, with two opposing open arms (50.8cm x 10.2 cm) and 2 opposing closed arms (50.8 cm x 10.2 cm x 40.6 cm) (ENV560; MedAssociates, Inc., St. Albans, VT, USA) and letting the animal freely explore it for 5 min. The ratio of time in the open arms and in the closed arms was used as a behavioural parameter of anxious-like behaviour.

Determination of estrous cycle stage

Vaginal smears were performed by inserting a drop of sterile 0.9% saline solution in the vagina with the help of a 1 ml syringe, collecting the cell suspension by inserting a small plastic inoculation loop and performing a smear into a glass slide. Smears were air-dried, fixed in alcohol 96% for 5 min and stained using the Papanicolaou protocol. Briefly, smears were hydrated in tap water, stained with Harris haematoxylin for 1 min, rinsed in running tap water for 2 min, regressively stained by a single-dip in a alcohol-acid solution, rinsed in tap water for 2 min, dehydrated in alcohol 96% for 1 min, stained with orange G for 1 min, washed in alcohol 96% for 1 min, stained with Eosine Azure (EA) 50 for 1 min, dehydrated in a decreasing series of alcohol concentration and cleared with xylene. Slides were analysed under a light microscope and the proportion of cornified epithelial cells, nucleated epithelial cells and leucocytes was used for the determination of the estrous cycle phases (Byers et al., 2012)

Primary glial cell culture

Glial cells were isolated from post-natal day 4 (P4) brains from newborn C57BL/6 mice (adapted from Salgado et al., 2009). The brains were dissected and after carefully removing the meninges, they were enzymatically digested (20 mg/ml DNase, 0.25% trypsin; both from Sigma-Aldrich, MO, USA), followed by strong mechanical dissociation. The resulting cell suspension was centrifuged at 800 rpm for 2 min, and the pellet was re-suspended in Dulbecco's modified eagle medium (DMEM) (supplemented with 10% FBS and 1% Penicilin/Streptomycin) (Sigma-Aldrich, MO, USA). After being counted using a Neubauer chamber, cells were then plated, on poly-D-lysine coated 12-wells plate, at a density of

250000 cells/well. Cells were left to grow for a week before the start of the experiment, in an incubator at 37 °C and 5% CO_2 content and the cell culture medium was exchanged every two days. Cells were stimulated with 17β-estradiol (Sigma-Aldrich, MO, USA) at a 10^{-9} M concentration.

Gene expression measurement by quantitative real-time polymerase chain-reaction (qPCR)

The prefrontal cortex (PFC), orbitofrontal cortex (OFC), dorsal and ventral hippocampus (dHip and vHip) were macrodissected and quickly frozen at -80 °C. mRNA levels of *Ifnγ, Il12 and Il4* genes were determined by qPCR. Total RNA was isolated using TRIzol-chloroform method (Life technologies, CA, USA). Total RNA was reverse transcribed into first strand cDNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as housekeeping gene. Oligonucleotide primers for *Il12* (sense 5'-GTGTCTTAGCCAGTCCCGAA-3', antisense 5'-TTCAAGTCCTCATAGATGCTACCAA-3'), *Ifnγ*, (sense 5'-CAACAGCAAGGCGAAAAAGG-3', antisense 5'- GGA CCACTCGGATGAGCTCA-3'), *Il4* (sense 5'- GTCACAGGAGAAGGGACGCCAT-3', antisense 5'- AGCCCTACAGACGAGCTCACTC-3') and *Gapdh* (sense 5'- GGGCCCACTTGAAGGGTGGA-3', anti-sense 5'- TGGACTGTGGTCATGAGCCCTT-3') were designed using the Primer3 software (Simgene, San Francisco, CA, USA). qPCR reactions were performed on a 7500 Api real-time PCR system instrument (Applied Biosystems, CA, USA), with the KAPA SYBR Fast (KAPABiosystems, MA, USA) according to the manufacturer's instructions and using ROX low as reference dye.

Enzyme-linked Immunosorbent assay (ELISA)

An in-house sandwich ELISA was performed for IFNγ protein levels determination. A 96-well plate was coated with the anti-mouse IFNγ (clone AN-18, eBioscience, CA, USA) capture antibody. Standards were made by serial dilution (1:2) of recombinant murine IFNγ (Peprotech, UK) in DMEM. After incubating both standards and samples, IFNγ was detected using a biotinylated anti-mouse IFNγ antibody (clone R4-6A2, eBioscience, CA, USA) followed by an incubation of streptavidin peroxidase (Sigma-Aldrich, MO, USA). After 30 min of incubation with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate, the reaction was stopped using 1N hydrochloric acid. The readings were performed at an optical density of 455 nm.

Statistical analysis

All values were presented as means + standard error of the mean (SEM). Data was analysed using two tail Student's T-test apart from the MWM data that was analysed using the ANOVA repeated measures

test. Differences were considered significant if $p<0.05$. Statistical analyses were performed with Graphpad Prism version 5.0b (La Jolla, San Diego, USA).

Results

Absence of IFNγ leads to an anxious-like phenotype on males, while leading to an enhanced cognitive phenotype on females

To explore if there were behavioural gender-differences in IFNγ KO mice, we compared the performance of male and female IFNγ KO mice in the EPM for anxious-like behaviour and the MWM for spatial learning and memory (Fig. 1). We observed that while male IFNγ KO mice exhibited an anxious-like behaviour relatively to controls ($t_{(16)}=2.042$; $p=0.0580$) (Fig. 1A), this phenotype was not detected in IFNγ KO female mice (Fig. 1B). On the other hand, while male IFNγ KO mice presented no differences on the MWM performance (Fig. 1 C), female IFNγ KO mice outperformed relatively to controls in the same task ($F_{(1,47)}=10.47$; $p=0.0022$) (Fig. 1D).

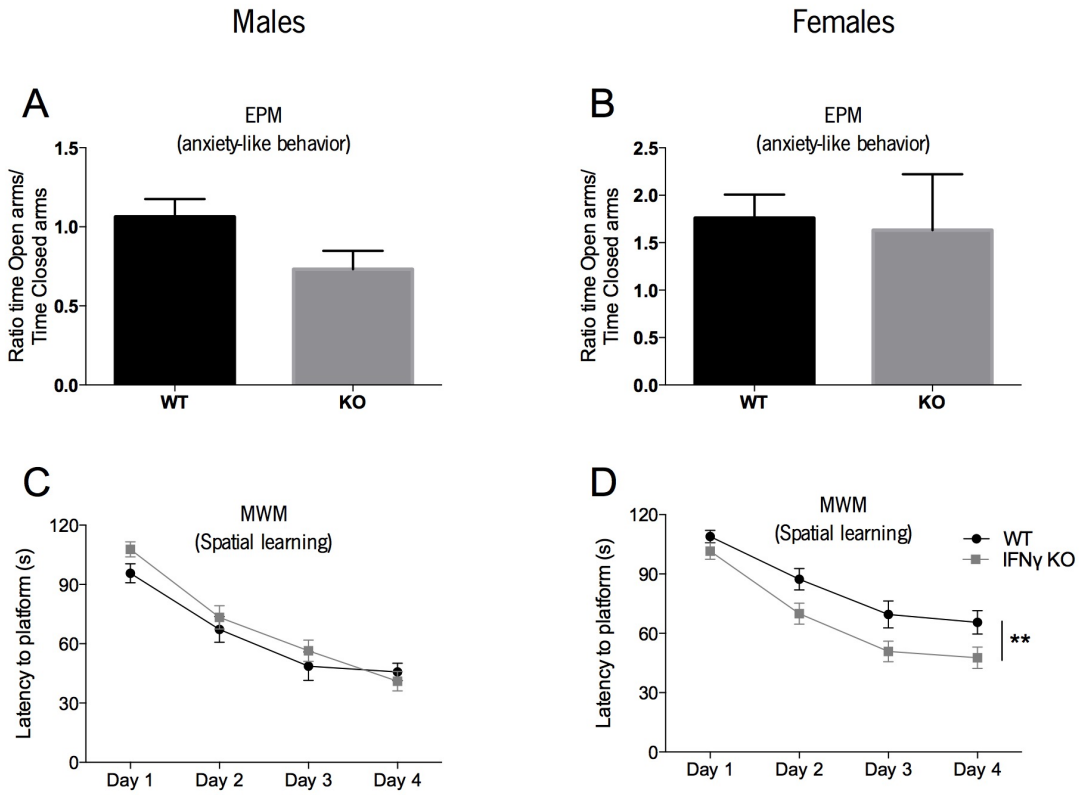

Fig. 1 – Gender differences of IFNγ KO mice behavioral phenotype. Male IFNγ KO mice exhibited an anxious-like behaviour (A) and no differences on spatial learning (C). Female IFNγ KO presented no differences on anxious-like behaviour (B) but an improved cognitive performance (D). Error bars denote mean +/-SEM. **p<0.01.

IFNγ is differentially expressed in the dorsal hippocampus of male mice

In order to test if there were gender-differences on endogenous IFNγ expression levels in the brain, we quantified by qPCR the expression in the prefrontal cortex and hippocampus from WT male and females mice; the females in proestrus phase (Fig. 2). We observed that while in the male brain, there was a tendency for a differential expression of IFNγ in the dorsal hippocampus (Fig. 2A); this peak was absent in the proestrus female mice brain (Fig. 2B).

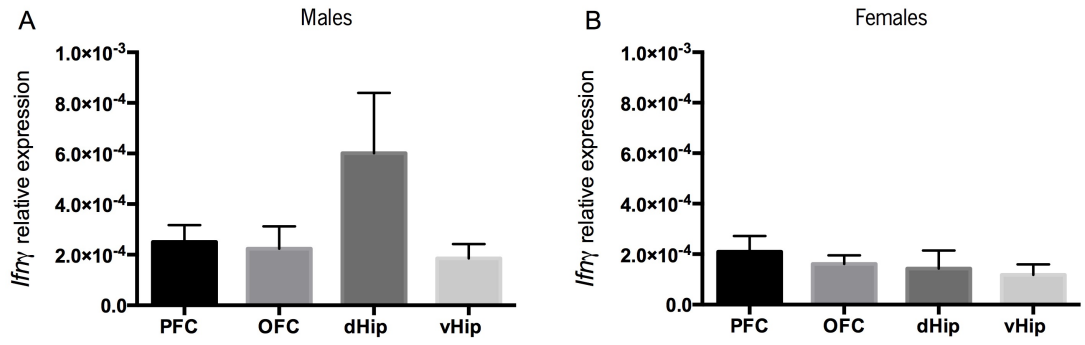

Fig. 2 – Gender differences on the pattern of endogenous IFNγ expression across brain regions. The expression pattern of endogenous IFNγ in the brain was suggested to be different between males and females (in proestrus) by a trend for increased levels in the dorsal hippocampus of male mice while in females the IFNγ expression levels were constant across brain regions. Error bars denote mean + SEM.

Estrogen-treated glial cells exhibited a decreased IFNγ secretion

In order to test whether estrogen could also modulate the IFNγ production profile by brain cells, we established a glial cell culture and stimulated for 24 h with estrogen. We analysed the impact on mRNA expression levels of *Il12*, *Ifnγ* and *Il4* and measured the IFNγ secreted levels in the cell culture media (Fig. 3). We observed that 24 h treatment with estrogen revealed a tendency to decrease *Il12* (Fig. 3A) and *Ifnγ* expression levels (Fig. 3C). Since interleukin (IL) 4 has been associated with improved cognition (Derecki et al., 2010), we also measured the effect of estrogen on *Il4* expression, and observed a trend for increased expression (Fig. 3B). In accordance with the decreased IFNγ mRNA expression (Fig. 3C) we also observed decreased IFNγ protein levels measured in the cell culture media from estrogen-stimulated glial cells ($t_{(4)}$=3.443; p=0.0262) (Fig. 3 D).

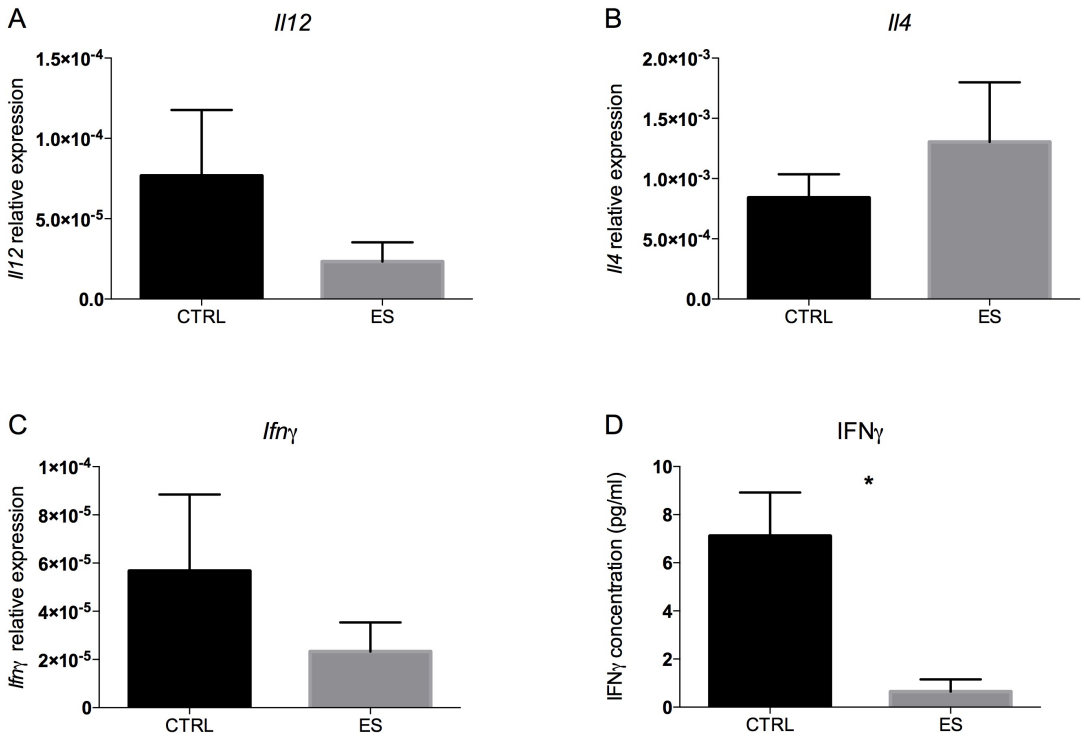

Fig. 3 – Effect of estrogen treatment on cytokine production by glial cells. *In vitro* estrogen treatment of glial cells led to a trend for decreased *Il12* (A) and *Ifnγ* (C) expression, and a mild trend for increased *Il4* expression (B). The secreted IFNγ levels in the media were decreased by estrogen treatment (D). Error bars denote mean + SEM. *p<0.05.

Discussion

In this sub-chapter we showed that there are gender-differences on cognitive and anxious-like behaviour of IFNγ KO mice. These differences might in fact explain our differential experimental results on these behavioural dimensions when using females (2nd Chapter) from the published data on males (Campos et al., 2014; Kustova et al., 1998; Litteljohn et al., 2014). Moreover, we observed that the IFNγ expression pattern across brain regions that are involved in the behaviour dimensions analysed, was also different between males and females.

The finding of gender-differences caused by the absence IFNγ on behaviour adds another level of complexity for the understanding on the role of this cytokine in higher brain function.

As a starting point for exploring the mechanism underlying these sex-differences, we hypothesize that estrogen could modulate the IFNγ production by brain cells. Although still preliminary, our results point to an involvement of estrogen in IFNγ levels variations, at least by glial cells.

For sure, there is still much to be explored in this context, from which we highlight the need to understand if the physiological estrogen levels cycling on females do impact IFNγ levels in the brain (and how), and more importantly, if there is indeed an association of these possible hormonal-induced variations on brain IFNγ levels and behaviour.

The ability of estrogen to modulate cytokine production by peripheral cells has been highly associated with sex differences on the prevalence of autoimmune diseases (Cutolo et al., 2006; Cutolo & Wilder, 2000; Guery, 2012; Ngo, Steyn, & McCombe, 2014). Likewise, the possibility of estrogen being able to modulate the production of IFNγ in the brain raises important questions, namely the possibility of this cytokine underlying gender-specific pathophysiological mechanisms in neuropsychiatric disorders, a subject that merits further investigation.

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4th CHAPTER

An Efficient Chronic Stress Protocol to Induce Stress-Related Responses in C57BL/6 Mice

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An efficient chronic unpredictable stress protocol to induce stress-related responses in C57BL/6 mice

Running title: Chronic stress model using C57BL/6

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Abstract

Exposure to chronic stress can have broad effects on health ranging from increased predisposition for neuropsychiatric disorders to deregulation of immune responses. The chronic unpredictable stress (CUS) protocol has been widely used to study the impact of stress exposure in several animal models and consists in the random, intermittent and unpredictable exposure to a variety of stressors during several weeks. CUS has consistently been shown to induce behavioral and immunological alterations typical of the chronic stress response. Unfortunately C57BL/6 mice, one of the most widely used mouse strains, due to the great variety of genetically modified lines, seem to be resistant to the commonly used 4-week-long CUS protocol. The definition of an alternative CUS protocol allowing the use of C57BL/6 mice in chronic stress experiments is a need. Here we show that by extending the CUS protocol to 8 weeks is possible to induce a chronic stress response in C57BL/6 mice, as revealed by abrogated body weight gain, increased adrenals weight and an overactive hypothalamic-pituitary-adrenal (HPA) axis with increased levels of serum corticosterone. Moreover, we also observed stress-associated behavioral alterations, including the potentiation of anxious-like and depressive-like behaviors and a reduction of exploratory behavior, as well as subtle stress-related changes in the cell population of the thymus and of the spleen.

The present protocol for C57BL/6 mice consistently triggers the spectrum of CUS-induced changes observed in rats and, thus, will be highly useful to researchers that need to use this particular mouse strain as an animal model of neuropsychiatric disorders and/or immune deregulation related to chronic unpredictable stress.

Keywords: chronic stress; CUS; neuropsychiatric disorders; immune dysfunction; anxiety; depressive-like behavior; social defeat

Introduction

Stressful life events can be triggering factors of numerous neuropsychiatric disorders namely anxiety, depression and dementia (McEwen, 2004), and many of these are accompanied by immune dysfunction (Glaser & Kiecolt-Glaser, 2005). Moreover, prolonged-stress-induced immune dysfunction itself is regarded as a contributing factor for the effects of stress on health (Haroon, Raison, & Miller, 2012). In contrast with chronic stress, the acute stress response is a beneficial event since it is an alarm reaction that prepares the body to a possible threat. This response is characterized by the secretion of stress mediators, such as glucocorticoids and epinephrine, which allows the stability of body function by adaptation to the stressor (McEwen, 2005). However, when this response persists in time, it might render the system unable to cope with the stressor, ultimately leading to chronic-stress-associated illness.

Neuropsychiatric alterations are the most widely described effects of chronic stress exposure and include anxious-like behavior (Bondi, Rodriguez, Gould, Frazer, & Morilak, 2008; McEwen, Eiland, Hunter, & Miller, 2012; Pego et al., 2008; Ventura-Silva et al., 2012), depressive-like behavior (Bessa, Mesquita, et al., 2009; Strekalova, Spanagel, Bartsch, Henn, & Gass, 2004) and cognitive deficits (Bondi et al., 2008; Cerqueira et al., 2007; Conrad, 2010; Dias-Ferreira et al., 2009; Morgado, Silva, Sousa, & Cerqueira, 2012). However, the effects of chronic stress are not only limited to behavioral changes. Immune cells express receptors for glucocorticoids and catecholamines (Dhabhar & McEwen, 1997; Padgett & Glaser, 2003), that can lead to alterations in gene transcription in response to stress (Dhabhar, Malarkey, Neri, & McEwen, 2012). In fact, it is generally accepted that chronic-stress-associated changes in the immune system alter the vulnerability to infectious disease and auto-immunity (Harpaz et al., 2013).

Stress exposure variables, such as duration, intensity and predictability, explain the spectrum of differential responses to stress but ultimately, the threshold in which the stress-response switches from physiological to deleterious is also dependent on neuroendocrine, neurochemical and genetic factors that are responsible for individual differences in stress perception and response (Franklin, Saab, & Mansuy, 2012).

Having this in mind, it seems logical that for the use of animal models, the chronic stress protocol needs to be adjusted to the animal species and even the strain used.

The most commonly used unpredictable chronic stress paradigms are the unpredictable chronic mild stress (uCMS) and the chronic unpredictable stress (CUS). Although both terms, uCMS and CUS, tend to be used indiscriminately nowadays and that both protocols are widely used to study depression, the

original purpose for which they were generated was quite distinct. uCMS paradigm have been long used to model depression, and consists in the continuous exposure of animals to stressful situations, usually for at least 4 weeks, including some stressors that involve water and/or food deprivation. In contrast, CUS was originally used to study mechanisms underlying the stress-response and involves the intermittent exposure to a daily stressful stimulus, lasting at least 4 weeks, being one of the main advantage of this protocol the absence of stressors that interfere with water and/or food deprivation which might better mimic everyday life stress.

Although rats are widely used as animal models of depression and other stress-related disorders, mice present advantages such as the availability of numerous genetically modified strains like transgenic and KO mice and the lower maintenance costs when compared to rats. Unfortunately, the most widely used inbreed strain of genetically modified mice, the C57BL/6, seems to be less vulnerable to stress than other mouse strains (Anisman, Hayley, Kelly, Borowski, & Merali, 2001; Anisman, Lacosta, Kent, McIntyre, & Merali, 1998; Parfitt, Walton, Corriveau, & Helmreich, 2007; Razzoli, Carboni, Andreoli, Ballottari, & Arban, 2011; Razzoli, Carboni, Andreoli, Michielin, et al., 2011; Savignac et al., 2011).

Our aim was to develop an improved CUS protocol to be used in C57BL/6 mice. In order to do so we modified the standard CUS protocol by including social defeat stress as one of the stressors and extending its duration to 8 weeks. By comparing the neuroendocrine, behavioral and immune changes induced by the unmodified 4-week long CUS exposure and the optimized 8-week long CUS protocol we, herein, show the advantages of later for C57BL/6 mice.

Material and methods

Animals

Male C57BL/6 mice (C57BL/6J JAX™ mice strain) were purchased from Charles River (Charles River Laboratories, Barcelona, Spain) and housed (5 animals per cage) under standard laboratory conditions (12 h light/12 h night cycles (8 h/ 20 h), 22-24 °C, relative humidity of 55% and *ad libitum* access to water and food. All procedures were carried out in accordance to EU directive 2010/63/EU and Portuguese national authority for animal experimentation, Direção Geral de Veterinária (ID:DGV9457) guidelines on animal care and experimentation.

Chronic unpredictable stress paradigm

One group of C57BL/6 animals was exposed to 4 weeks of CUS and compared to a control group that was subjected to gentle handling, twice a week, for the same period. Another group was exposed to 8 weeks of CUS and compared to other control group that was subjected to gentle handling, twice a week, for the same period. Mice were 8-week old when the CUS protocol was initiated. Each group consisted of 10-15 male C57BL/6 mice. We run two independent experiments to confirm our findings: data from the first, representative of our findings, are presented in the main paper, whereas data from the second experiment are shown as a supplementary figure (Supplementary Fig 1 and Table 1).

Briefly, the CUS paradigm consisted in exposure, once daily, to one of the following aversive stressors: **restraint** – mice were placed in a 50 ml plastic tube (Falcon) with openings in both sides for breathing, for 1 h; **shaking** – groups of 5 mice were placed in a plastic box container and placed in an orbital shaker for 1 h at 150 rpm; **social defeat** – mice were introduced in a cage of an aggressive mice and after being defeated, they were placed in a transparent and perforated plastic container, to avoid further physical contact, inside the resident homecage for 30 min (Golden, Covington, Berton, & Russo, 2011); **hot air stream** – mice were exposed to a hot air stream from a hairdryer for 10 min; **overnight illumination –** mice were exposed to regular room light during the night period; **inverted light cycle** – regular room light was off during daytime and on during nighttime for 2 d; **tilted cage** – homecages were tilted in a 45° angle during 1 h. Stressors were presented in a random order in an unpredictable fashion (see table 1). The stressors distribution for the group submitted to 4 weeks of CUS is a truncated version of table 1. Body weight was monitored once a week and *postmortem* thymus and adrenal weight were recorded.

Table 1 – Example of stressors distribution.

	Mon	**Tue**	**Wed**	**Thu**	**Fri**	**Sat**	**Sun**
Week 1	BW hot drier	shaking	restraint	social defeat	restraint	restraint	tilted cage
Week 2	BW restraint	shaking	social defeat	restraint	shaking	social defeat	restraint
Week 3	BW restraint	social defeat	restraint	BC-*zenith*	social defeat	restraint	shaking
Week 4	BW BC - *nadir* social defeat	EPM OF restraint	FST TST Social defeat	Shaking	restraint	shaking	hot drier
Week 5	BW restraint	Sacrifice 4weeks Tilted cage	Cytometry hot drier	restraint	social defeat	inverted light	inverted light
Week 6	BW hot drier	shaking	restraint	inverted light	overnight illumination	restraint	hot drier
Week 7	BW restraint	social defeat	restraint	BC-*zenith*	shaking	restraint	overnight illumination
Week 8	BW BC-*nadir*	EPM OF restraint	FST TST Social defeat	MWM Shaking	MWM restraint	MWM restraint	MWM Shaking
Week 9	BW	Sacrifice 8weeks	Cytometry				

BW – body weight measurement; BC – Blood collection; EPM – elevated plus maze; OF – open field; FST – forced swimming test; TST – tail suspension test; MWM – Morris water maze test.

Corticosterone quantification

Blood was collected through the tail by venopuncture within a maximum 120 s period since removal of each mouse from its homecage to the end of blood collection. Sera were separated by centrifugation at 13000 rpm, during 5 min and stored at -80 °C. Serum corticosterone levels were measured on sera collected at *nadir* phase (9 a.m.) and at *zenith* phase (8 p.m.) using a commercial radioactive immunoassay kit (MP Biomedicals, California, USA).

Behavioral assessment

Mice were transported and left for habituation to the testing room for 1 h prior to the behavioral test. The order of the behavioral tests was: elevated plus maze (EPM) and open field (OF) (Day 1), forced swimming test (FST) and tail suspension test (TST) (Day 2) and Morris water maze (MWM) (Day 3-7).

Elevated-plus maze

Anxious-like behavior was assessed using the EPM test (Walf & Frye, 2007). Briefly, this test consists on placing each mouse in the hub of a plus-like apparatus elevated 72.4 cm from the floor, with two opposing open arms (50.8 cm x 10.2 cm) and two opposing closed arms (50.8 cm x 10.2 cm x 40.6 cm) (ENV560; Med Associates Inc, Vermont, USA) and letting the animal freely explore it for 5 min. Time in the open arms and in the closed arms was used as a behavioral parameter of anxious-like behavior. EPM data from one animal from each group was not included in the analysis due to failure of the video recording system.

Open Field

Locomotor and exploratory activities were assessed using the OF. Each mouse was left in the center of a squared arena (43.2 cm x 43.2 cm) which the mouse was free to explore for 5 min. This arena is equipped with infrared beams for activity detection (Med Associates Inc, Vermont, USA). Data was collected using the activity monitor software (Med Associates Inc, Vermont, USA). Distance traveled was used as a measure of locomotor activity and the number of vertical counts as a measure of exploratory activity.

Forced swimming test and Tail-suspension test

Depressive-like behavior was assessed through the FST as described by (Can et al., 2012) and through the TST (Steru, Chermat, Thierry, & Simon, 1985). Briefly, in the FST each mouse was placed in an inescapable transparent cylindrical tank filled with water (± 24 °C), for 6 min. In the TST each animal was suspended by the tip of its tail for 6 min. The activity of each mouse, in both tests, was recorded using a videocamera. Latency (time to the first stop), mobility and immobility times were scored manually by an investigator blind to the experimental conditions, using Etholog 2.2 software (Ottoni, 2000), and used as a measure of behavioral despair. TST data from one animal was not included in the analysis due to failure of the video recording system.

Morris water maze

In order to assess spatial reference memory, mice were tested in a white circular pool (170 cm diameter) filled with water (24-25 °C) placed in a dimly lit room. Spatial cues were placed in the walls around the pool (square, stripes, triangle and a cross). The pool was divided in 4 imaginary quadrants and a hidden transparent platform was placed in 1 of the quadrants. Data was collected by a fixed

camera placed in the ceiling and connected to a video-tracking system (Viewpoint, Champagne-au-Mont-d'Or, France).

Mice had to learn the position of a hidden platform over a period of 4 d. In each day, mice were placed facing the wall of the pool at different quadrants (north, west, south and east) as a starting point for each trial. Each trial was completed whenever the mouse reached the platform or when 120 s elapsed. Latency to reach the platform (escape latency) was recorded for each trial during the 4 d.

In the fifth day, the platform was removed and a single trial of 60 s was performed (probe trial). The percentage of time that each mouse swam in each quadrant was recorded to confirm the acquisition of platform location through reference memory.

Flow Cytometry

Thymus and spleen (8-10 animals per group) were dissected and homogenized in supplemented Dulbecco's modified eagle medium (DMEM) with 10% heat inactivated FCS, 10 mM HEPES buffer, 1 mM sodium pyruvate, 2 mM l-glutamine, 50 μg/mL streptomycin, and 50 U/mL penicillin, (all from Invitrogen, CA, USA) in order to obtain single-cell suspensions. Splenic erythrocytes were depleted by incubating for 5 min with a hemolytic solution (155 mM NH_4Cl, 10 mM $KHCO_3$, pH 7.2). To analyze the main cell populations in the thymus, the cells ($1x10^6$ cell) were stained with APC anti-mouse CD3 (clone 145-2C11, Biolegend, San Diego, California, USA), V450 anti-mouse CD4 (clone RM4-5, BD Pharminogen, San Jose, California, USA), V500 anti-mouse CD8 (clone 53-6.7, BD Pharminogen, San Jose, California, USA). Splenocytes ($1x10^6$ cell) were stained with APC anti-mouse CD3 (clone 145-2C11, Biolegend, San Diego, California, USA) for T-lymphocytes, PE.Cy5.5 anti-mouse CD19 (clone 6D5, Biolegend, San Diego, California, USA) for B-lymphocytes, V450 anti-mouse CD4 (clone RM4-5, BD Pharminogen, San Jose, California, USA) for T helper cells, V500 anti-mouse CD8 (clone 53-6.7, BD Pharminogen, San Jose, California, USA) for T cytotoxic cells and FITC anti-mouse NK1.1 (clone PK136, Biolegend, San Diego, California, USA) for natural killer cells. To analyze myeloid cell populations, splenocytes ($1x10^6$ cell) were stained with PE anti-mouse CD11b (clone M1/70, Biolegend, San Diego, California, USA), and PE.Cy7 anti-mouse Gr1 (clone RB6-8C5, Biolegend, San Diego, California, USA). Cells were first gated for singlets (FSC-H vs FSC-A) and viable cells (FSC-H vs SSC-H). Myeloid cells were selected using the gating strategy described previously (Rose, Misharin, & Perlman, 2012). Briefly, myeloid cells were gated as $CD11b^+$ cells excluding the $NK1.1^+$ cells. Macrophages/dendritics were selected as the population $Gr1^+SSC^{low}$, neutrophils were selected as $Gr1^+SSC^{high}$ and eosinophils as $Gr1^-SSC^{high}$ (Fig. 7C). After staining cells were fixed in 2% paraformaldehyde

for 20 min. Cell surface staining was acquired (100000 events) in an eight-color LSRII flow cytometer (BD, Pharminogen, San Jose, California, USA) and analyzed with FlowJo software version 7.6.4.

Statistical analysis

All values were calculated as means ± SEM. Kolmogorov-Smirnov normality test was used to analyze if values departed from an approximate Gaussian distribution. Body weight, serum corticosterone levels and reference memory task data were compared between groups using ANOVA repeated-measures on the average results of each week/phase/day, respectively. When the main effect was significant, post-hoc Bonferroni test was performed in order to assess whether means differed significantly from each other. For all the other data, the differences among groups were analyzed using Student's T-test. Differences were considered significant if $p<0.05$. Statistical analysis was performed with Graphpad Prism version 5.0b (La Jolla, San Diego, USA).

Results

Biometric parameters and corticosterone measurements

Body weight gain, *post-mortem* thymus and *post-mortem* adrenal weight and serum levels of corticosterone were monitored to control for stressors efficacy (Fig. 1 and supplementary Fig. 1). In the group submitted to the 4-weeks protocol of CUS, both time ($F_{(4,72)}=23.85$; $p<0.0001$) and exposure to CUS ($F_{(1,18)}=11.94$; $p=0.003$) had a significant impact on body weight (Fig. 1A). Moreover, there was a significant interaction between these factors ($F_{(4,72)}=23.85$; $p<0.0001$) with stressed animals gaining significantly less weight over time (Fig. 1A). In the group submitted to the 8-weeks protocol, repeated-measures ANOVA has shown again a significant effect of both time ($F_{(8,144)}=80.13$; $p<0.0001$) and exposure to CUS ($F_{(1,18)}=63.43$; $p<0.0001$) on body weight (Fig. 1B). There was also a significant interaction between these factors ($F_{(8,144)}=34.17$; $p<0.0001$) with stressed animals gaining significantly less weight over time (Fig. 1B). CUS had no significant effect on thymus weight nor on thymic cell number, both in the group exposed to the 4- and the 8-weeks protocol of CUS (Fig. 1C and D). CUS exposure during 4 weeks had no effect on adrenals weight, while exposure to CUS for 8 weeks led to a significant increase on adrenals weight ($t_{(18)}=3.449$; $p=0.003$) (Fig.1E and F). There were no statistically

significant changes on corticosterone levels in the group submitted to 4 weeks of CUS, both at *nadir* and *zenith.* Repeated-measures ANOVA has shown a significant effect of exposure to 8 weeks of CUS on corticosterone levels ($F_{(1,18)}$=21.99; p=0.0002). Post-hoc test has shown a statistically significant increase of corticosterone levels in the *zenith* phase of the day, in the group submitted to 8 weeks of CUS ($t_{(18)}$=4.113; p<0.001) (Fig.1G and H).

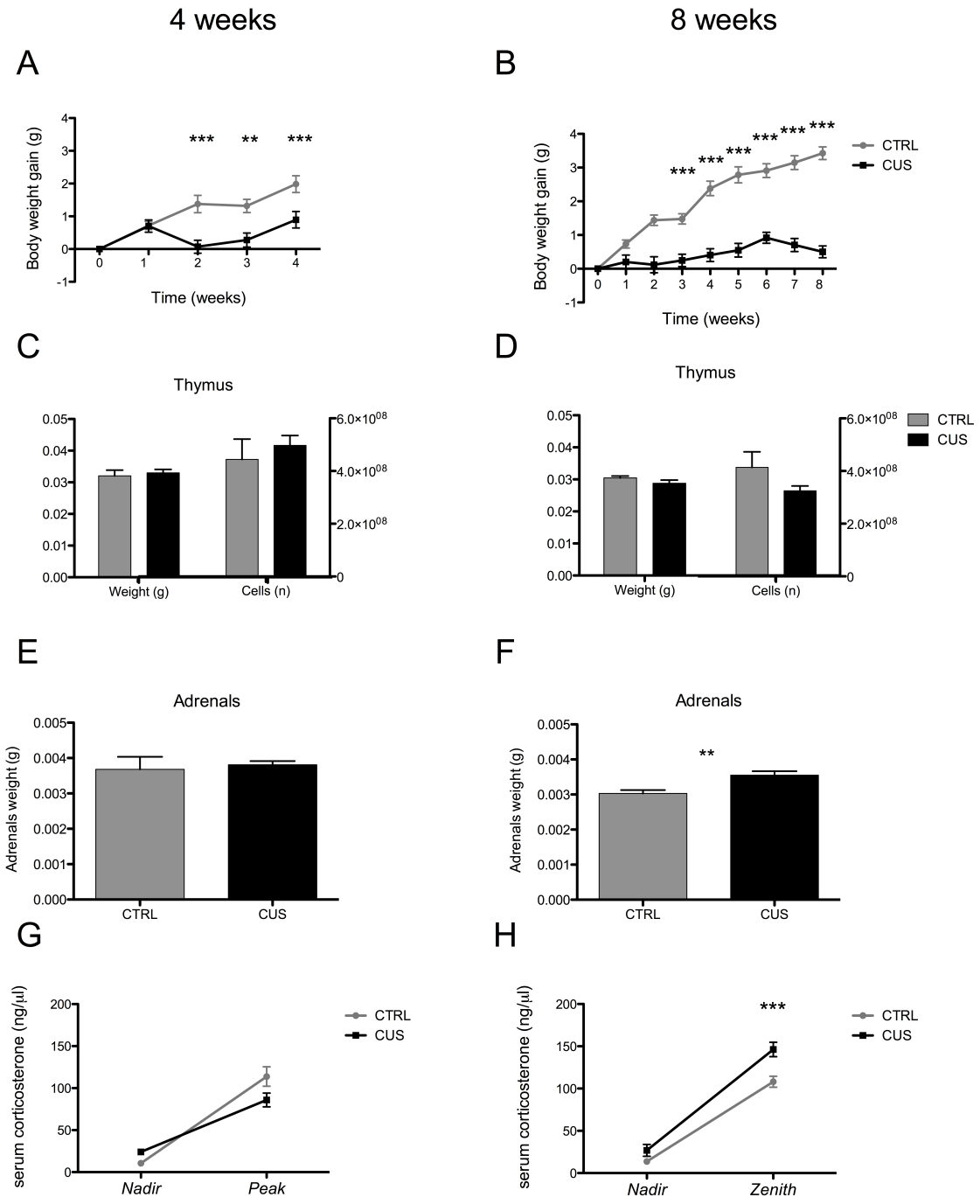

Fig. 1 – Effect of 4 weeks versus 8 weeks of CUS on biometric parameters. Body weight gain for animals submitted to 4 (A) and 8 (B) weeks of CUS. Thymus weight and cellularity after

exposure to 4 (C) and 8 (D) weeks of CUS. Adrenals weight after 4 (E) and 8 weeks of CUS (F). Corticosterone levels in the serum of animals from the group submitted to 4 (G) and 8 (H) weeks of CUS. Each bar/point represents the mean +/- SEM from 10 animals per group. ** $p<0.01$, *** $p<0.001$.

Exposure to 8 weeks of CUS leads to altered emotional behavior but not to memory impairments

There was a significant effect of exposure to 8 but not 4 weeks of CUS on anxious-like behavior, measured by a decreased time spent on the open-arms of the EPM ($t_{(16)}=2.401$; $p=0.029$) and an increased time spent in the closed arms ($t_{(16)}=2.176$; $p=0.045$) (Fig. 2A and B; and supplementary Fig. 1I and J) by the 8-weeks CUS group when compared to controls. Exposure to CUS did not alter locomotor activity, assessed by the OF, both on the group exposed to 4 and 8 weeks of CUS (Fig. 2C and D; and supplementary Fig. 1K and L), therefore validating behavioral tests that are dependent on an intact locomotor function. CUS had an impact on the exploratory activity, measured by a decrease on the number of rearings in the OF test, both in the group submitted to 4 ($t_{(18)}=2.743$; $p=0.013$) (Fig. 2E and supplementary Fig. 1M) and 8 weeks of CUS ($t_{(18)}=2.308$; $p=0.033$) (Fig. 2F and supplementary Fig. 1N).

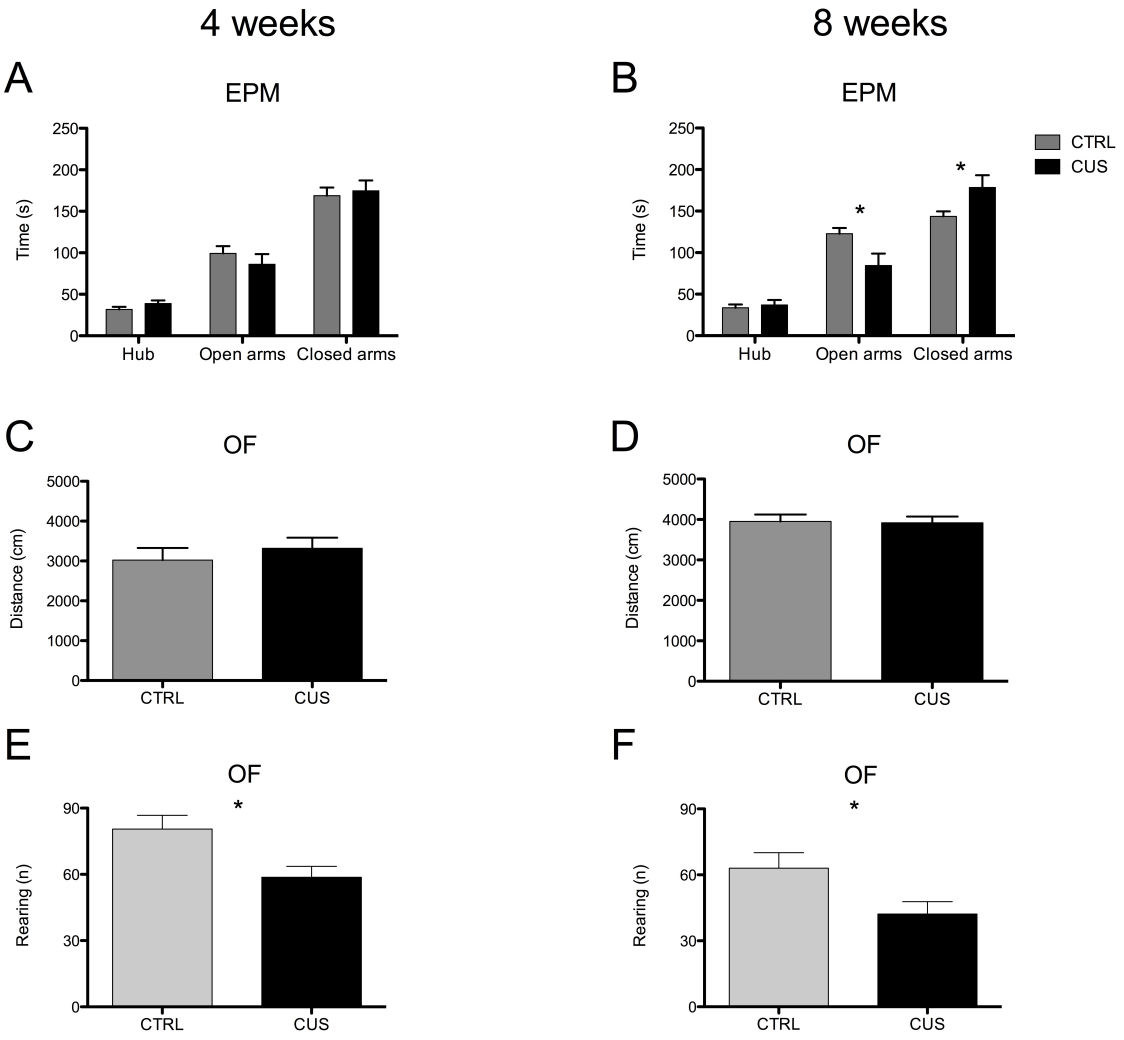

Fig. 2 – Impact of 4 weeks and 8 weeks of CUS on anxious-like and locomotor behavior and exploratory activity. Behavioral performance of mice exposed to 4 (A) and to 8 weeks of CUS (B) in the EPM. Locomotor function of mice submitted to 4 (C) and 8 weeks (D) of CUS measured in the OF. Exploratory activity of mice submitted to 4 (E) and 8 weeks (F) of CUS measured in the OF. Each bar represents the mean +/- SEM from 8-10 animals per group. * $p<0.05$.

Animals exposed to 4 weeks of stress did not show any major differences in the FST when compared to controls (Fig. 3A). The group of animals exposed to 8 weeks of CUS exhibited decreased mobility time ($t_{(18)}=2.741$; $p=0.013$) and increased immobility time ($t_{(18)}=2.310$; $p=0.033$) in the FST when compared to controls (Fig. 3B). In the TST, the group submitted to 4 weeks of CUS did not show any major differences when compared to controls (Fig. 3C), while the group submitted to 8 weeks of CUS exhibited a increased immobility time ($t_{(17)}=3.710$; $p=0.002$) and an decreased mobility time ($t_{(17)}=3.873$; $p=0.001$) (Fig. 3D and supplementary Fig. 1O); a typical phenotype of depressive-like behavior. No differences on latency time were found at any time point, both in the FST and TST (Fig. 3).

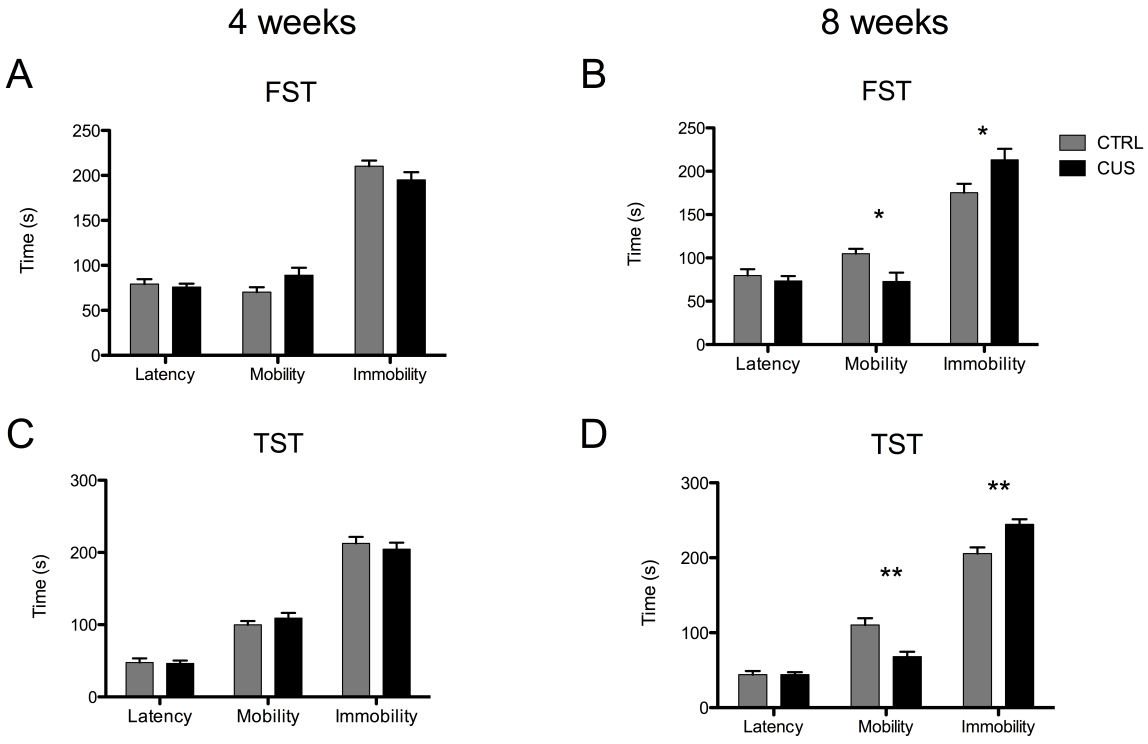

Fig. 3 – Impact of 4 weeks versus 8 weeks of CUS on depressive-like behavior. Behavioral performance of mice submitted to 4 (A) and 8 weeks of CUS (B) in the FST. Behavioral performance of mice submitted to 4 (C) and 8 weeks of CUS in the TST (D). Each bar represents the mean +/- SEM from 9-10 animals per group.* $p<0.05$, ** $p<0.01$.

The impact of different exposures to CUS was also tested in the MWM task in order to investigate whether the cognitive dimension was also affected. Although there was a slight tendency for a faster learning curve of the control group, especially on day 2 and 3, in comparison to CUS exposed animals, the ANOVA repeated measures test revealed that there were no significant differences between groups, meaning that, at the end of the learning task, both CUS and control groups were able to successfully learn the task therefore exhibiting an intact spatial learning ability (Fig. 4 and supplementary Fig. 1P).

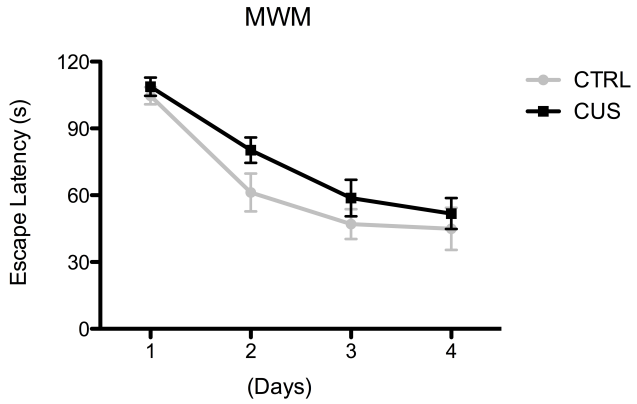

Fig. 4 - Impact of 8 weeks of CUS on cognition. Behavioral performance of mice exposed to 8 weeks of CUS in the MWM. Each point represents the mean +/- SEM from 10 animals per group.

Thymic and splenic cell population changes by exposure to 8 weeks of CUS

It is known that thymocytes are sensitive to stress hormones, such as glucocorticoids, which modulate several processes along their differentiation within the thymus (Berki, Palinkas, Boldizsar, & Nemeth, 2002). Due to this well-known susceptibility to stress hormones, the thymus weight and cellularity have been widely used as indirect measures of stress. Thymocytes might be divided in four main differentiation populations depending on the expression of the CD4 and CD8 co-receptors ($CD4^-CD8^-$ double negative – DN; $CD4^+CD8^+$ double positive - DP; $CD4^+CD8^-$ single-positive CD4 - SPCD4; and $CD4^-CD8^+$ single-positive CD8 - SPCD8 cells). We therefore studied the major thymic subsets to determine if our CUS protocols had a differential impact on them. We observed that 4 weeks of CUS did not alter the proportion of the 4 main thymocyte subsets (Fig. 5A) while 8 weeks of stress led to an increase of the DN thymocytes proportion ($t_{(18)}$=2.681; p=0.020) (Fig. 5B and C).

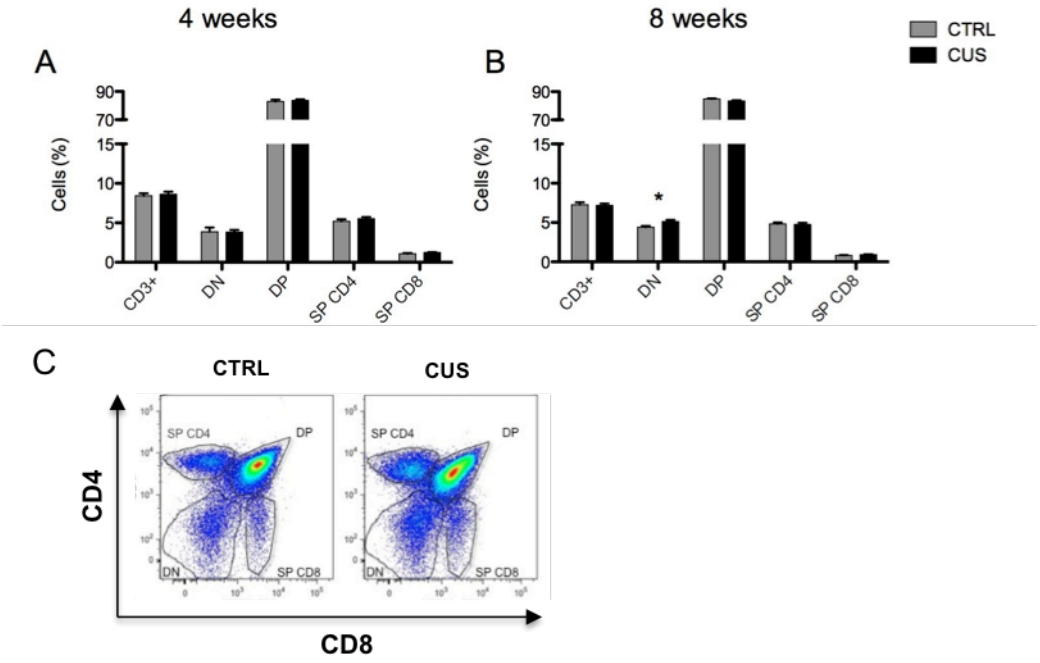

Fig. 5 - Impact of 4 weeks versus 8 weeks of CUS on thymocyte subsets. Percentage of main cell populations in thymus after exposure to 4 (A) and 8 (B) weeks of CUS. Flow cytometry plot showing the gating strategy for thymocyte subsets (gate) (C). DN – double-negative thymocytes; DP – double-positive thymocytes; SPCD4 and SPCD8 – single-positive CD4 and CD8 thymocytes, respectivly. Each bar represents the mean +/- SEM from 10 animals per group..* $p<0.05$.

Since prolonged stress is known to influence the peripheral immune system we consider of relevance to investigate potential alterations caused by CUS on major lymphoid cell populations in the spleen, one of the most important lymphoid organs of the immune system. Animals exposed to 4 or 8 weeks of CUS did not show any differences on the percentage of splenic T and B cells (Fig. 6A and B) nor in the $CD4^+$ and $CD8^+$ subsets among the T cells (Fig. 6C and D). On the contrary, while animals exposed to 4 weeks of CUS did not show any major differences on the percentage of splenic eosinophils, neutrophils and macrophages/dendritic cells (Fig. 7A), the 8-week long CUS protocol led to an increased percentage of macrophages/dendritic cells ($t_{(14)}=2.188$; $p=0.046$) and neutrophils ($t_{(14)}=3.327$; $p=0.005$) in the spleen (Fig. 7B and C).

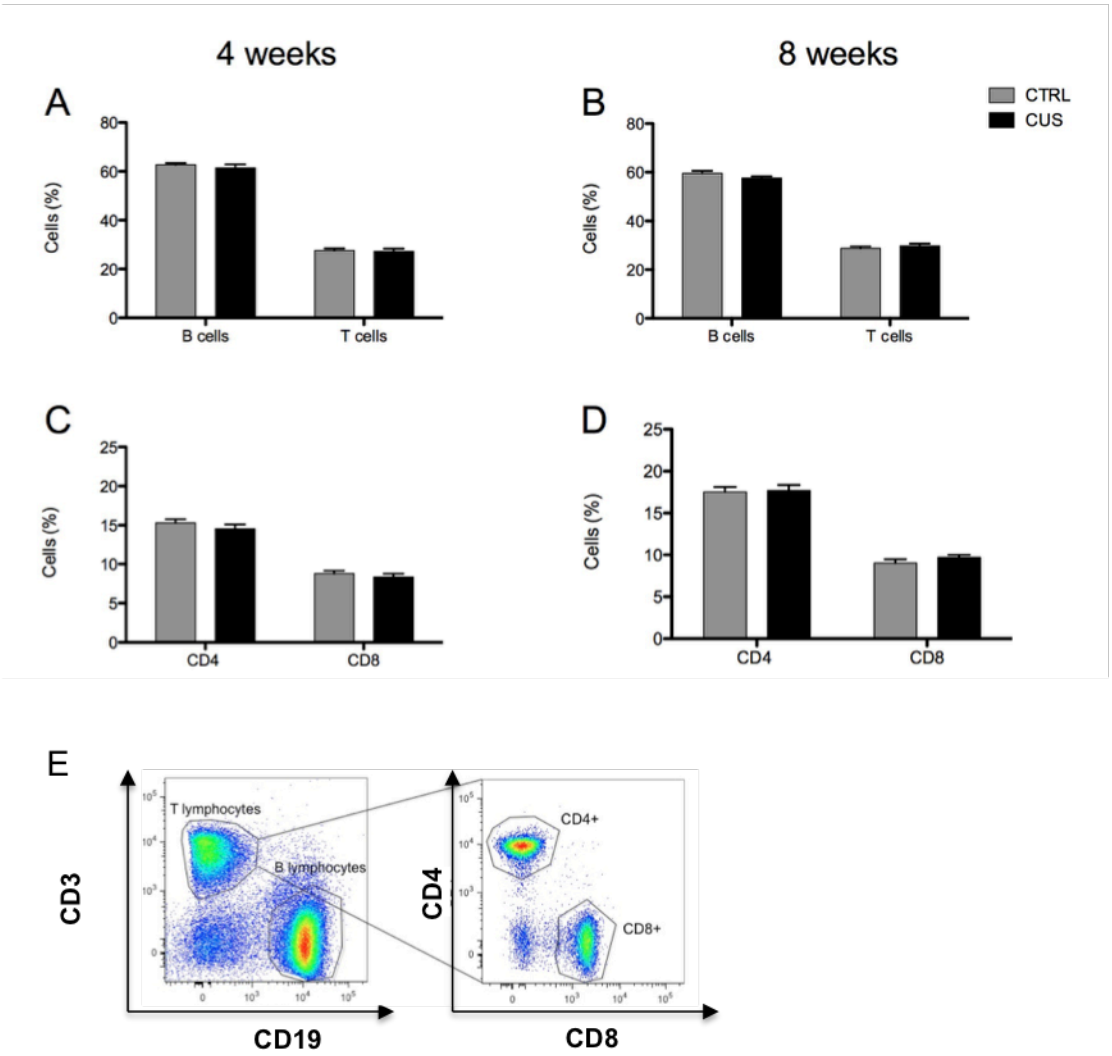

Fig. 6 - Impact of 4 versus 8 weeks of CUS on lymphoid cellular populations in the spleen. Percentage of T and B cells in spleen after exposure to 4 (A) and 8 (B) weeks of CUS. Percentage of $CD4^+$ and $CD8^+$ T cells in spleen after exposure to 4 (C) and 8 (D) weeks of CUS. Flow cytometry plot showing the gating strategy for T and B lymphocytes (gates) (E). Each bar represents the mean +/- SEM from 8 animals per group.

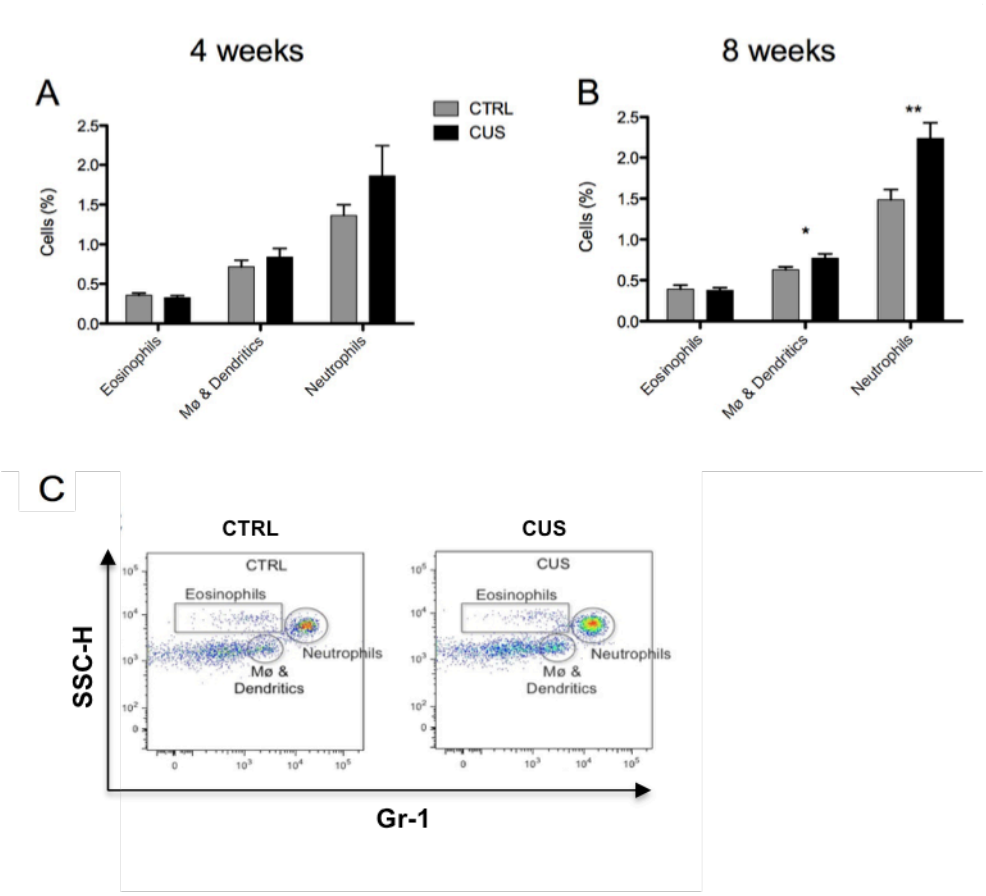

Fig. 7 - Impact of 4 versus 8 weeks of CUS on myeloid cellular populations in the spleen. Percentage of eosinophils, macrophages/dendritics and neutrophils in spleen after exposure to 4 weeks (A) and 8 (B) weeks of CUS. Flow cytometry plot showing the gating strategy of myeloid splenocytes subsets (gates) (C). Each bar represents the mean +/- SEM from 8 animals per group.* $p<0.05$, ** $p<0.01$.

Discussion

In the present work, we have optimized a CUS protocol that results in a consistent stress-response in C57BL/6 mice. Published protocols on how to induce chronic stress on rodents are diverse and generate inconsistencies in their behavioral and immunological outcomes (Nestler & Hyman, 2010). Among the main reasons for such inconsistencies are strain inherent differences of stress susceptibility/resistance to distinct protocols. In mice, specifically, the C57BL/6 strain seems to be more resistant to chronic unpredictable stress than other strains and/or other species (Anisman et al., 2001; Anisman et al., 1998; Parfitt et al., 2007; Razzoli, Carboni, Andreoli, Ballottari, et al., 2011; Razzoli, Carboni, Andreoli, Michielin, et al., 2011; Savignac et al., 2011). Yet, it is by far the most used mouse strain for genetic manipulations. This, and the fact that unpredictable chronic stress exposure is often used as a model of neuropsychiatric disorders, renders an effective CUS protocol in C57BL/6 mice, such as the one herein described, an important addition to the field.

Besides strain considerations, the type, diversity of stressors applied and stress exposure length are also critical determinants of the impact of chronic stress. Some protocols use a single stressor, e.g. 6 h of daily restraint stress for a 4-week period (Voorhees et al., 2013; Yun et al., 2010), which, despite being simpler to apply, have several limitations due to lack of unpredictability or the prolonged removal of animals from their homecages with no access to food or water for half of their resting period. On the other hand, reducing restraint stress to 1 h per day in order to overcome this difficulty results in a mild stress protocol.

Other widely used chronic stress protocol consists in the exposure to repeated bouts of social defeat stress, which have shown to induce a stressed phenotype in some C57BL/6 mice. However, both restraint and social defeat stress paradigms are characterized by repeated exposure to a single stressor, which lacks the variability of psychological and physical stressors generally encountered in life. Taking the aforementioned into account, we designed a CUS protocol, based on the appliance of a variety of stressors, presented randomly once per day, in an intermittent and unpredictable fashion, mimicking the variability of stressors encountered on everyday life (construct validity). Although not often used in mice, CUS protocols are widely used in rats and were shown to be highly effective in inducing a stress-related phenotype (Bessa, Ferreira, et al., 2009; Cerqueira et al., 2007; Dias-Ferreira et al., 2009; Pego et al., 2008). In addition, by extending this protocol to 8 weeks, instead of the usual 4 weeks, we were able to reach the point where this particular strain of mice clearly and consistently

exhibits a maladaptive response to chronic stress with behavioral and immunological alterations (face validity).

One of the main advantage of this 8-week long CUS protocol is that there is no stressor that implies the disturbance of food and/or water consumption, which is of particular importance for metabolism studies, for example. Moreover, in this protocol FST or TST are not used as stressors, as used in some published protocols (Strekalova et al., 2004), which means that in our protocol these tests can still be used as behavioral measures.

Reduction on body weight gain, thymic involution (Ashwell, Lu, & Vacchio, 2000; Dominguez-Gerpe & Rey-Mendez, 1997; Zivkovic, Rakin, Petrovic-Djergovic, Kosec, & Micic, 2005) and increased adrenals weight (Ulrich-Lai et al., 2006) are typically used as markers of stressors efficacy. We have observed that although behavioral and immunological alterations were only evident after exposing mice to 8 weeks of stress, suppression of body weight gain was observed as early as after 2 weeks of exposure and was maintained throughout the duration of CUS. These findings suggest that, as a read-out of the maladaptive response to stress, body weight gain has a lower threshold than other changes and is not a good marker of the stress-impact in behavior and/or immunity. Moreover, we did not observe a consistent reduction on thymus weight; although we cannot discard the possibility of being unable to detect small differences of thymus weight, specially given that mice were previously transcardially perfused with 0.9% saline. Nevertheless, the concomitant lack of differences in thymic cellularity favors our observation that, in C57BL/6 mice, our CUS protocol does not impact thymus weight significantly. This observation strengths the idea that C57BL/6 are more resistant to the effects of chronic stress than other mouse strains.

An overactive HPA axis is also a feature of a maladaptative response to chronic stress (Sousa & Almeida, 2012). In fact, resistance to chronic stress can be associated with an effective negative feedback system that is able to shut down the excessive production of glucocorticoids occurring in response to stress (Taliaz et al., 2011). We observed that the 8-week long CUS protocol was the only one that led to a persistent increase on circulating corticosterone levels and increased adrenals weight, features consistent with a hyperactive HPA axis. Of note, based on corticosterone levels at peak we identified a reduced number of resistant animals (2 out of 10 in one of the experiments and 2 out of 10 in the replicated experiment), a proportion of stress-resistance very similar to what already have been described in other models of chronic stress (Golden et al., 2011). Accumulating evidence shows that glucocorticoids modulate the behavioral effects of chronic stress (Cerqueira et al., 2005; Sousa, Cerqueira, & Almeida, 2008). In accordance, we observed that only the 8-week long CUS protocol, the

only that induced a hyperactive HPA axis, had a negative impact on emotional behavior. Specifically, we observed an enhanced anxious-like behavior, revealed by an increased time spent in the closed arms, and a decreased time in the open arms of the EPM. These animals also displayed behavioral despair, a symptom of depressive-like behavior, as they spent more time immobile in the FST. Of notice, this was further confirmed by performing the TST, another validated test for depressive-like behavior assessment.

Despite the emotional changes caused by 8 weeks of CUS exposure, cognitive functioning, namely spatial learning, seems to be intact, confirming data from other model of chronic stress (uCMS) (Bessa, Mesquita, et al., 2009). In fact, we observed that although stressed animals at the end of 4 d of MWM training were able to learn task at the same level as controls, there was a tendency for a slower learning progression on day 2 and 3. This type of learning pattern was previously shown using rats submitted to CUS (Cerqueira et al., 2007), therefore emphasizing that the effects of CUS on spatial learning are more subtle than those on emotional behaviors. Contrary to the above mentioned effects, chronic stress triggers a decreased exploratory behavior of mice, both at 4 and 8 weeks of CUS, which might not be dependent on increased levels of corticosterone.

Although we cannot completely discard the possible confounder effect from performing two behavioral tests in the same day, we believe that data from the OF and TST was not significantly affected by acute stress caused by prior testing; indeed motor function (measured by OF) is not known as a target of acute stress, whereas data from TST was confirmed by the findings of the FST.

Glucocorticoids play a crucial role on thymopoiesis (Berki et al., 2002; Zivkovic et al., 2005), a process that occurs in the thymus in which immature precursor cells differentiate into mature T cells. In accordance, it was previously shown that rats exposed to chronic stress, with increased levels of circulating corticosterone levels, exhibit an increase in the percentage of DN thymocytes, while the percentage of SPCD4 was decreased (Zivkovic et al., 2005). In our model, an increase in the percentage of DN thymocytes was observed. Still, contrary to the previously described (Zivkovic et al., 2005), we did not observe any differences on the SPCD4 and SPCD8 populations of thymocytes, which may be due to the stress resistance inherent to this particular strain of mice. T and B lymphocytes in the spleen were not altered by exposure to chronic stress. However, we observed that exposure to 8 weeks of CUS (and not to 4 weeks) led to alterations in the cell composition of the spleen, characterized by an increased percentage of myeloid cells (macrophages/dendritic cells and neutrophils), in agreement with previous reports in both mice (Heidt et al., 2014a; Wohleb, Powell, Godbout, & Sheridan, 2013) and humans (Heidt et al., 2014a). Glucocorticoids were shown to inhibit neutrophils'

apoptosis which may explain the persistent presence of these cells with short life span (Saffar, Ashdown, & Gounni, 2011) in the spleen of chronically stressed animals. Moreover, it was shown that stress, through norepinephrine signalling from sympathetic nerve fibers, increased the proliferation of hematopoietic progenitors in the bone marrow giving rise to an increase on disease-promoting monocytes and neutrophils output (Heidt et al., 2014a). Stress was also shown to increase monocyte recruitment to the brain by increased expression of cytokines and chemokines in specific brain regions. And more importantly, this monocyte recruitment to the brain was shown to be essential for the development of anxiety behavior induced by stress (Wohleb et al., 2013).

The absence of neuroendocrine, major behavioral and immunological alterations seen in the 4-week CUS exposed group could reflect the temporal dynamics of the stress-response rather than a failure to respond to stress. In fact, it should be noted that stress did impact the body weight gain (Fig.1A) and exploratory behavior (Fig. 2E) on this group. This absence of major alterations resembles the Hans Selye's resistance phase of the so-called "syndrome of adaptation" (Selye, 1936) in which adaptative processes reinstall homeostasis during stress, including the normalization of glucocorticoid secretion. Therefore the 4-week CUS protocol may be preferable to studies that target this specific stage of the stress-response like for example those that want to show a negative impact of a particular treatment on the stress-response, since the 8-week CUS alterations may approach a "ceiling effect". In contrast, the alterations observed in the 8-week version of CUS are consistent with phase 3 of this syndrome, where the system is no longer able to cope with stressors and is exhausted, which renders this version a robust model of the maladaptative response to chronic stress.

The establishment of a robust mouse model of stress-related disorders on C57BL/6 background represents a valuable research tool endowing the study of different genetic contributions to chronic stress responses which may enhance current knowledge on the neurobiology and immunology of complex neuropsychiatric and other stress-related disorders.

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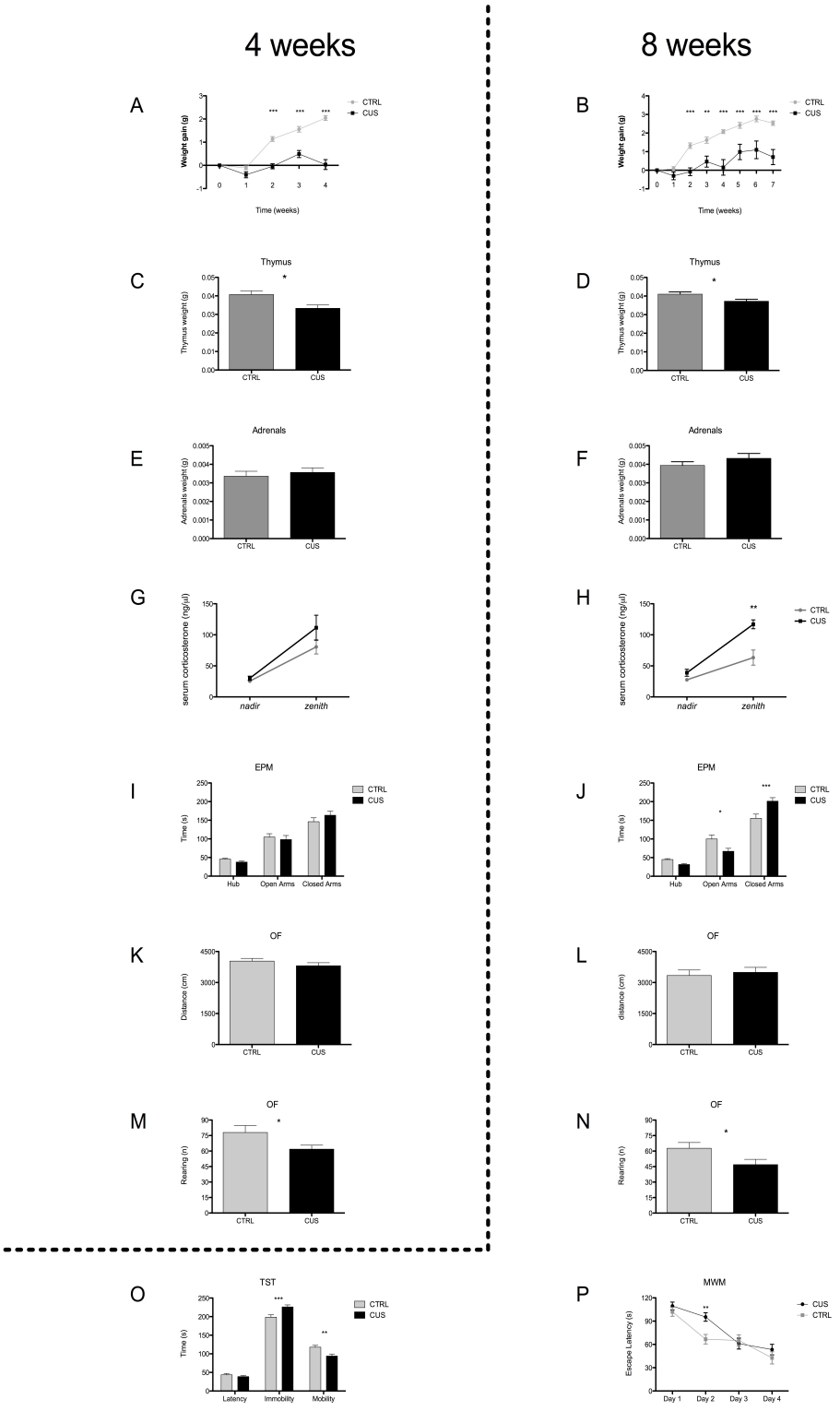
Supplementary Data

The full data set of the replicated experiment is depicted below (supplementary figure 1). We also added a table with the stressors sequence and layout of the replication experiment (supplementary table 1).



Supplementary Figure 1. Replication experiment data set. Body weight gain for animals submitted to 4 (A) and 8 (B) weeks of CUS. Thymus weight after exposure to 4 (C) and 8 (D) weeks of CUS. Adrenals weight after 4 (E) and 8 weeks of CUS (F). Corticosterone levels in the serum of animals from the group submitted to 4 (G) and 8 (H) weeks of CUS. Behavioral performance of mice exposed to 4 (I) and to 8 weeks of CUS (J) in the EPM. Locomotor function of mice submitted to 4 (K) and 8 weeks (L) of CUS measured in the OF. Exploratory activity of mice submitted to 4 (M) and 8 weeks (N) of CUS measured in the OF. Behavioral performance of mice submitted to 8 weeks of CUS in the TST (O) and MWM (P). *p<0.05, ** p<0.01, *** p<0.001

Body weight gain, *post-mortem* thymus and adrenal weight, serum levels of corticosterone, performance in the elevated plus maze (EPM), open field (OF), tail suspension test (TST) and morris water maze (MWM) were monitored in the replicated experiment to control for stressors efficacy (Supplementary Fig.1). In the group submitted to the 4-weeks protocol of CUS, both time ($F_{(4,112)}$=73.92; p<0.0001) and exposure to CUS ($F_{(1,28)}$=32.59; p=0.0001) had a significant impact on body weight (supplementary Fig. 1A). Moreover, there was a significant interaction between these factors ($F_{(4,112)}$=27.33; p<0.0001) with stressed animals gaining significantly less weight over time (supplementary Fig. 1A). In the group submitted to the 8-weeks protocol, repeated-measures ANOVA has shown again a significant effect of both time ($F_{(7,182)}$=48.68; p<0.0001) and exposure to CUS ($F_{(1,26)}$=19.21; p=0.0001) on body weight (supplementary Fig. 1B). There was also a significant interaction between these factors ($F_{(7,182)}$=9.81; p<0.0001) with stressed animals gaining significantly less weight over time (supplementary Fig. 1B). CUS has slightly decreased the thymus weight, both in the group exposed to the 4- and the 8-weeks protocol of CUS ($t_{(23)}$=2.762, p=0.01; $t_{(24)}$=2.259, p=0.03) (supplementary Fig. 1C and D). CUS exposure had no effect on adrenals weight, while exposure to CUS for 8 weeks led to a tendency for a increase on adrenals weight (supplementary Fig.1E and F). There were no statistically significant changes on corticosterone levels in the group submitted to 4 weeks of CUS, both at *nadir* and *zenith.* Repeated-measures ANOVA has shown a significant effect of exposure to 8 weeks of CUS on corticosterone levels ($F_{(1,18)}$=14.54; p=0.001). Post-hoc test has shown a statistically significant increase of corticosterone levels in the *zenith* phase of the day, in the group submitted to 8 weeks of CUS ($t_{(18)}$=4.956; p<0.001) (supplementary Fig.1G and H).There was a significant effect of exposure to 8 but not 4 weeks of CUS on anxious-like behavior, measured by an increased time spent in the closed arms ($t_{(22)}$=2.418; p=0.024) (supplementary Fig. 1I and J) by the 8-weeks CUS group when compared to controls. Exposure to CUS did not alter locomotor activity, assessed by the OF, both on the group exposed to 4 and 8 weeks of CUS (supplementary Fig. 1K and L). CUS had an impact on the exploratory activity, measured by a decrease on the number of rearings in the OF test, both in the group submitted to 4 ($t_{(27)}$=2.122; p=0.043) (supplementary Fig. 1M) and 8 weeks of CUS ($t_{(25)}$=2.086;

p=0.047) (supplementary Fig. 1N). In the TST the 8 week CUS exposure has led to an increased time immobile ($t_{(26)}$=3.285; p=0,003) and decreased time mobile ($t_{(26)}$=3.255; p=0.003) (supplementary Fig. 1O). There was no statistically differences observed in the MWM test between exposure to 8 weeks of CUS and controls (supplementary Fig. 1P).

	Mon	**Tue**	**Wed**	**Thu**	**Fri**	**Sat**	**Sun**
Week 1	Bw basal restraint	restraint	shaking	social defeat	hot drier	restraint	tilted cage
Week 2	Bw 1st restraint	shaking	social defeat	hot drier	restraint	social defeat	overnight illumination
Week 3	Bw 2nd hot drier	social defeat	restraint	Blood collect. *zenith*	social defeat	inverted light	shaking
Week 4	Blood collect. nadir Bw 3rd social defeat	EPM&OF restraint	FST&TST Social defeat	shaking	restraint	restraint	hot drier
Week 5	BW 4th week restraint	Sacrifice 4w Tilted cage	Cytometry hot drier	restraint	social defeat	hot drier	inverted light
Week 6	BW 5th week hot drier	social defeat	restraint	inverted light	restraint	social defeat	hot drier
Week 7	BW 6th week restraint	social defeat	restraint	blood collect. zenith	shaking	restraint	overnight illumination
Week 8	BW 7th wk Blood collect.nadir	EPM&OF restraint	FST&TST Social defeat	MWM Shaking	MWM restraint	MWM restraint	MWM Shaking
Week 9	BW 8th wk	Sacrifice 8w	Cytometry				

Supplementary Table 1 – Stressors distribution and layout of the replication experiment.

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5th CHAPTER

Maladaptive immune response to chronic stress: focus on IFNγ

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Maladaptive immune response to chronic stress: focus on IFNγ

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Abstract

Chronic stress is known to lead to a maladaptive response involving multiple systems. One of these is the immune system, which, under chronic stress, exhibits a dysfunctional response implicated in the development not only of inflammatory and autoimmune diseases but also of neuropsychiatric disorders. In the present chapter, we explored the impact of chronic unpredictable stress (CUS) on splenic immune cells and adrenergic signaling of the spleen, and also on the brain cytokine profile. Finally, we also studied the specific role of the pro-inflammatory cytokine IFNγ in the immune maladaptive response to chronic stress in the spleen.

Our results show that, in the spleen, cells of the innate immune system, but not the adaptive immune cells, are significantly more affected by chronic stress exposure, which triggers an increase in neutrophils and a decrease in monocytes/macrophages. Of note, these changes seem to result from an increase in adrenergic signalling, since they were accompanied by a higher density of splenic adrenergic fibers in chronically stressed animals. The inflammatory phenotype caused by chronic stress was also extended to the brain, with an increase on *Ifnγ* mRNA levels in the mPFC and OFC. Interestingly, it seems that this pro-inflammatory cytokine contributs to the immune maladaptive response in the spleen, since mice lacking the expression of IFNγ did not display the stress-related alterations on the percentage of splenic neutrophils and monocytes/macrophages populations.

Collectively these data shows that the spleen is an important target of chronic stress exhibiting increased adrenergic signaling and pro-inflammatory cellular alterations. Moreover, the pro-inflammatory cytokine IFNγ that has been involved in the maladaptive behavioural response to stress seems to also be an important mediator of the immune alterations caused by chronic stress at the spleen.

Introduction

Although it refers to a primarily adaptive and beneficial response, the term *"Stress"* implicitly has a negative undertone due to an association with psychiatric disorders and other health problems related to a deregulation of the immune system. Anxiety, depression and difficulties with learning and memory are the most evident forms of the maladaptive response to stress (Sousa & Almeida, 2012). Nonetheless, deregulation of immunity due to stress (Curtin, Boyle, Mills, & Connor, 2009; Dhabhar, 2014; Flint et al., 2011; Glaser & Kiecolt-Glaser, 2005; Heidt et al., 2014b) is gaining increasing attention not only due to its potential contributory role for the development of inflammatory and auto-immune disorders (Padgett & Glaser, 2003) but also because it can itself precipitate neuropsychiatric alterations (McKim et al., 2015; Wohleb et al., 2013).

Indeed, both arms of the stress-response, the hypothalamic-pituitary-adrenal (HPA) axis through glucocorticoid production and the sympathetic nervous system (SNS) through release of adrenaline/noradrenaline at the fiber terminals innervating lymphoid organs (Kin & Sanders, 2006), represent important pathways on the neural control of immunity. As an example, cathecolamines secreted by adrenergic fibers in the thymus were shown to modulate the immune response to interferon therapy (Cavallotti, Artico, Iannetti, & Cavallotti, 2002). Moreover, it was demonstrated that adrenergic fibers regulate the rhythmicity of leukocyte recruitment to the tissues, a function long-though to be attributed solely to signals from the inflammatory response (Scheiermann et al., 2012). In addition, lymphoid organs' innervating fibers seem to mediate some of the chronic stress-induced alterations on immunity. For example, chronic variable stress, through noradrenergic fibers, was shown to lead to the activation of hematopoietic stem cells in the bone marrow promoting an increase on the neutrophils output (Heidt et al., 2014b). Importantly, this stress-induced increase on neutrophils production was correlated with increased susceptibility to develop atherosclerosis (Heidt et al., 2014b). Furthermore, repeated social defeat stress led to an increase of circulating monocytes and promoted their mobilization to the brain (Wohleb et al., 2013). More importantly, this recruitment of monocytes was required for the development of the stress-related anxiety-like behavior (Wohleb et al., 2013). Apart from cell mobilization and recruitment, adrenergic signaling also affects cytokine production, as shown in Powell et al. which analyzed the blood mononuclear cells transcriptome after repeated social defeat stress, pointing out for an increased activity of transcription factors involved in early myeloid lineage differentiation and pro-inflammatory effector function. These effects were absent when antagonists of β-adrenoreceptors were administrated (Powell et al., 2013).

The association of pro-inflammatory cytokines and alterations in behaviour has been extensively discussed. Chronic stress promotes an increase of pro-inflammatory cytokines, and particularly IL-6 and IL1β, both produced peripherally and in the brain, were shown to mediate some of the dysfunctional stress-related behavioural alterations (You et al., 2011). IFNγ is also a pro-inflammatory cytokine that has been shown to impact synaptic plasticity and neuronal dendritic arborizations, important mechanisms underlying behaviour. Indeed, in the study by Litteljohn and colleagues, the behavioural response to stress was ameliorated in mice with a targeted deletion of the pro-inflammatory cytokine IFNγ (Litteljohn et al., 2010; Litteljohn et al., 2014), suggesting that this cytokine may play a role in the maladaptive behavioural response to chronic stress.

Here we aimed at further exploring the impact of chronic stress on immunity by analysing cellular changes in the spleen cell composition, splenic adrenergic signalling and cytokine production in different areas of the brain, in mice submitted to a chronic unpredictable stress (CUS) paradigm known to induce the maladaptive behavioural response (Monteiro et al., 2015). To explore the specific role of IFNγ in the maladaptive immune response in the spleen, we exposed both WT and mice that do not express IFNγ (IFNγ KO) mice to CUS and analysed the main cellular populations.

Material and methods

Animals

Male C57BL/6 mice (C57BL/6J JAX™ mice strain) purchased from Charles River (Charles River Laboratories, Barcelona, Spain) and IFNγ KO mice on C57BL/6 background were housed (5 animals per cage) under standard laboratory conditions (12 h light/12 h night cycles (8 h/ 20 h), 22-24 °C, relative humidity of 55% and *ad libitum* access to water and food. All procedures were carried out in accordance to EU directive 2010/63/EU and Portuguese national authority for animal experimentation, Direção Geral de Veterinária (ID:DGV9457) guidelines on animal care and experimentation.

Chronic unpredictable stress (CUS) protocol

3 month-old male C57BL/6 (WT) mice were exposed to 8 weeks of CUS (Monteiro et al., 2015) and compared to a control group that was subjected to gentle handling, twice a week, for the same period. Briefly, the CUS paradigm consisted in exposure, once daily, to one of the following aversive stressors: restraint – mice were placed in a 50 ml plastic tube with openings in both sides for breathing, for 1 h; shaking – groups of 5 mice were placed in a plastic box container and placed in an orbital shaker for 1 h at 150 rpm; social defeat – mice were introduced in a cage of an aggressive mice and after being defeated, they were placed in a transparent and perforated plastic container, to avoid further physical contact, inside the resident home cage for 30 min (Golden et al., 2011); hot air stream – mice were exposed to a hot air stream from a hairdryer for 10 min; overnight illumination – mice were exposed to regular room light during the night period; inverted light cycle – regular room light was off during daytime and on during nighttime for 2 d; tilted cage – homecages were tilted in a 45° angle during 1 h. Stressors were presented in a random order in an unpredictable fashion. For controlling stressors efficacy, body weight was monitored once a week in the same day of the week and at the same time of the day.

Flow Cytometry

Mice were deeply anaesthetized with 20% sodium pentobarbital and were transcardially perfused with 0.9% saline. Spleen was dissected and homogenized in supplemented Dulbecco's modified eagle medium (DMEM) with 10% heat inactivated FCS, 10 mM HEPES buffer, 1 mM sodium pyruvate, 2 mM l-glutamine, 50 μg/mL streptomycin, and 50 U/mL penicillin, (all from Invitrogen, CA, USA) in order to obtain single-cell suspensions. Splenic erythrocytes were depleted by incubating the spleen cell

suspension for 5 min in a hemolytic solution (155 mM NH_4Cl, 10 mM $KHCO_3$, pH 7.2). Splenocytes ($1x10^6$ cell) were incubated with respective antibodies to analyze both lymphoid and myeloid lineages. For lymphoid lineage analysis, splenocytes were stained with PE anti-mouse CD3 (145-2C11 clone, Biolegend, San Diego, California, USA) and PerCP-Cy5.5 anti-mouse CD19 (clone 6D5, Biolegend, San Diego, California, USA). For myeloid lineage analysis, splenocytes were stained with PE anti-mouse CD11b (clone M1/70, Biolegend, San Diego, California, USA), APC anti-mouse CD11c (clone N418, Biolegend, SanDiego, California) and FITC anti-mouse Gr-1 (clone RB6-8C5, Biolegend, San Diego, California, USA). The gating strategy for lymphoid lineage analysis was as follow: cells were first gated for singlets (FSC-H vs FSC-A) and viable cells (FSC-H vs SSC-H), then T lymphocytes were considered the $CD3^+$ population and B lymphocytes the $CD19^+$ population. The gating strategy for myeloid cells was adapted from Rose et al. (Rose et al., 2012). Briefly, after gating for singlets and viable cells as described above, $CD3^+$ cells were gated out, and dendritic cells were gated as the $CD11b^+CD11c^+$ cells. Gated in $CD11b^+CD11c^-$ and by comparing SSC scatter to Gr-1 expression, eosinophils were identified as $SSC^{high}Gr\text{-}1^{low}$, neutrophils as $SSC^{int}Gr\text{-}1^{high}$ and monocytes/macrophages as $SSC^{low}Gr\text{-}1^{lo\text{-}neg}$ and $SSC^{low}Gr\text{-}1^+$. These two different populations according to Gr-1 expression represent different maturation stages of monocytes/macrophages, being the Gr-1+ the less differentiated cells (Rose et al., 2012; Swirski et al., 2009). After staining, cells were fixed in 2% paraformaldehyde for 20 min. Cell surface staining was acquired (100000 events) in an eight-color LSRII flow cytometer (BD, Pharminogen, San Jose, California, USA) and analyzed with FlowJo software version 7.6.4. One sample from the IFNγ KO group has to be excluded from the analysis due to technical problems in the single-cell suspension preparation.

Immunofluorescence

Spleens were dissected and fixed in formaldehyde 10% for 48 h. They were dehydrated in an increasing series of alcohols and cleared in xylene. Spleens were then mounted in paraffin and serial sections (4 μm) were cut in a microtome and collected in slides. After being deparaffinized and re-hydrated, sections were stained for tyrosine hydroxylase (TH) (rabbit polyclonal TH, AB152, 1:1000, Millipore, Darmstadt, Germany) followed by staining for neutrophils (rat anti-mouse Ly-6G, 1:200, 1A8, BD Biosciences, Franklin Lakes, NJ, USA). Sections were then incubated with secondary fluorescent antibodies (anti-rabbit 488 and anti-rat 594, Invitrogen, CA, USA). Finally, for nuclear staining all sections were incubated with 4',6- diamidino-2-phenylindole (DAPI) (1:1000, Sigma-Aldrich, St. Louis, MO, USA). Sections were observed under a fluorescence microscope (BX61;Olympus, Japan).

The analysis of TH-positive fibers was performed using StereoInvestigator software (MicroBrightfield, Williston, VT, USA) and a camera (DXC-390, Sony, Japan) attached to a motorized microscope (BX51, Olympus, Japan). The density of TH-positive fibers was estimated using a ''staggered'' cycloid test system (Baddeley, Gundersen, & Cruz-Orive, 1986; Emre, Heckers, Mash, Geula, & Mesulam, 1993; K. R. Isaacs, Sirevaag, Marks, Chang, & Greenough, 1993), using a cycloid' width of 26,5 μm, length of 52,1 μm and a test point separation of 70,1 μm (total number of counted points between 150-250 per slice). The total number of intersections of the cycloid arcs with the stained fibers, divided by the section area, was obtained on randomly selected sections of the spleen in a total of 20 sections per group. To minimize bias, each slide was coded to keep the experimenter blind to the genotype.

Western Blot

Spleens were homogenized in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.2% Triton™ X-100, 25 mM EDTA, supplemented with complete protease inhibitor (Roche, Indianapolis, IN, USA)) and homogenised using a 1ml syringe and a 20g needle; extracts were sonicated and centrifuged (15.000 rpm; 25 min; 4 °C) and their protein contents were estimated Bradford protein assay (Bio-Rad, Hercules, CA, USA). Samples were diluted in Laemmli buffer (250 mM Tris–HCl, pH 6.8, containing 4% sodium dodecyl sulfate, 10% glycerol, 2% β-mercaptoethanol and 0.002% bromophenol blue) at a final concentration of 5mg/ml. Samples were heated for 5 min at 100°C and loaded (20 μl) in 10% SDS-PAGE minigels. The blots were blocked in 2.5% dry milk in PBS-Tween 0.1% before incubation overnight at 4 °C with the primary antibody: tyrosine hydroxylase (TH) antibody (anti-rabbit, clone AB152, 1:1000, Millipore) and actin (DSHB 1:200). Secondary antibodies were incubated for 1 hour at room temperature. Membrane was incubated with ECL solution for 5 min protected at dark. Signal bands were quantified using the Image J Software.

Gene expression measurement by quantitative real-time polymerase chain-reaction (qPCR)

The prefrontal cortex (PFC), orbitofrontal cortex (OFC), dorsal and ventral hippocampus (dHip and vHip) were macrodissected and quickly frozen at -80 °C. mRNA levels of *Ifnγ and Il4* genes were determined by qPCR. Total RNA was isolated using TRIzol-chloroform method (Life technologies, CA, USA). Total RNA was reverse transcribed into first strand cDNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as the reference gene. Oligonucleotide primers for *Ifnγ*, (sense 5'-CAA CAG CAA GGC GAA AAA GG-3', antisense 5'- GGA CCA CTC GGA TGA GCT CA-3'), *Il4* (sense 5'- GTC ACA GGA GAA GGG ACG CCA T-3',

antisense 5'- AGC CCT ACA GAC GAG CTC ACT C-3') and *Gapdh* (sense 5'- GGGCCCACTTGAAGGGTGGA-3', anti-sense 5'- TGGACTGTGGTCATGAGCCCTT-3') were designed using the Primer3 software (Simgene, San Francisco, CA, USA). qPCR reactions were performed on a 7500 Api real-time PCR system instrument (Applied Biosystems, CA, USA), with the KAPA SYBR Fast (KAPABiosystems, MA, USA) according to the manufacturer's instructions and using ROX low as reference dye.

Statistical analysis

All values were calculated as means + standard error of the mean (SEM). Data was analysed using two tail Student's T-test. Differences were considered significant if $p<0.05$. Statistical analyses were performed with Graphpad Prism version 5.0b (La Jolla, San Diego, USA).

Since the focus of the experiment using IFNγ KO mice was to dissect the role of this cytokine in the stress-response and not to compare basal differences between genotypes (even though there were no major basal differences), we normalized the stress data to genotype-matched control groups. To do so, we calculated a "difference score" by dividing each value from the animals that were exposed to CUS to the average mean of the control group and dividing it by the standard deviation of the control group ((stress value – mean control group)/standard deviation control group).

Results

In the spleen, chronic stress increases adrenergic input and alters the composition of the leukocyte pool

Stress exposure altered the composition of the leukocyte pool in the spleen in a specific way (Fig. 1). Indeed, while neutrophils were more abundant in stressed animals than in controls ($t_{(12)}$=2.565; p=0.0248) (Fig. 1 D) and the percentage of monocytes/macrophages was smaller in stress-exposed mice ($t_{(12)}$=2.825; p=0.0153) (Fig. 1 F), dendritic cells ($t_{(12}$)=0.2597; p=0.7995) (Fig. 1 E) and eosinophils ($t_{(12)}$=0.4801; p=0.6398) (Fig. 1 C) were unaltered after stress exposure. We further divided the monocyte/macrophages pool according to Gr-1 expression, into two cellular subsets representing different stages of differentiation (Rose et al., 2012; Swirski et al., 2009). Interestingly, the less differentiated pool, the $SSC^{low}CD11b^{+}Gr\text{-}1^{+}$ cells, from animals exposed to chronic stress present a tendency for being increased after stress exposure ($t_{(12)}$=0.8932; p=0.3893) (Fig. 1 G) which goes in line with the already reported increased numbers of monocytes in the spleen after stress exposure (McKim et al., 2015; Wohleb et al., 2013). The percentage of $SSC^{low}CD11b^{+}Gr\text{-}1^{lo\text{-}neg}$ cells, most probably representing tissue resident macrophages and more differentiated monocytes, presented a tendency for being decreased after CUS exposure (t(12)=2.109; p=0.0566) (Fig. 1 H). Interestingly, no major alterations were observed in cells from the lymphoid lineage, including T lymphocytes ($t_{(12)}$=1.756; p=0.1046) and B lymphocytes ($t_{(12)}$= 1.623; p=0.1306) (Fig. 1 A and B).

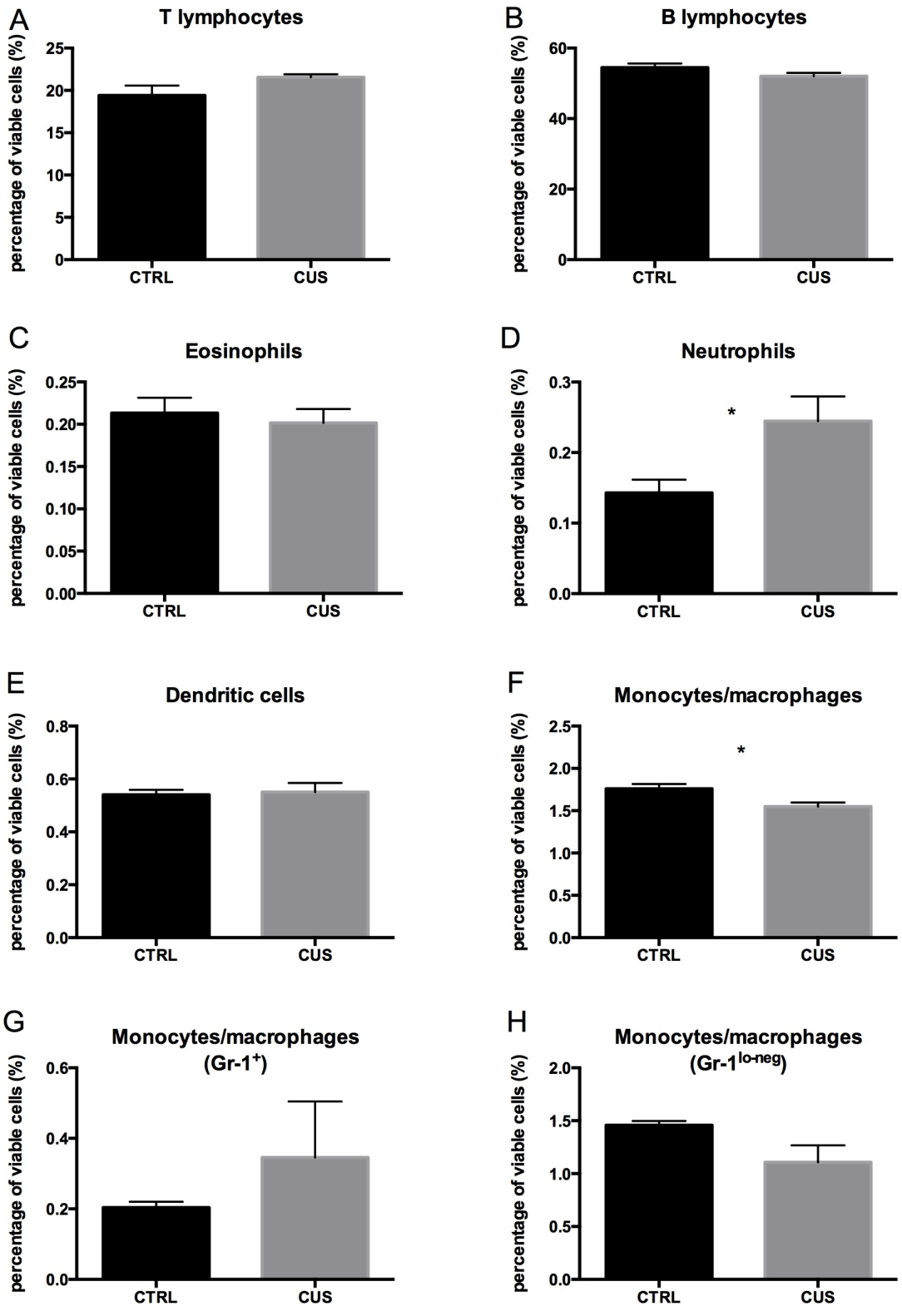

Fig. 1 – Chronic stress led to an increased percentage of neutrophils and differentially affected the monocyte/macrophage pool in the spleen. Chronic stress did not impacted on T lymphocytes (A) nor B lymphocytes (B) percentages, but increased the percentage of neutrophils (D) and decreased that of monocytes/macrophages (F). The impact of chronic stress seems to be differential within the monocytes/macrophages population. While the percentage of the less

differentiated subset (Gr-1^{+}) exhibited a trend for being increased by CUS (G), the more differentiated pool (Gr-1 $^{lo\text{-}neg}$) presented a tendency for being decreased after CUS exposure (H). The percentage of eosinophils (C) or dendritic cells (E) was not affected. $n_{(control)}=7$, $n_{(CUS)}=7$. *p<0.05. Data expressed as S.E.M.

The spleen is innervated by cathecolamine terminals from the sympathetic nervous system, which might be involved in the regulation of immune cell populations (Mina-Osorio et al., 2012; Rosas-Ballina et al., 2008; Wan et al., 1993). Accordingly, both the density of adrenergic (TH-positive) fibers innervating the spleen ($t_{(37)}=3.306$; p=0.0021) (Fig. 2 A) and the levels of TH in this lymphoid organ ($t_{(2)}=4.783$; p=0.0410) (Fig. 2 B) were increased after chronic stress.

To further clarify these findings, we assessed the spatial distribution of Ly6-G positive cells (mainly neutrophils, Fig. 2C) in relation with the localization of adrenergic fibers (Fig. 2D). As can be gleaned from the merged picture (Fig. 2E), although most neutrophils are spread throughout the tissue, some are in close vicinity with the nervous terminals, making these potential sites of neuroimmune communication.

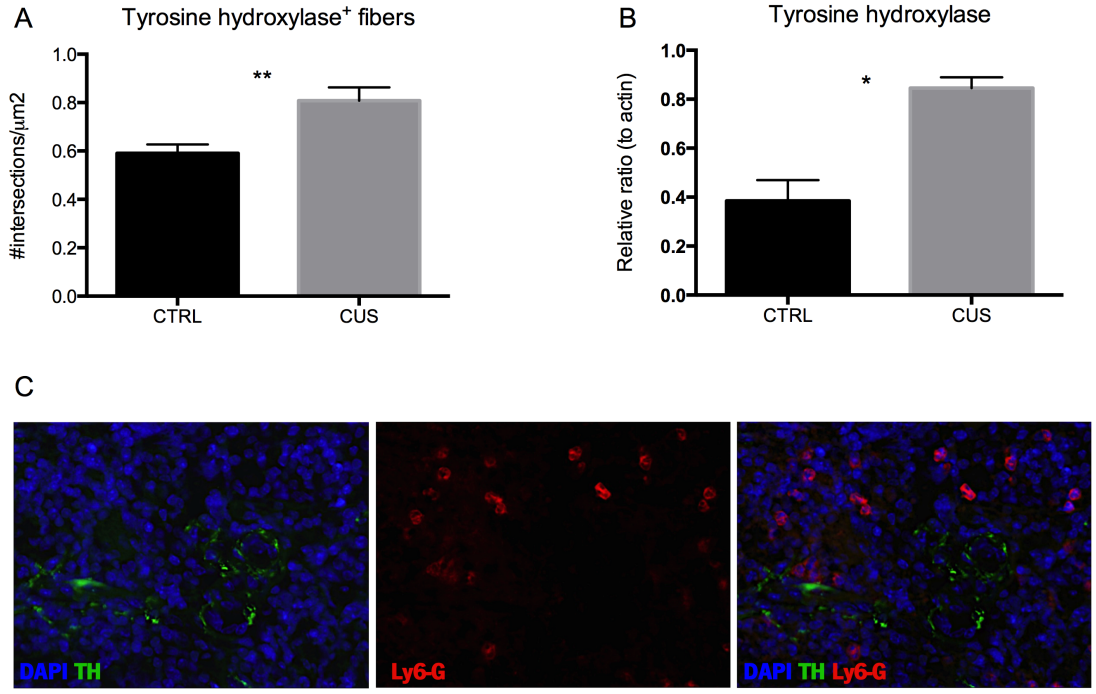

Fig. 2 - Chronic stress increased the density of adrenergic fibers innervating the spleen. Chronic stress led to an increase in the density of TH-positive fibers in the spleen (A) $n_{(control)}=20$, $n_{(CUS)}=19$, and an increase on TH levels (B) $n_{(control)}=2$, $n_{(CUS)}=2$. Immunostained splenic TH-positive fibers (green) (C) and neutrophils (red) (D). **p<0.01,*p<0.05. Data expressed as S.E.M.

In the brain, chronic stress increased the expression of Ifnγ but not of Il4

After submitting C57BL/6 mice to 8 weeks of chronic unpredictable stress (CUS), we analysed the variations on mRNA levels of *Ifnγ* and *Il4*, cytokines that have been shown to modulate behaviour (2nd Chapter of this thesis and (Derecki et al., 2010)), in the medial prefrontal cortex (mPFC), orbitofrontal cortex (OFC), dorsal hippocampus (dHip) and ventral hippocampus (vHip) (Fig. 3)

Ifnγ mRNA expression levels were increased after chronic stress in the mPFC ($t_{(8)}$=2.351; p=0.0466) (Fig. 3 A) and the OFC ($t_{(8)}$=3.302; p=0.0108) (Fig. 3 C), but not in the hippocampus (dHip $t_{(8)}$=0.2287; p=0.8248; vHip $t_{(8)}$=0.6614; p=0.5269) (Fig. 3 E and G). This effect seemed rather specific and did not extended to *Il4* mRNA expression in any of the analysed areas (mPFC $t_{(8)}$=1.417; p=0.1942; OFC $t_{(8)}$=1.823; p=0.1058; dHip $t_{(8)}$=0.1464; p=0.8872; vHip $t_{(8)}$=0.6731; p=0.5199).

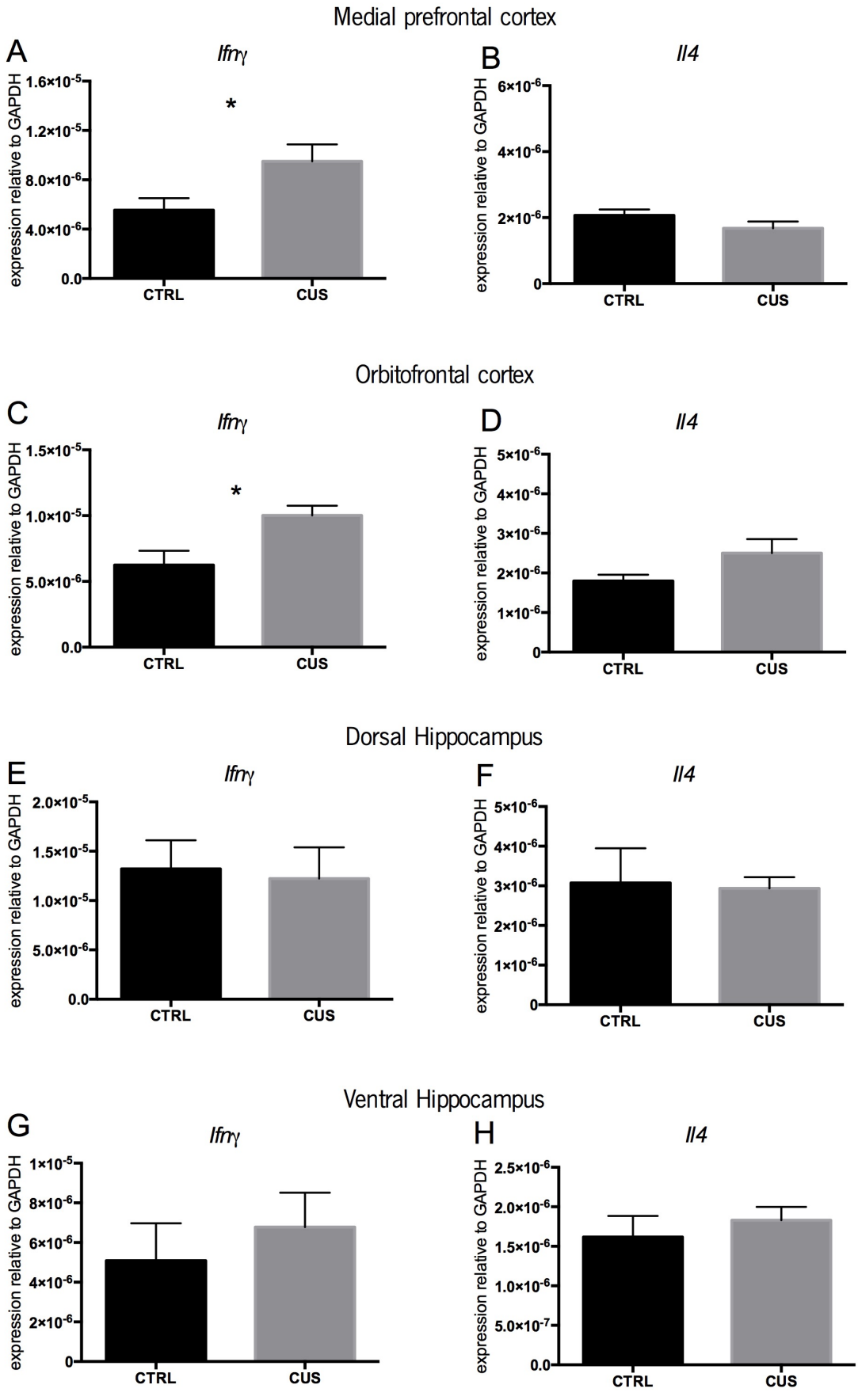
Medial prefrontal cortex
A
Ifnγ
*
expression relative to GAPDH
1.6×10-5
1.2×10-5
8.0×10-6
4.0×10-6
0.0
CTRL
CUS
B
Il4
expression relative to GAPDH
6×10-6
4×10-6
2×10-6
0
CTRL
CUS
Orbitofrontal cortex
C
Ifnγ
*
expression relative to GAPDH
1.5×10-5
1.0×10-5
5.0×10-6
0.0
CTRL
CUS
D
Il4
expression relative to GAPDH
5×10-6
4×10-6
3×10-6
2×10-6
1×10-6
0
CTRL
CUS
Dorsal Hippocampus
E
Ifnγ
expression relative to GAPDH
2.0×10-5
1.5×10-5
1.0×10-5
5.0×10-6
0.0
CTRL
CUS
F
Il4
expression relative to GAPDH
5×10-6
4×10-6
3×10-6
2×10-6
1×10-6
0
CTRL
CUS
Ventral Hippocampus
G
Ifnγ
expression relative to GAPDH
1×10-5
8×10-6
6×10-6
4×10-6
2×10-6
0
CTRL
CUS
H
Il4
expression relative to GAPDH
2.5×10-6
2.0×10-6
1.5×10-6
1.0×10-6
5.0×10-7
0.0
CTRL
CUS

Fig. 3 Chronic stress selectively increased brain regional expression of Ifnγ while Il4 expression remained unaltered. In the mPFC, *Ifnγ* mRNA levels were increased (A) while *Il4* were not (B). In the OFC, *Ifnγ* mRNA levels were increased (C) while *Il4* were not (D). No differences were observed in *Ifnγ* (E and G) and *Il4* (F and H) mRNA levels in the dorsal and ventral hippocampus. $n_{(control)}=5$, $n_{(CUS)}=5$. $p<0.05$. Data expressed as S.E.M.

The impact of chronic stress in the spleen is blunted in IFNγ KO animals

In the spleen, constitutional deletion of IFNγ prevented stress-induced changes in leukocyte populations (neutrophils: $t_{(10)}=2.281$, $p=0.0457$; macrophages/monocytes: $t_{(10)}=3.858$; $p=0.0032$) (Fig. 4 D and F), with all other populations remaining unaltered (T cells: $t_{(10)}=1.747$, $p=0.1112$; B cells: $t_{(10)}=0.1224$, $p=0.9050$; eosinophils: $t_{(10)}=0.2606$, $p=0.7997$; dendritic cells: $t_{(10)}=1.636$; $p=0.1330$; monocytes/macrophages ($Gr\text{-}1^{+}$): $t_{(10)}=0.7458$; $p=0.4729$ and monocytes/macrophages ($Gr\text{-}1^{lo\text{-}neg}$): $t_{(10)}=2.204$; $p=0.0521$) (Fig. 4 A-C; E; G and H).

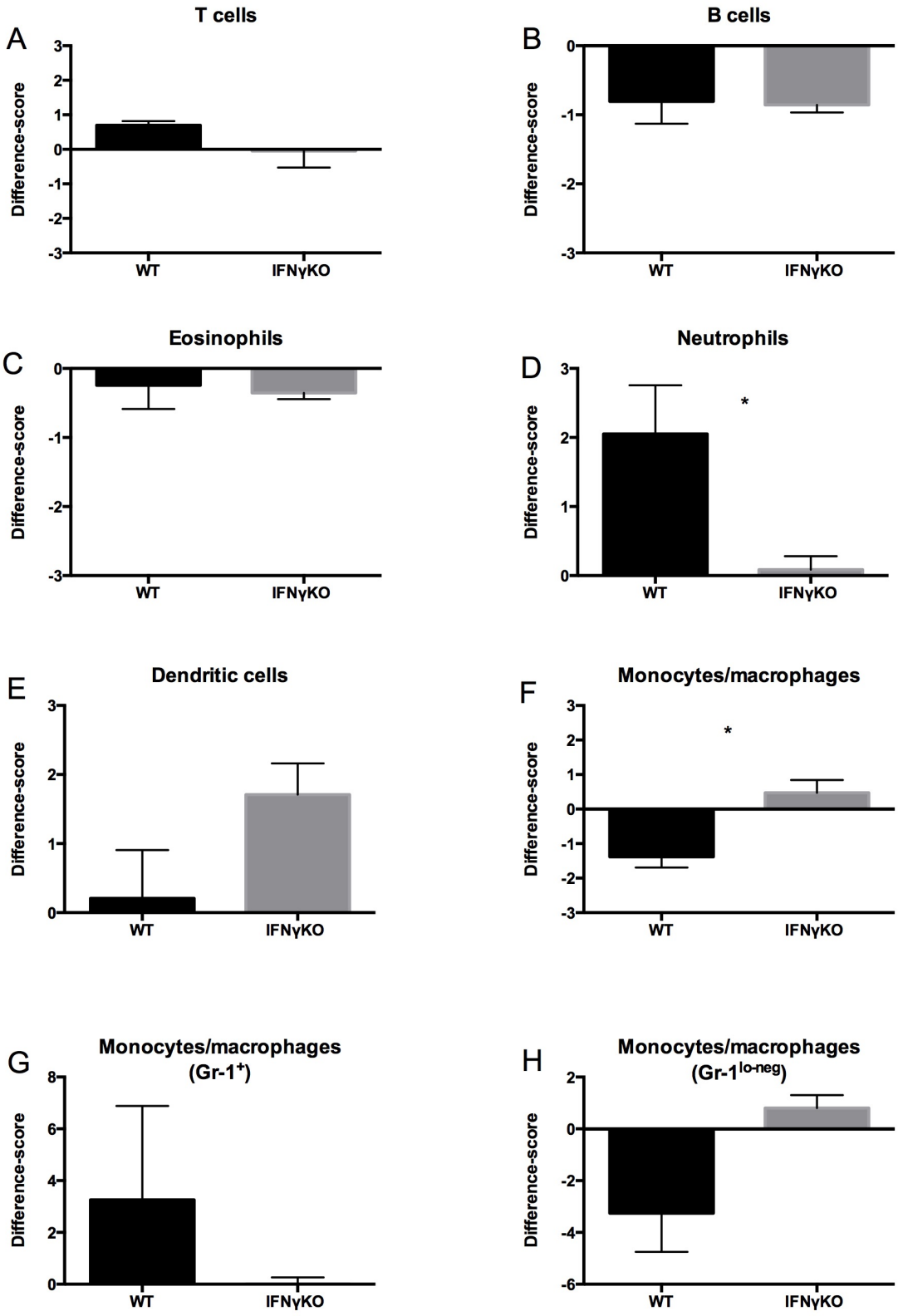

Fig. 4 - Absence of IFNγ blunted the chronic stress-related alterations on the percentage of neutrophils and monocytes/macrophages populations in the spleen. The stress-response in the absence of IFNγ was no different from that of the WT in what concerns the percentage of T cells (A), B cells (B) and eosinophils (C). However, the stress-response in the absence of IFNγ did not exhibit

the increase on the percentage of neutrophils caused by chronic stress observed in the WT group (D). Although it seems that the percentage of dendritic cells were increased by stress in the IFNγ KO mice, this was no statistically different from the stress-response in the WT group (E). While WT exposed to chronic stress exhibited a decrease on the percentage of monocytes/macrophages population, stressed IFNγ KO displayed a tendency for increased percentage on this type of cell population (F). The stress-impact observed in WT animals in the percentage of the different subsets of monocytes/macrophages according to their differentiation state, was also blunted in the IFNγ KO mice stress-response (G and H) $n_{(WT)}=8$, $n_{(IFN\gamma\ KO)}=5$. $p<0.05$. Data expressed as S.E.M.

Discussion

In this chapter, we initially explored the neuroimmune changes under chronic stress in both the spleen and in the brain.

The spleen has been shown to be an important stress-target, exhibiting both structural changes (Hernandez et al., 2013) and altered proportion of immune cells (Monteiro et al., 2015). We here further demonstrate that the main cellular alterations caused by chronic stress are associated with innate immunity, namely with an increase on neutrophils percentage and a decrease on monocytes/macrophages percentage. Instead, adaptive immune cells proportion seems to be unaltered with the percentage of T and B lymphocytes population not exhibiting major changes after chronic stress exposure. An increase on neutrophils after chronic stress exposure was previously reported, although it was observed in the bone marrow in response to the noradrenaline secreted by innervating fibers (Heidt et al., 2014b). And importantly, this increase on neutrophils output promoted by the secretion of stress-cathecolamines was associated with disease-promoting activity by increasing susceptibility to develop atherosclerosis (Heidt et al., 2014b) and also impairing wound healing (M. H. Kim et al., 2014).

The spleen is also innervated by adrenergic fibers that secrete both adrenalin and noradrenaline (Elfvin, Johansson, Hoijer, & Aldskogius, 1994; Felten, Ackerman, Wiegand, & Felten, 1987), indeed the splenic nerve was shown to be able to mediate immune function of the spleen (Mina-Osorio et al., 2012; Rosas-Ballina et al., 2008; Wan et al., 1993). We here demonstrated that adrenergic signalling in the spleen is enhanced by chronic stress exposure, both by an increase on TH-positive fibers density and by an increase on TH levels. However, although tempting to admit since we demonstrate that neutrophils are spatially located in privileged site to receive inputs from these fibers, we cannot assure at this point that the cellular changes observed are due to the enhanced adrenergic signalling. Moreover, we cannot exclude the possibility that the raised percentages of neutrophils seen in the spleen are a reflection of increased activity from progenitor cells after chronic stress in the bone marrow already reported by Heidt et. al.(Heidt et al., 2014b).

Still, the spleen displays an important role on mediating the stress-induced alterations since surgical removal of the spleen in mice previously submitted to repeated social stress prevented the recurrence of anxiety-like behaviour observed after a new exposure (McKim et al., 2015).

Intriguingly, in this study we observed a reduction on monocytes/macrophages population after chronic stress, which seems in contradiction with the reported increase on monocytes in the spleen after chronic stress exposure (McKim et al., 2015; Wohleb et al., 2013). This may be related to the lack of a

specific marker for the monocyte population in our analysis or simply because this particular stress paradigm does not induce this immune alteration. However, when dividing the monocytes/macrophages population into two different subsets according to their differentiation status, we observed that the percentage of the less differentiated population ($SSC^{low}CD11b^{+}Gr\text{-}1^{+}$ cells) most likely corresponding to monocytes, presented a tendency for being increased after chronic stress. Indeed, the stress-increase on monocytes was reported to be exposure dependent (Wohleb et al., 2013), so is likely that in our analysis this tendency for increased monocytes represent initial alterations of stress-induced increase on monocytes.

Paralleling with the increased inflammatory cellular changes observed in the spleen, the brain also presented an increased pro-inflammatory cytokine profile. The pro-inflammatory cytokine - IFNγ expression levels were increased in both mPFC and OFC, areas highly associated with cognitive behaviours such as executive function and attention. Indeed, IFNγ has been shown to impair synaptic plasticity phenomena underlying cognitive behaviours. For instance, intracerebroventricular infusion of IFNγ has been shown to disrupt long-term potentiation (LTP) (Maher et al., 2006), or *in vitro* administration of IFNγ inhibited synapse formation and induced dendritic retraction (I. J. Kim et al., 2002). Therefore, it is feasible to hypothesize that the increased levels of IFNγ may partially mediate some of the behavioural alterations caused by chronic stress. Of note, the effects of stress seemed rather specific, not only in what regards affected regions (with the frontal areas more susceptible than the hippocampus) but also pro-inflammatory cytokines, as Il4 levels were not altered upon stress exposure. In our opinion, this specificity highlights a close relationship between stress and IFNγ that merits further consideration in future studies.

In line with the close relationship highlighted above, IFNγ absence was able to blunt the impact of chronic stress in the leukocyte populations of the spleen, preventing the increase in neutrophils and the decrease in monocytes/macrophages observed in chronically stressed animals. In fact, IFNγ is able to regulate neutrophils recruitment to the tissue in the context of infection (Bonville et al., 2009; McLoughlin, Lee, Kasper, & Tzianabos, 2008), but whether this function also applies in non-infectious conditions, including upon chronic-stress exposure, is still a matter of debate.

Curiously, although the main effector molecules of the stress response, corticosteroids and cathecolamines, are reported to have acute anti-inflammatory properties, it seems that the chronic exposure to stress actually triggers a pro-inflammatory response, specifically mediated by IFNγ, in both the spleen and the brain.

Here we demonstrated that chronic-stress induced important alterations in the splenic innate immunity that may be associated to the increased adrenergic signalling in this lymphoid organ and that these changes were absent in animals lacking the expression of IFNγ. In addition, we showed that chronic stress exposure selectively increased the expression levels of IFNγ (but not IL4) in the PFC and OFC (but not the hippocampus), highlighting the special relationship between stress and this particular pro-inflammatory cytokine. As more light is shed into the influence of components of the immune system in normal brain function, our results bring to light potential novel mechanisms that might contribute to stress induced cognitive deficits and could be the target of novel immune-based therapies for stress-related disorders.

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SECTION III – DISCUSSION AND CONCLUDING REMARKS

6th CHAPTER

Overall discussion and conclusions

General discussion and conclusions

In recent years, the way that the immune system is being regarded in what concerns its role in the brain is suffering profound changes. We moved from the initial perception - where immune cells were regarded as nearly inactive under physiological conditions being their excessive activity associated with pathology; to the concept of a well-adjusted immune activity as being essential for maintaining healthy brain function (Michal Schwartz, 2015).

The recent studies, showing that cognitive training recruits T cells and shapes them into a pro-cognitive phenotype, together with the observation that ablating T cells, or T-cell derived cytokines (IL-4) (Baruch & Schwartz, 2013; Brynskikh et al., 2008; Derecki et al., 2010; Derecki et al., 2011; Gadani, Cronk, Norris, & Kipnis, 2012; Kipnis, Gadani, & Derecki, 2012b; Ziv et al., 2006) leads to deficits in higher brain function, were seminal for the current concept that a tightly regulated immune activity is indeed a requirement for healthy brain function. And importantly, these studies have opened avenues for exploring similar roles for other immune components.

IFNγ is a cytokine mostly produced by T cells, which altered levels in chronic stress, aging, depression and schizophrenia (Maes et al., 1994; Maes et al., 1998; B. J. Miller, Buckley, Seabolt, Mellor, & Kirkpatrick, 2011; Oxenkrug, 2011) have been implicated in the neuroinflammatory components of these disorders. As discussed on the 1st Chapter, the vast majority of the work involving IFNγ in the CNS was framed in pathological contexts (Arolt et al., 2000; Maes et al., 1994; Maes et al., 1999; O'Connor, Andre, et al., 2009), since the prevailing view was that activity from immune mediators (neuroinflammation) in the CNS was mainly associated with damage to neuronal activity (Crutcher et al., 2006; Haroon et al., 2012; Najjar, Pearlman, Alper, Najjar, & Devinsky, 2013; M. Schwartz, Butovsky, & Kipnis, 2006; Yirmiya & Goshen, 2011). As so, many of these studies reflect the effect of abnormally elevated levels of IFNγ in the brain, using either infections or direct administration of this cytokine in the brain or brain cell cultures. While, in one sense there is no doubt that excessive IFNγ production is, in general, deleterious to the brain, the contribution of normal levels of this cytokine to brain function is yet to be fully understood.

In this context, we took advantage of knockout mice lacking IFNγ expression in order to dissect the contributory role of this cytokine for higher brain function in physiological conditions, and to understand the possible contributing value of IFNγ to the pathophysiology of stress-related disorders.

IFNγ as a negative regulator of cognitive function in the healthy brain

For testing the hypothesis that IFNγ may play a role in healthy brain functioning (2nd Chapter), we submitted IFNγ KO mice (and respective littermate controls) to behavioural paradigms assessing emotional, motor and cognitive domains. Based on the observation that these mice outperformed in cognitive tasks while no differences were observed on other behavioural dimensions, the data suggested that at least in the healthy brain, the function of this cytokine is specific for cognitive domains. Interestingly, only cognitive behaviours dependent on hippocampal functioning were enhanced, while behavioural flexibility, a prefrontal cortex (PFC)-dependent behaviour, seems to not be regulated by endogenous levels of this cytokine. Certainly, more experiments targeting PFC-behaviours need to be performed to assure the lack of involvement of IFNγ in this type of behaviours.

Less ambiguous was the involvement of IFNγ in hippocampal-dependent behaviours, which drove us to further explore the neuroplastic events known to be on the basis of cognitive behaviour in this particular brain region. Our results showed that IFNγ KO mice exhibited increased neurogenesis and pyramidal neurons with larger dendritic arborisations in CA1 region. Larger dendritic trees may potentially represent more synaptic contact points, improving synaptic transmission necessary for backup an enhanced cognitive processing. In support of a boosted synaptic connectivity activity, we also observed that IFNγ KO exhibit decreased paired-pulse facilitation, a feature that has been associated with enhanced pre-synaptic mechanisms (Ho et al., 2006; Larson et al., 1999). Curiously, long-term potentiation (LTP), a mechanism known to be on the basis of learning and memory was unaffected by IFNγ absence, suggesting that the enhanced performance of these mice may rely more on neurogenesis, dendritic remodelling and other aspects of structural synaptic plasticity rather than on LTP.

Still puzzling, was the finding of a brain regional selectivity for endogenous IFNγ action. An interesting hypothesis for the selective impact of IFNγ in hippocampal functioning is that IFNγ is differentially expressed in the healthy brain. In support of this hypothesis, according to the *Human Protein Atlas*, the hippocampus is the only brain region where at basal conditions IFNγ expression is detected by immunohistochemical techniques (Atlas, 2014). Another appealing hypothesis is that IFNγ absence impacts on mechanisms that are specific for the hippocampus. Indeed, in line with the role of IFNγ as an anti-proliferative molecule, we observe that IFNγ KO mice presented increased numbers of newly-

born neurons from the total pool of proliferating cells at the subgranular zone of the dentate gyrus. The commitment of neural precursors cells to the neuronal line is an important step of neurogenesis, being followed by the maturation into functional neurons and the integration into neuronal circuits. In the hippocampus lays an important adult neurogenic niche, the subgranular zone of the dentate gyrus that together with the subventricular zone constitutes the two recognised regions of adult neurogenesis. An important feature of hippocampal adult neurogenesis that distinguishes it from the subventricular zone adult neurogenesis, is that the former is associated with learning and memory (Akers et al., 2014; Lepousez, Nissant, & Lledo, 2015), while there is no known behavioural outcome, in the healthy brain, for the latter. The specific association of adult hippocampal neurogenesis to learning and memory, together with the observation that the cognitively enhanced IFNγ KO mice present behavioural and structural alterations that are specific for the hippocampus, make it tempting to speculate that neurogenesis is the mediator mechanism underlying the effects of IFNγ, or its absence, on cognition, although further experiments are needed to prove this causality.

The lack of a significant effect on anxious-like behaviour seems to be in contradiction with the already reported anxiety-like behaviour of these animals (Campos et al., 2014). However, as shown in 3rd chapter, there are important gender differences in the actions of IFNγ. Indeed, as pointed out in that chapter, this cytokine seems to preferentially impact anxiety-related behaviours in males and cognitive abilities in females. Curiously, the hormonal variations characteristic of females can play a role on this gender effects (Mukai et al., 2010; Vegeto et al., 2001). Indeed, estrogen was shown to have anti-inflammatory properties (Vegeto et al., 2001), and has been shown to modulate IFNγ production in the periphery, an observation that might be transversal to the CNS. We are currently exploring the role of estradiol in the modulation of IFNγ actions in the brain to confirm this hypothesis. In line with it, our preliminary data, also presented in the 3rd chapter, suggest that this hormone decreases production of this cytokine by glial cells in a primary culture.

Moreover, the absence of effects on anxiety on our behavioural experiments is supported by the structural data. Indeed, it was noted an increase on dorsal areas of hippocampus contrasting with an absence of volumetric differences in the ventral part. This divided analysis permitted a better understanding of the structural findings since it has been recently shown that hippocampal connectivity, function and biochemistry varies along the dorsal-ventral axis. While the function of the dorsal hippocampus is more associated with cognitive behaviours, the ventral part is mainly linked to emotional behaviours and the effects of stress (Fanselow & Dong, 2010).

Although ablating IFNγ expression in the normal brain seems to benefit hippocampal neurogenesis and enhance dendritic and synaptic plasticity, it is still to clarify if this is a direct effect from the deletion or a result of neurodevelopmental changes and life-long enhancing phenomena on learning and memory. During the embryonic stage, IFNγ is not found in the brain (Li et al., 2010), which suggests that at least in this period it is unlikely that IFNγ absence impacts on brain development. However at postnatal day 2, a period in which the brain is still extremely plastic to environmental changes, the brain exhibits a 2.5 fold change increase of IFNγ expression relatively to the adult brain (Li et al., 2010). For this reason, we cannot at this moment exclude that the absence of IFNγ in the postnatal period led to structural changes that dictated life-long behavioural differences in cognition. Even though, this hypothesis merits further investigation due to a suggestive involvement of IFNγ on neurodevelopmental disorders that are associated with some degree of cognitive impairment (Hallam et al., 2000; Maroun, 1996; Nateghi Rostami et al., 2012). Moreover, another important unresolved question is still whether peripheral immune system or CNS immune system is involved in this cognitive enhancement, an issue to be further explored.

A final note on the cognitive-enhancement caused by IFNγ absence in the healthy brain concerns its possible clinical implications. Although blocking IFNγ expression may at first look as an attractive cognitive enhancer strategy, we are aware that in clinical terms, this would lead to a catastrophic outcome, since our body is constantly being exposed to insults and IFNγ is an extremely important cytokine of the immune system defence. Instead, we think that the insights coming from studying the individual impact of each immune component on the healthy brain may potentially contribute for the development of therapeutical strategies aiming at re-establishing the immune function balance required to optimal cognitive function.

Overcoming a stress-resistant mice strain for modelling stress-related disorders

The impact of chronic stress is quite universal since it targets the brain but also other systems, such as the immune system.

Following our novel finding that IFNγ is a negative regulator of cognitive function in the healthy brain, and since this is a behavioural domain impaired in many neuropsychiatric disorders, we then aimed at studying the role of this cytokine in the effects of chronic stress - a triggering factor for these disorders (Sousa & Almeida, 2012).

The use of the IFNγ KO mice in a C57BL/6 background implied that the current available CUS paradigms would lead to the same stress-related phenotype in our mouse model as the one seen in rats. Unfortunately this was not the case since this particular strain of mice is more resistant to stress (4th Chapter). Therefore, as reported in 4th Chapter, we moved to optimise a CUS protocol targeting both the behavioural and immune dysfunction in this strain by doubling the exposure time to 8 weeks and comparing to the classical 4 weeks-long paradigm.

Exposing C57BL/6 mice to the optimized 8 weeks of CUS led to a constellation of biometric, endocrine, behavioural and immune alterations consistent with stress-related disorders. Importantly, while exposure to 4 weeks only led to mild alterations, the comparison of the read-outs from the two different stress-exposure-periods permitted taking some insights on the temporal dynamics of the stress-response. Of note, we observed that body weight reduction is a main target of stress being primarily visible after as soon as 2 weeks of CUS exposure and maintained throughout the 8 weeks. The hallmark of the stress-response, a hyper productive HPA axis is detectable at the 8 weeks timepoint (but not at the 4 weeks timepoint) of CUS exposure. However, the body weight loss, a decreased exploratory behaviour and a tendency for increased neutrophils in the spleen are already observed at 4 weeks of CUS exposure suggesting that at least for these alterations, excessive glucocorticoid production seems to not be involved. Curiously, neutrophilia is a hallmark of the immune stress-response, a finding that was attributed to the glucocorticoid-related decrease on adhesion molecules in the endothelial wall leading to the re-entry of the marginated neutrophils pool into circulation (Nakagawa et al., 1998). Intriguingly, a tendency for increased splenic neutrophils after 4 weeks of CUS exposure was detecetd while no elevation on glucocorticoid levels were observed at this stage, strongly suggesting the contribution of other arms of stress-response for the stress-related neutrophilia.

The sympathetic nervous system plays also a crucial role in the stress response by producing cathecolamines, adrenalin and noradrenalin, both systemically by the adrenal gland through the sympathetic-medullary axis (SMA) axis or locally by adrenergic fibers innervating peripheral organs (lymphoid organs included). The finding of suggestive initial alterations on splenic neutrophils in a stage that preceded the detectable dysfunction of the HPA axis, has prompted us to further explore the contribution of the sympathetic nervous system in the stress-induced alterations on splenic immunity (5th Chapter).

IFNγ as a contributor for the development of the splenic immune maladaptive response to chronic stress

One of the major apparent paradoxes related to the chronic stress-response is the activation of the immune system, leading to a pro-inflammatory status, while the main secreted stress-hormone – corticosterone - is indeed an anti-inflammatory molecule.

While the acute stress-immune response is characterized by a downregulation of inflammatory components, chronic exposure to stress has been shown to lead to a certain degree of desensitization of cells of the immune system to this hormone therefore favouring the development of a pro-inflammatory profile (Cohen et al., 2012; G. E. Miller, Cohen, & Ritchey, 2002). IFNγ was described as being increased in the serum of medical students with high perceived stress before an exam (Maes et al., 1998) suggesting that this cytokine may be altered in stressful situations, which together with our data from the 2nd chapter, showing IFNγ' role on cognitive function, makes it an attracting candidate for underlying both the behavioural and immune alterations caused by chronic stress.

In the 5th chapter of this thesis, we show that mice submitted to chronic stress display both central and peripheral pro-inflammatory alterations. In the CNS, we found an elevation on mRNA expression levels of IFNγ in the PFC. Curiously, although in the healthy brain IFNγ seem to have an important role in the dorsal hippocampus, no alterations on the expression levels of this cytokine were observed in this region in response to chronic stress. In fact, we show in the 4th chapter of this thesis that this paradigm of chronic stress does not significantly impact the spatial learning performance in the Morris Water maze, a function mainly attributed to the hippocampus. Curiously, the PFC has been shown to be a target for the stress-related increase of pro-inflammatory cytokines (Audet, Jacobson-Pick, Wann, & Anisman, 2011), but the specific finding for IFNγ is novel. Moreover, the PFC was previously shown to also be a target of chronic stress leading to an impairment of PFC-associated behaviors (Cerqueira et al., 2007). As discussed in the introduction (1st chapter), and also shown in the 2nd chapter, IFNγ has an important impact on neuroplastic phenomena that are on the basis of learning and memory. Therefore, it is likely that the increased expression of IFNγ in the PFC may contribute for some of the stress-related behavioural alterations. In agreement, an enhancement of cognitive behaviour induced by chronic stress in IFNγ KO mice were already reported (Litteljohn et al., 2010; Litteljohn et al., 2014), being still to understand what is the mechanism through which chronic stress triggers a even better cognitive performance relatively to unstressed controls. One speculative hypothesis is that since IFNγ KO mice lack one of the most important cytokine of the Th1 response, chronic stress skews the

immune response of these mice into a Th2-like response, which has been associated with a better cognitive outcome (Derecki et al., 2010).

The pro-inflammatory alterations caused by chronic stress were also extended to the spleen where we show an altered distribution of immune cells, with an increase of neutrophils and decreased monocytes/macrophages. Intriguingly, we did not observe an increase of monocytes (monocytosis) typically found after chronic stress exposure. This could be due to the lack of a specific marker for monocytes in our flow cytometry phenotyping experiments and, most likely, by the fact that our populations are contaminated with other cellular subsets. Furthermore, since neutrophilia precedes the stress-related monocytosis, it is still possible that our analysis targeted a stage where monocytosis was not yet established. However, the neutrophilia observed in the spleen was an interesting finding to further explore.

Since in the 4th chapter, we show initial suggestive alterations in the neutrophils population in the spleen in the group submitted to 4 weeks of CUS, a stage where no elevations on corticosterone production were yet detected, we explored the role of other stress-response arm – the sympathetic nervous system.

We observed an increase on the adrenergic signalling of the spleen, as shown by an increase on adrenergic fibers density together with an increase on the quantity of the rate-limiting enzyme for cathecolamine synthesis, tyrosine hydroxylase. The adrenergic innervation of lymphoid organs has previously been shown to modulate the immune response in the thymus (Cavallotti et al., 2002), bone marrow (Heidt et al., 2014b) and also in the spleen (Rosas-Ballina et al., 2011). We here show that in response to chronic stress, these fibers are increased. Our results suggest that it may potentially be involved in the neutrophilia observed in the spleen since neutrophils possess adrenergic receptors (M. H. Kim et al., 2014) and furthermore neutrophils are spatially located in privileged sites to receive inputs from these fibers terminals.

An interesting finding was that IFNγ KO mice do not display the stress-related neutrophilia, a fact that is driving us to further investigate the adrenergic signalling on these mice. Indeed, it is known that the sympathetic nervous system is able to stimulate IFNγ production in arthritis, or that noradrenaline stimulates Th1 differentiation in the spleen with an increase on IFNγ production (Swanson, Lee, & Sanders, 2001), which suggests that is possible that IFNγ is a mediator of the sympathetic increase on neutrophils.

Still, there are other possibilities for the role of IFNγ in the stress-related neutrophilia. For instance, it is possible that the absence of IFNγ leads to an increased *de novo* production of neutrophils and

respective egress from the bone marrow, since IFNγ is involved in the inhibition of hematopoietic stem cells proliferation in the bone marrow (de Bruin et al., 2013). Another interesting possibility for the stress-related increase on neutrophils in the spleen is that IFNγ may act locally as an inflammatory cue for the recruitment of circulating neutrophils into this organ, a function already attributed to IFNγ in case of infections (Bonville et al., 2009; McLoughlin et al., 2003).

Although the raise of neutrophils after chronic stress has been associated with the increased susceptibility to develop inflammatory diseases (Glaser & Kiecolt-Glaser, 2005), still to understand is if these neutrophils, as an analogy to what was already proven with monocytes (Wohleb et al., 2013), could also be associated with the development of the behavioural dysfunction. This may be an appealing hypothesis to further explore based on the observations that the increase of neutrophils precedes the first signs of behavioural alterations (4th Chapter) and also since this neutrophilia is not observed in the IFNγ KO (5th Chapter) that presented an ameliorated behavioural phenotype.

The present thesis demonstrates that the pro-inflammatory cytokine IFNγ, besides its classical role in the immune system, also has the ability to modulate higher brain function. Indeed, in the healthy brain, this cytokine is a negative regulator of hippocampal plasticity and cognitive function. In addition, under chronic stress, a trigger for many neuropsychiatric disorders, this cytokine has revealed an important, but still enigmatic role on the development of the immune stress-response, with possible implications for the stress-related disorders.

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7th CHAPTER

Future perspectives

Future Perspectives

"Science, in the very act of solving problems, creates more of them"

Abraham Flexner, 1930

Although the results included in the present thesis have helped on the understanding of the IFNγ role in higher brain function, they have also raised a number of important questions that merit further investigation.

1. *Which timepoints and IFNγ producing cells are key to the herein observed actions?*

The majority of the findings described in the present thesis are related to the use of a knock-out animals (IFNγ KO). While the complete deletion of the gene may be well informative for the identification and characterization of affected systems, it is also possible that a life-long period without expression of this cytokine may trigger compensatory mechanism therefore confounding the results. In the future, the same hypothesis concerning cognitive modulation by this cytokine will be tackled but taking advantage of conditional ablation models in which expression of this cytokine is supressed and restored at different timepoints and in different cell types, in order to understand: 1) what is the time window across lifespan in which IFNγ is important and 2) is the peripheral or central absence of IFNγ that is implied in cognitive modulation?

2. *What is the role of IFNγ production in other locations?*

Our starting point was to look for alterations in the brain parenchyma and in lymphoid organs; however, considering that the immune response is compartmentalized, it will be also interesting to explore the IFNγ action on higher brain function from new locations such as the dCLN, the choroid plexus, CSF and meningeal spaces.

3. *What is the role of estrogen in modulating IFNγ actions in females?*

As we found gender differences in cognitive behaviours of IFNγ KO mice, and since estrogen is capable of modulating IFNγ expression, it would be particularly interesting to study a possible association between the herein described IFNγ-induced enhancement of cognitive performance in females and estrogen. Specifically, it remains to be clarified if physiological levels of estrogen impact IFNγ

expression and if the hormonal-control of IFNγ expression in the brain could underlie the behavioural gender diferences observed.

4. *What is the role of the fibers innervating the spleen in stress-related immune alterations?*

Our data highlight the possible importance of the innervating fibers of the spleen in mediating stress-related immune alterations. Mechanistically evidence for this association will also be possibly targeted in future experiments.