Exercise has persistent effects on learning and memory throughout the lifespan: analysis of the underlying mechanisms

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Thesis submitted for the degree of Doctor of Philosophy at the University of Dublin, Trinity College Dublin.

Submitted April 2018

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I. Declaration

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II. Summary

Ageing is a complex process associated with a decline in organism functions. Ageing affects several cellular and molecular brain mechanisms and is commonly associated with decline in brain function, such as cognition and emotion Moreover, age is characterised by the presence of chronic and low-grade inflammation, which is associated with decreased responsiveness of immune cells to stress and exacerbated inflammatory response. However, sometimes, even though morphological and physiological changes are observed in the brain of aged individuals, they may not experience the cognitive decline usually associated with ageing. In an attempt to explain this discrepancy between an individual's measured level of brain pathology/or age-related changes and the functional and/or cognitive deficits that are expected to result from that pathology or from normal ageing, the cognitive reserve hypothesis was proposed. Evidence suggest that life exposure and experiences can build brain reserve, by increasing brain resources and making the brain more flexible and capable of recruiting theses resources. In this context, exposure to physical exercise is associated with positive inflammatory modulation and increased brain plasticity, thereby contributing to reduce the unfavourable effects of ageing.

Therefore, the overall aim of this study is to investigate the effects of forced exercise on memory and learning throughout the mouse lifespan, investigating the cellular mechanisms involved and the reserve hypothesis. Firstly, we investigated the effects of long-term forced exercise, during youth and middle-age, on learning and memory throughout the lifespan of the mouse, and its effects on age-related anxiety and depression like behaviour, investigating the mechanisms involved in these effects. Secondly, as inflammation was demonstrated to be participating in the pro-cognitive effects of exercise, we decided to address the effects of short-term forced exercise on memory, inflammatory responses and activation of isolated populations of microglia and astrocytes, in a mouse model of neuroinflammation. To respond our first question, young male mice underwent treadmill running for a period of 8 months until middle-age, when they stopped exercising. After that, mice were housed in the absence of exercise for 10 more

months until old age. All mice were tested every 2 months for non-spatial (NOR) and spatial memory (OD) and at old age they were tested in the MWM and underwent analysis of anxiety and depression-like behaviour. Subsequently, structural MRI was performed, tissue was collected, and glial cells were isolated from the brain tissue. Assessment of BrdU labelling and cell phenotype by immunohistochemistry were used to investigate neurogenesis and assay of mRNA and protein expression of different targets of interest, focusing mainly in inflammatory markers, were carried out. To answer our second question, mice underwent 9 consecutive days of treadmill running prior to a single intraperitoneal injection of a sub-septic dose of lipopolysaccharide. Four hours later, mice were tested for spatial learning and memory and brain tissue was removed for analysis of inflammatory response and glia cells activation.

Our results demonstrated that exercise has an important effect in enhancing cognition and emotional behaviour as well as protecting against cognitive impairments in non-spatial and spatial memory, even months after exercise cessation. Moreover, exercise protected against LPS-induced impairment in spatial memory. We demonstrated that these cognitive improvements were mediated in large part by the anti-inflammatory effects of exercise, which positively modulated the brain environment protecting against the disruption of brain mechanism structure and function induced by neuroinflammation and age. The anti-inflammatory effect of exercise was shown to be directly involved in the preservation and enhancement of the mechanisms of hippocampal neurogenesis and synaptogenesis induced by exercise in our study and consequently the protection of the integrity of important brain regions. We propose that collectively these changes contribute to building a cognitive reserve and rendering the brain more resilient, thereby mediating protection against cognitive decline during the ageing process.

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IV. Acknowledgments

I would first and foremost like to thank my supervisor Professor Áine Kelly for all her help, guidance, encouragement and support over these years. It has been an absolute pleasure to work and learn with you in these years and I am sincerely grateful for everything. I wish you all the best in the future Professor Kelly.

I wish to express my gratitude to all the member of Physiology Department, specially Dr. Aedin Minogue for her direct help in the execution of this project and for teaching me so much in these years and Dr Eric Downer for his contribution to my project in the continuation transfer. To the technical and administrative department support for their help over the past few years, specially Noreen, Aidan, Kieran, Alice, Quentin and Christine for the availability and for helping me all the times I needed (even when I was very annoying). I would like to extend my acknowledgments to all the members of Comparative Medicine Unit, for their help with care of the animals over these years.

To all members of TCIN, especially, to Dr Charlotte O´Callaghan, for being so kind and helpful with the behaviour tasks and also to Dr Allison McIntosh and Rustam Rakhmullin for their help with the MRI study. To all MAL and VAC lab members thanks for all the help over these years, specially to Virginia Rivas for the friendship and collaboration in research as well. To members of the Kelly lab and to my officemates, Orla, Steven, Michael, Séan, thanks for everything, for the company and friendship over these years. A special thanks to Dr Sinead Ryan for all the knowledge transmitted and support and to my undergrad and MSc students, thanks for your patience and for teaching me as much as I taught you. And a very big thank you to my best, Ruth Hennessy, thanks for being with me these years, laughing and crying, but always there…your friendship was one of the best things of my PhD, I miss you.

I wish to thank to the “Brazilian community” from TCIN and Dublin, Lucas, Elaine, Cris, Rena, Dé, Petrya and Matheus. To Matheus, my most sincere gratitude for the daily chat, the funny lunch times and for all the support and friendship, I wish you the greatest success in your future, and I will miss you a lot my friend. To the

new member of this community Rogério (Roger), thank you for the help with the thesis in these final weeks and for dealing for me with the annoying thesis formatting part. To all my friends in Brazil, specially to the big family BIOEX, thanks for the friendship, even though far away you were always present, I miss you guys.

Last but certainly not least, a huge thank you to all my family, that from miles away and an ocean of distance were always sending me love and support. Specially, to my grandmothers Roma and Tetê, Mon Ana, Dad Carlos, Elisa, Júlio, Zuzu, Chico and Nina, without you none of this would have been possible, thank you for everything. My special gratitude to my second family Sandra, Fernando, Maria Fernanda, Picky and Panda, thank you for all the love and support. And the most special appreciation is for you Luiz, I have no words to thank you for everything you have been doing for me these past years. Thank you for all the support, friendship, encouragement and love. Undoubtedly, this path would have been much harder without you by my side…we passed through this, together - Te amo! To these great and loved ones, I dedicate this thesis.

V. List of Abbreviations

AD	Alzheimer disease
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-Isoxazolepropionic acid
ANOVA	Analysis of variance
APP	Amyloid precursor protein
APS	Ammonium persulfate
Arg1	Arginase-1
Aβ	Reduced β-amyloid
Bak	Pro-apoptotic protein regulator
Bax	Pro-apoptotic protein regulator
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
Bcl-2	Anti-apoptotic proteins regulator
Bcl-w	Anti-apoptotic proteins regulator
Bcl-Xl	Anti-apoptotic proteins regulator
BDNF	Brain-derived neurotrophic factor
BR	Brain reserve
BrdU	5'-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
Btg1	Anti-proliferative gene
CA	Cornu ammonis
CA1	Cornu ammonis 1
CA2	Cornu ammonis 2
Ca^{2+}	Calcium
CA3	Cornu ammonis 3
CA4	Cornu ammonis 4
$CaCl_2$	Calcium chloride
Calbindin	calcium-binding proteins
CaMKII	Ca/calmodulin-dependent kinase
cAMP	Cyclic adenosine monophosphate

CD11b	Cluster of differentiation molecule 11B
CD44	Cell surface adhesion molecule
cDNA	Complimentary deoxyribonucleic acid
CNS	Central nervous system
CR	Cognitive reserve
CREB	cAMP response element-binding protein
CRP	C-reactive protein
CSF	Cerebrospinal fluid
Cx3cl1	Fractalkine
Cx3C1	Fractalkine receptor
DA	Diacylglycerol
DAMPs	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DCX	Doublecortin
DG	Dentate gyrus
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
EC	Entorhinal cortex
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
E-LTP	Early-phase Long-term potentiation
eNOS	Endotelial nitric oxide synthase enzyme
EPM	Elevated plus maze
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
EX	Exercise group
FOV	Field of view
FST	Forced swim test
GAB1	GRB2 Associated Binding Protein 1
GCL	Granular cell layer
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein

Grb2	Growth factor receptor-bound protein 2
H_2SO_4	Sulfuric Acid
HA	Hyaluronan
HBSS	Hank’s Balanced Salt Solution
HCl	Hydrochloric acid
HD	Huntington's disease
HRP	Horseradish peroxidase
IκB	Inhibitors of kappa B proteins
i.p.	Intraperitonial
Iba-1	Ionized calcium-binding adapter molecule 1
IFN-α	Interferon alpha
IGF-1	Insulin-like growth factor 1
IGF1R	Insulin Like Growth Factor 1 Receptor
IGF-2	Insulin-like growth factor 2
IgG	Immunoglobulin G
IL-1R	Interleukin 1 receptor
IL-10	Interleukin-10
IL-13	Interleukin-13
IL-18	Interleukin 18
IL-1ra	Interleukin-1 receptor antagonist
IL1β	Interleukin 1 beta
IL-4	Interleukin-4
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
IQ	Intelligence quotient
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate monobasic
L-LTP	Late-phase Long-term potentiation
LPP	Lateral performant pathways
LPS	Lipopolysaccharide
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MCH-II	Major histocompatibility complex-II

MDB	Membrane-desalting buffer
MEK	Mitogen activated protein kinase kinase
MIPAV	Medical Image Processing, Analysis, and Visualization
MPP	Medial performant pathways
Mrc-1	Mannose receptor C1
MRI	Magnetic resonance image
MWM	Morris water maze
Na2HPO4	Disodium hydrogen phosphate
Na_3VO_4	Sodium Orthovanadate
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NeuN	Neuronal nuclei
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NLRs	Nucleotide oligomerization domain-like receptors
NMDA	N-methyl D-aspartate
nNOS	Neuronal nitric oxide synthase enzyme
NO	Nitric oxide
NOR	Novel object recognition
NOS	Nitric oxide synthase enzyme
NPCs	Neural progenitor cells
NSCs	Neural stem cells
NT3	Neurotrophin 3
NT4	Neurotrophin 4
NT5	Neurotrophin 5
OCT	Optimum cutting temperature compound
OD	Object displacement
p42 MAPK	Isoforms of Erk 2
p44 MAPK	Isoforms of Erk 1
p75	Pan-neurotrophin receptor 75
PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PD	Parkinson disease
PDK1	Phosphoinositide- dependent kinase-1
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein kinases A
PKC	Protein kinases C
PLCγ	Phospholipase C gamma
proBDNF	Pro brain-derived neurotrophic factor
PRRs	Pattern-recognition receptors
PSD-95	Postsynaptic density protein 95
RNS	Reactive nitrogen species
ROI	Region of interest
ROS	Reactive oxygen species
Rpm	Rotations per minute
RQ	Relative quantity
RT-PCR	Real time polimerase chain reaction
SDS	Sodium dodecyl sulphate
SED	Sedentary group
SGZ	Subgranular zone
Shc	Protein adaptor
SOS	Sons of sevenless
SVZ	Subventricular zone
TBI	Traumatic brain injury
TBS	Tris buffered saline
TBS-T	Tris buffered saline-Tween
TE	Echo time
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor

TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	Tumor necrosis factor alpha
TR	Repetition time
Tris-HCl	Tris hydrochloride
Trk	Tyrosine kinase receptor
TrkA	Tyrosine kinase receptor A
TrkB	Tyrosine kinase receptor B
TrkC	Tyrosine kinase receptor C
TST	Tail suspension test
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organization
Ym1	Chitinase-3-like-3

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“Success is not final, failure is not fatal:
it is the courage to continue that counts”

- Winston Churchill -

Chapter 1

Introduction

Chapter 1: Introduction

1.1 Ageing

1.1.1 World population ageing

Worldwide, life expectancy has increased due to improvements in longevity and consequently, the world population is rapidly ageing. According to the last United Nations report, around 607 million people were aged 60 or older (~8% of the world's population) in 2000. Moreover, between 2015 and 2030, the number of people in the world aged 60 years or over is projected to grow from 901 million in to 2015 to 1.4 billion in 2030. By 2050, this number is expected to almost double, reaching nearly 2.1 billion people, representing 16% of the world's population. The oldest population, people aged 80 years or over, is also growing faster globally. According the United Nations, 125 million people were aged 80 years or over in 2015, and this number is projected to triple in 2050, reaching 434 million of people worldwide.

In developed countries, the percentage of people aged 60 years or over was approximately 12% in 1950, increased to 23% in 2013 and is expected to reach 32% in 2050 (petrikJohn R. Beard, 2011). However, still according to the United Nation report, in the developing regions, the growth of the population aged 60 years or over is accelerating. It is projected to 2050 that 1.7 billion of people aged 60 years or over (approximately 80% of the older population) will live in the less developed countries (Figure 1.1). Moreover, specifically in Ireland, according to the 2016 Census, there was a huge increase in the number of people aged 65 years or over since the 2011 Census. This population was approximately 102,000 people in 2011 and raised to up to 637,000 people in 2016.

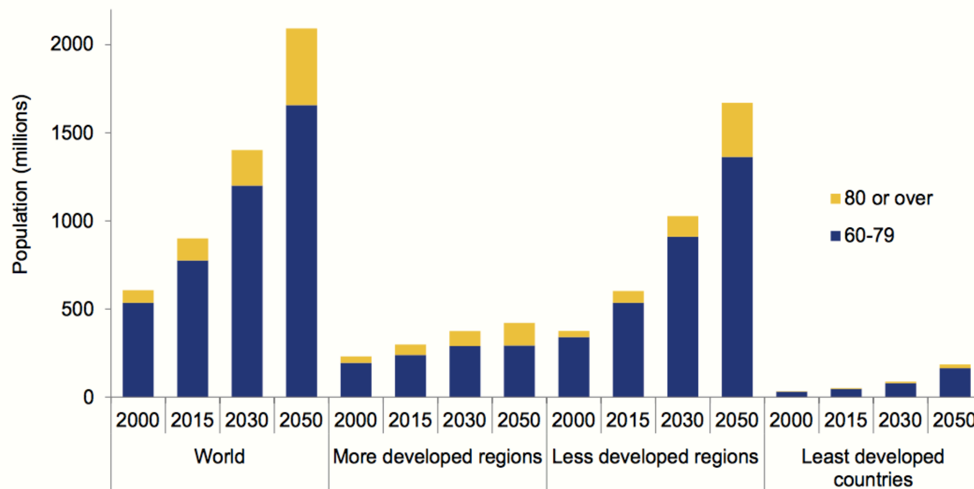


Figure 1.1 - Ageing population worldwide

World population aged 60–79, and 80 years and over, by development group 2000, 2015, 2030 and 2050 (Source: United Nations. *World Population Ageing 2015*).

1.1.2 Age-related diseases

Ageing is a multifactorial process associated with a decline in organism functions. The most evident characteristic of ageing is the reduction in the ability of the body to maintain homeostasis (Seidler et al., 2010, Comfort, 1979). Among the alterations related to ageing, one of the most striking is the decline in brain function, bringing increased morbidity, functional limitations, loss of autonomy and consequently reducing the quality of life of this population.

According to the World Health Organization (WHO), Alzheimer's disease (AD) and other dementias are listed as one of the ten leading causes of disability globally among women and men aged 60 years or over. Reports from the Alzheimer's Association show that approximately 20% of people aged 65 years or over have Mild Cognitive Impairment (Roberts et al., 2014), a potential precursor for AD and other dementias (Mitchell et al., 2009). Also, according to the WHO, there are approximately 47.5 million cases of dementia, and AD accounts for approximately 65% of these (WHO statistics, 2016). Thus, due to the increase in the elderly population and the consequences associated with ageing, this subject has become

a public health concern and efforts in research, aiming to ameliorate the quality of life of this population, have increased substantially.

1.2 Cognitive function

1.2.1 Learning and memory

One of the most intriguing faculties of the brain is its ability to acquire and store information from an experience and to recall that information at a later point. For this reason, the process of learning and memory is integral and crucial to functional life. We can define learning as the ability to acquire knowledge and change behaviour as a result of an experience (Kolb and Whishaw, 2014) and memory as the processes of encoding, storage and retrieval of learned information (Bunke and Kandel, 2000). Over the past years, studies in animals and humans have significantly enhanced our understanding about how the brain accomplishes these processes (Han and Stevens, 1999).

1.2.2 Memory classification

Memory is composed of distinct systems and can be classified in two sub-types; short-term (information is retained over a few seconds) and long-term (information is retained from minutes to years) (Squire, 1986). Long-term memory can be subdivided into explicit (or declarative) memory and implicit (procedural or non-declarative) memory (Figure 1.2). Explicit memory refers to the conscious retention of available information and can be further divided into episodic memory (or contextual; capacity to re-experience personal events within a specific spatial and temporal context; e.g. a memory related to a place, object or people) and semantic memory (or non-contextual; capacity to recall facts and knowledge about the world; e.g. memory of an abstract fact). Although explicit (declarative) memory is commonly tested via verbal-based tests, over the years, several cognitive tests have been designed and employed to assess this component in animals (Eichenbaum, 1997). In contrast, implicit memory Is procedural memory and refers to an unconscious memory, e.g., learning to play a musical instrument, driving a car (Dharani, 2015).

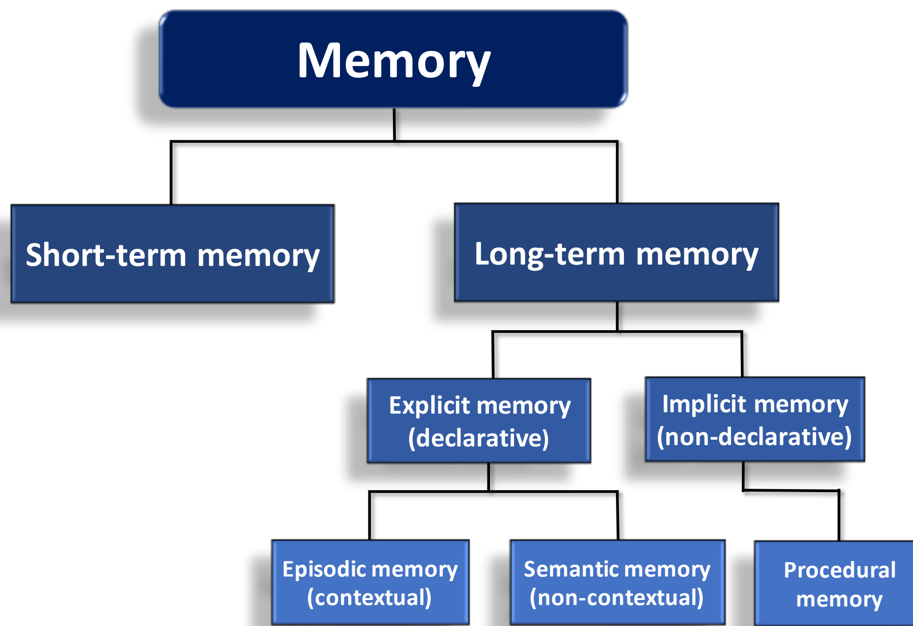


Figure 1.2 - Types of memory

Schematic diagram of the classification of memory (based on Squire, 1986).

Over the years, several studies have contributed new insights to help identify the brain structures and cellular mechanisms involved in memory. In this context, one important difference between these forms of memory seems to be the brain structures involved (Squire, 2004). It has been demonstrated that implicit memory involves areas such as the cerebellum, and basal ganglia, while explicit memory requires areas of the medial temporal lobe, such as entorhinal and perirhinal cortex and, particularly, the hippocampus (Squire, 2004, Squire, 1992).

1.2.3 Spatial and recognition memory

This thesis investigates two types of memory in mice: spatial memory and object recognition (non-spatial) memory. Spatial memory is the ability to encode, store and retrieve information about the spatial arrangement of objects or place (Kessels et al., 2001). This memory is a component of several of the categories in the memory system mentioned previously, involving aspects of both short and long-term memory, as well as procedural (non-declarative), semantic and episodic (declarative) memory (Moscovitch et al., 2006).

In rodents, several spatial tasks that are used are based on maze navigation, including the Morris Water Maze (MWM), which is widely employed to test the ability to learn and remember routes and paths using spatial cues (Vorhees and Williams, 2006). The Object Displacement (OD) task is another test used to evaluate spatial memory (object-location memory) and also requires the creation of a spatial layout (Kessels et al., 2001). Studies have demonstrated the hippocampus to be vital for learning, memory, and spatial navigation (Preston and Eichenbaum, 2013)

Recognition memory is the ability to distinguish between a familiar item (previously presented) and a novel item. This type of memory is one of the main components of the declarative memory category (Squire et al., 2007). The most common task used to assess recognition memory, in rodents, is the Novel Object Recognition (NOR) task, which relies in innate preference of the rodent to explore the novel object (Leger et al., 2013). The perirhinal cortex area has been shown to be crucial for remembering and recognition of the features of objects. However, although recognition memory is often described as a hippocampus-independent memory, compelling evidence suggests that the hippocampus plays an important role in processing information related to the familiarity of objects (Buckley, 2005).

1.3 The hippocampus

1.3.1 The hippocampal formation

The hippocampus is a bilateral brain structure located in the medial temporal lobe and consists of four subregions: the dentate gyrus (DG), the hippocampus proper, the subiculum and the entorhinal cortex. The hippocampus proper is subdivided into four subfields, the *cornus ammonis* (CA) regions: CA1, CA2, CA3 and CA4 (hilus) (Figure 1.3) (Afifi and Bergman, 2005). The DG and the hippocampus proper are composed of three layers; a polymorphic layer, molecular layer, and pyramidal/granular layer, while the subiculum has a polymorphic layer of interneurons and a pyramidal layer (Knierim, 2015). The basic hippocampal arrangement was originally described by Cajal (1901) and since then, genomic and anatomic studies have supported and helped to re-evaluate and to define the

hippocampal cytoarchitecture. These findings strongly support a functional segmentation of the hippocampus into two main parts, the dorsal (posterior in humans) and the ventral (anterior in humans) portion of the hippocampus (see Figure 1.3) (Fanselow and Dong, 2010, Strange et al., 2014).

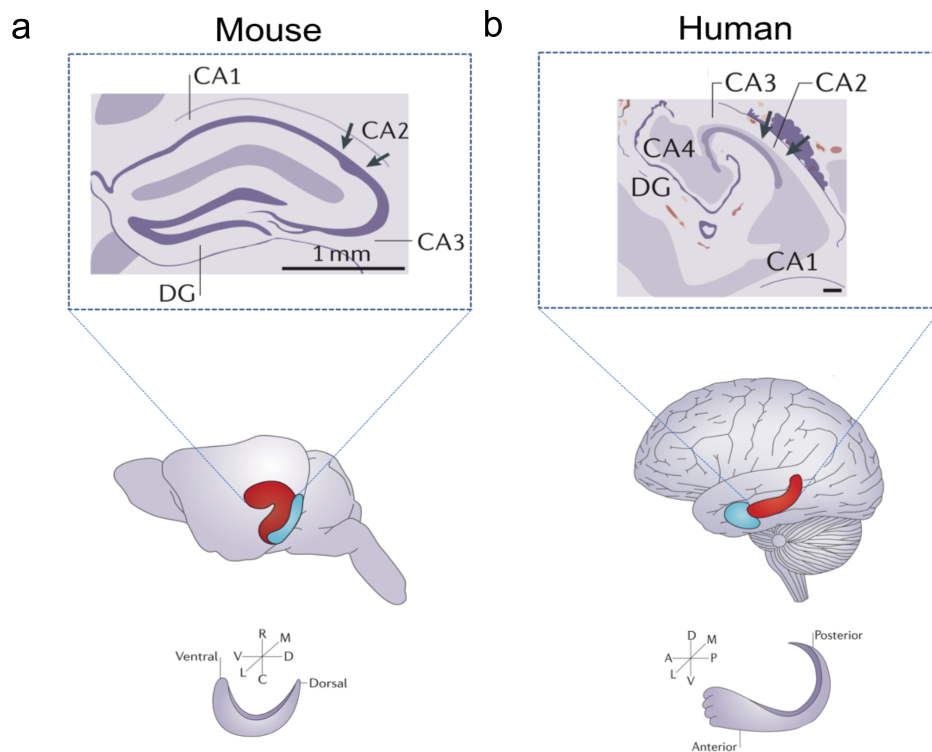


Figure 1.3 - The hippocampus

Hippocampal formation (in red) in the rodent **(a)** and human **(b)** brain. Hippocampal axis in rodents is ventro-dorsal **(a)** and in humans is antero-posterior **(b)**. Representative coronal section of the hippocampal formation showing CA1-CA4 and dentate gyrus regions in both the rodent **(a)** and human **(b)** brain (adapted from Strange et al., 2014).

The hippocampus exhibits three major excitatory pathways, with the main input running via the entorhinal cortex to the DG, CA3, CA1 and returning to the entorhinal cortex (Anderson et al., 1987, Deng et al., 2010). This is called the tri-synaptic pathway and consists of the perforant pathway, the mossy fibres pathway and the Schaffer collateral pathway (see Figure 1.4). Briefly, the hippocampus receives input from axons in the entorhinal cortex to the granule cells of the DG via the perforant pathway. The granule cells from the DG projects axons to the pyramidal cells in the CA3, via the mossy fibres pathway. Finally, the pyramidal

cells from the CA3 region send projections to the CA1 pyramidal cells, via the Schaffer collateral pathway, with subsequent CA1 connection to the subiculum (Deng et al., 2010). This elaborated neural circuitry is the basis to our understanding of the role of hippocampus in learning and memory.

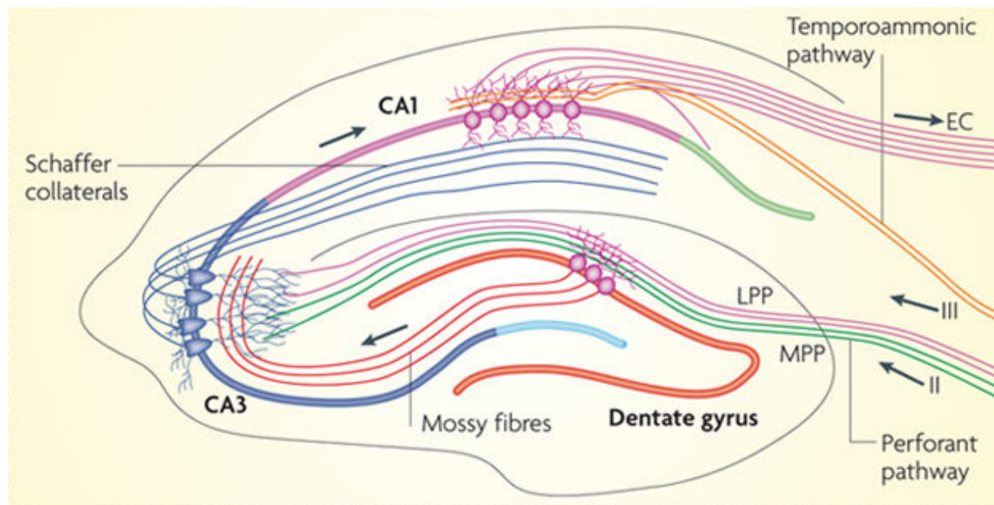


Figure 1.4 – Subfields and neural circuitry of the hippocampus in rodent

The main inputs to the hippocampus from the perirhinal and parahippocampus originate from neurons in the entorhinal cortex (EC), which project their axons to the dentate gyrus through the medial (MPP - II) and lateral (LPP - III) perforant pathways. The mossy fibres connect the cells from the dentate gyrus to CA3, while the Schaffer collaterals connect CA3 pyramidal neurons to CA1 pyramidal neurons. The main output from the hippocampus is from CA1 to the EC via subiculum (Deng et al., 2010).

1.3.2 Role of the hippocampus

Although different brain structures are involved in the process of learning and memory, the hippocampus plays a crucial role in formation of explicit memory (Lynch, 2004). The first insights to critical role of hippocampus in learning and memory was in 1950's with the famous study of Scoville and Milner (1957), when in an attempt to alleviate a patient's debilitating seizure, surgical lobotomy was performed (bilateral medial lobe removal, including hippocampal formation, amygdala and entorhinal cortex). As result of the surgery, a profound amnesia for all events following surgery (anterograde amnesia) was observed in the patient, demonstrating that the explicit (declarative) memory was affected by hippocampal

region removal, whereas his implicit (non-declarative) memory, such as motor skill abilities and semantic memories was spared (Scoville and Milner, 1957, Corkin, 2002).

Since then, several studies, in humans (Burgess et al., 2002) and animals (Martin and Clark, 2007) provided further evidence of the central role of the hippocampus in the process of learning and memory. In particular, the DG has received special attention for its role in memory formation. It has been demonstrated that lesion of DG blocks memory acquisition, but does not alter retrieval of hippocampal memory (Lee and Kesner, 2004, Madronal et al., 2016). Also, many of the cellular and molecular mechanisms associated with learning and memory, such as long-term potentiation, morphological changes and neurogenesis, are well-established in the DG (Jonas and Lisman, 2014). Furthermore, as the hippocampus is part of the limbic system it plays an important role controlling emotions. Moser and Moser (1998), suggested that dorsal (posterior in humans) and ventral (anterior in humans) portions of hippocampus have different roles (Moser and Moser, 1998). Several lesion studies demonstrated that spatial learning and memory appear to depend on the dorsal portion, while the ventral portion is associated with stress responses and emotional behaviour (Moser et al., 1995, Henke, 1990).

The discovery of the role of the hippocampus in learning and memory led to the search for the cellular and molecular changes underlying this process. Since then, many studies have assessed hippocampal changes in synaptic strength, in the form of brain plasticity and changes in the number of cells, in the form of adult neurogenesis. In addition, this important structure is highly susceptible to the effects of aging and neurodegenerative diseases; studies have demonstrated a reduction in the volume of hippocampal tissue in the elderly, AD and in depression, contributing to cognitive impairments (Convit et al., 2003, Burke and Barnes, 2006, Driscoll et al., 2006).

1.4 Cognitive function and ageing

Ageing is characterized by several morphological, chemical and biological changes in brain structures, which are often manifested as deterioration in cognitive function (Bishop et al., 2010). There is an abundance of evidence of age-induced cognitive decline in the literature, in humans (Albert et al., 1995, Atkinson et al., 2007) and in rodents (Means et al., 1993, Latimer et al., 2014). It has been demonstrated that the cognitive decline associated with ageing is due to several changes in specific brain areas and alteration in learning and memory related mechanisms, such as changes in synaptic plasticity and neurogenesis. Specific regions of the brain shrink due ageing, mainly the prefrontal cortex and the hippocampus, both important areas to learning and memory and other complex mental activities (Peters, 2006). Also, age-related changes in behaviour are not limited to cognitive function; studies have also demonstrated increased anxiety in aged mice and mouse models of neurodegenerative disease (Flint et al., 2010). In addition, ageing is associated with inflammation, reduced brain plasticity in the form of either impaired LTP or decreased neurogenesis, and neurodegenerative disease (Grady, 2012).

1.5 Cellular and molecular mechanisms of learning and memory

1.5.1 Synaptic plasticity

Synaptic plasticity can be defined as an activity-dependent modification of the strength or efficacy of synaptic transmission at pre-existing synapses (Bunke and Kandel, 2000). The existence of synaptic plasticity was first described by Cajal (1913) and later by Hebb (1949). Nowadays, these changes in synapse strength and efficacy are believed to represent one of the most important cellular mechanisms associated with the process of learning and memory (Berlucchi and Buchtel, 2009).

The most investigated form of synaptic plasticity is long-term potentiation (LTP), and was first recorded and described in 1973, by Bliss and Lomo (Bliss and Lomo, 1973). They demonstrated that a high-frequency stimulation of the perforant path

resulted in a sustained increase in the synaptic response of the granule cells in the DG and since then, LTP has been widely studied and described in the brain across different species (Artola and Singer, 1993, Bruel-Jungerman et al., 2007). Induction and maintenance of LTP requires the activation of specific receptors and molecular signalling. The most studied form of LTP is N-methyl D-aspartate (NMDA) receptor dependent-LTP and it is divided into three phases: initial, early-phase (E-LTP) and late-phase (L-LTP). The initial phase, also called short-term potentiation, can last from seconds to minutes and requires the release of glutamate neurotransmitter from the pre synaptic terminal due to an increase in presynaptic levels of Ca^{2+}, in response to an action potential. This initial phase acts as a prelude to the two subsequent phases, the E-LTP and L-LTP, that are described to be related mainly with post-synaptic signalling (Sweatt, 1999).

The next phase, the E-LTP, can be sustained from minutes to hours and involves the activation of NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the post-synaptic neuron, increasing the influx of Ca^{2+} into the cell and leading to a subsequently activation of Ca^{2+}-dependent kinases, such as Ca/calmodulin-dependent kinase (CaMKII) and protein kinases A and C (PKA and PKC) (Mayford, 2007). This cascade is essential to the induction of E-LTP, once activated Ca^{2+} dependent kinases phosphorylate the post-synaptic AMPA receptors, making them sensitive to glutamate (Sweatt, 2001, Lynch, 2004). The last phase, known as L-LTP, can last from hours to weeks *in vivo* (Lynch, 2004) and requires the activation of intracellular signalling and synthesis of new proteins. The kinases activated in E-LTP trigger signalling pathways, which lead to the activation of transcription factors, such as cAMP response element binding protein (CREB) and consequent synthesis of new proteins, including AMPA receptor that are inserted in the post-synaptic membrane and increase the sensitivity of the cell to glutamate (Linden and Routtenberg, 1989, Kelleher et al., 2004).

Impairment in LTP results in spatial and recognition memory deficits (Morris et al., 1986, Molinaro et al., 2011). Also, neurotrophins, which are presented in the next sections, are considered to play an important modulatory role in synaptic plasticity; for example, brain derived neurotrophic factor (BDNF) can trigger LTP in the rodent

hippocampus (Ying et al., 2002, Kuipers et al., 2016). Furthermore, a study from our laboratory showed exercise to increase expression of BDNF in the DG together with enhanced LTP and recognition memory (O'Callaghan et al., 2007).

1.5.2 Synaptogenesis

Synaptic organization and remodelling of neural circuits are important mechanism for the acquisition and storage of new memories (Bruel-Jungerman et al., 2007). Synaptogenesis includes the changes in the number of synapses and change in the number, size and density of dendritic spines. Enlargement of existing spines can be observed in minutes (Tanaka et al., 2008), while insertion of AMPA receptors in these spines, important to switch synapses form silent to functional, can take hours (Park et al., 2006b) and it can take days to result in fully functional synapses (Nagerl et al., 2007).

Synaptogenesis has been linked with enhanced cognitive function (Markham and Greenough, 2004, Birch et al., 2013) and it has been shown that exercise can induce the generation of new synapses and up-regulate synaptic proteins in the hippocampus (Shih et al., 2013). Exercise increases the levels of presynaptic proteins, such as synapsin-1 and post-synaptic proteins, such as PSD-95, and it has been linked to increase in BDNF levels and improvements in spatial memory (Vaynman et al., 2003, Siette et al., 2013, Shih et al., 2013). Thus, evidence suggests that remodelling of synapses has a stable and persistent effect on learning and memory.

1.5.3 Synaptic plasticity, synaptogenesis and ageing

Age-related decline in cognitive function can be explained in part by changes in the mechanisms of synaptic plasticity (Burke and Barnes, 2006). Accumulating evidence has established that LTP is impaired with age (Lynch and Voss, 1994, Murray and Lynch, 1998a, Ryan et al., 2015), but the underlying mechanisms involved remain unclear. Neurochemical and neurophysiological changes are observed in the aged brain, such as, reduction in the level of neurotransmitter and alterations in the balance between inhibitory and excitatory neurotransmission,

which are often related to cognitive impairments and dementia (Loerch et al., 2008, Jouvenceau et al., 1998).

A significant decrease in the expression of synaptic genes with ageing has also been reported, contributing to altered connectivity and integration of brain networks in ageing (Lu et al., 2004). Moreover, a dysfunction in Ca^{2+} homeostasis can be observed in the aged brain, which can also contribute to alterations in synaptic plasticity and consequent cognitive impairment (Yankner et al., 2008). In addition, age-related changes in the structure of neuronal synapses have also been reported, characterized by alterations in dendritic branching and reduction in the number of synapses as well as decreased expression of pre-post/synaptic structural proteins (such as synaptophysin, synapsin-1 and PSD-95) and progressive loss of synaptic density (Burke and Barnes, 2006, Ojo et al., 2012). Interestingly, many of these age-related changes in cellular mechanisms and morphology are tightly associated with impairments in learning and memory (Erickson and Barnes, 2003). Taken together, these finds strongly suggest that synaptic plasticity is affected by ageing and linked with cognitive impairments observed in ageing.

1.5.4 Adult Hippocampal Neurogenesis

Neurogenesis is a term used to describe the proliferation, differentiation, integration and survival of newborn neurons. In 1965, Altman & Das (1965) were the first to suggest that neurogenesis could happen in the adult mammalian brain (Altman and Das, 1965). However, only in the 1990's, investigations were made that would finally support the existence of neurogenesis in the adult mammalian brain (Gould et al., 1992, Eriksson et al., 1998, Gage, 2000). In 2000, Gage showed that the neural stem cells (NSCs) were present not only in the developing mammalian brain but also in the adult mammalian brain, including humans. Also, this study has shown that the NSCs from the adult hippocampus were capable of producing new neurons, astrocytes and oligodendrocytes, under the right conditions (Gage, 2000).

Nowadays, a wide body of evidence indicates that new neurons are continually being generated in the adult brain. Actively dividing NSCs have been discovered in two specific “neurogenic niches” in the mammalian brain, which are the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the DG in the hippocampus (Doetsch and Alvarez-Buylla, 1996, Deng et al., 2010). In the SVZ, adult born neurons differentiate and migrate to become periglomerular and granular neurons of the olfactory bulb (Deng et al., 2010). In contrast, the NSCs originating in the SGZ of DG migrate to the granule cell layer (GCL) and incorporate into local neural circuits as granular cells of the dentate gyrus (van Praag et al., 2002, Duman et al., 2001b).

1.5.4.1 Timeline of hippocampal neurogenesis

The process of adult hippocampal neurogenesis has been divided into a sequence of complex processes, which begins with the proliferation and differentiation of neural progenitor’s cells (NPCs) in the SGZ of DG, followed by migration and maturation of newborn neurons. There are four types of NPCs in the DG, which are the type-1, type-2a, type-2b and type-3 NPCs (Figure 1.5). They can be distinguished from each other based on morphology, proliferation rate and protein expression (Lledo et al., 2006), 2006). Type-1 cells are radial glial-like cells and express the astrocytic protein glial fibrillary acid protein (GFAP), SOX2 and the intermediate filament protein nestin. Type-1 cells have a low proliferation rate and can give rise to Type-2a NPCs (Farioli-Vecchioli et al., 2014). Type-2a cells do not express GFAP, however the nestin expression is maintained. This type of cell is characterized by high proliferation rate and, under favourable conditions, can generate type-2b NPCs (Farioli-Vecchioli et al., 2014, Lledo et al., 2006).

Neuronal determination occurs at this stage; type-2b NPCs express the immature-neuron specific protein doublecortin (DCX) and also maintain nestin expression. The type-2b cells are known for their high proliferation rate and as they initiate the neuronal differentiation they migrate from SGZ to the GCL of DG and are characterised by the expression of ki67. The fourth type of NPCs is the type-3 cell, which maintains DCX expression. These cells have a low proliferation rate and they continue to migrate within the GCL. Afterwards, they are transformed from an

immature neuron to a fully mature neuron transiently expressing the calcium binding protein calretinin, followed by more mature neuronal markers such as neuronal nuclei (NeuN) and calbindin (Steiner et al., 2004).

Newborn neurons become functionally integrated into existing DG circuitry within 3 weeks, extending their axons to CA3 region of hippocampus, as indicated by morphological and electrophysiological studies (Hastings and Gould, 1999, Esposito et al., 2005), Once mature, these newborn neurons can be integrated into neuronal network and form synapses with interneurons, mossy cells and CA3 pyramidal neurons (Toni et al., 2008). In this context, these newborn neurons can develop an important role in learning and memory. However, it has been shown that some endogenous and exogenous factors can affect the proliferation, differentiation and survival of the NCSs, including growth factors, neurotransmitters, hormones, transcription factors and inflammation (Klempin and Kempermann, 2007).

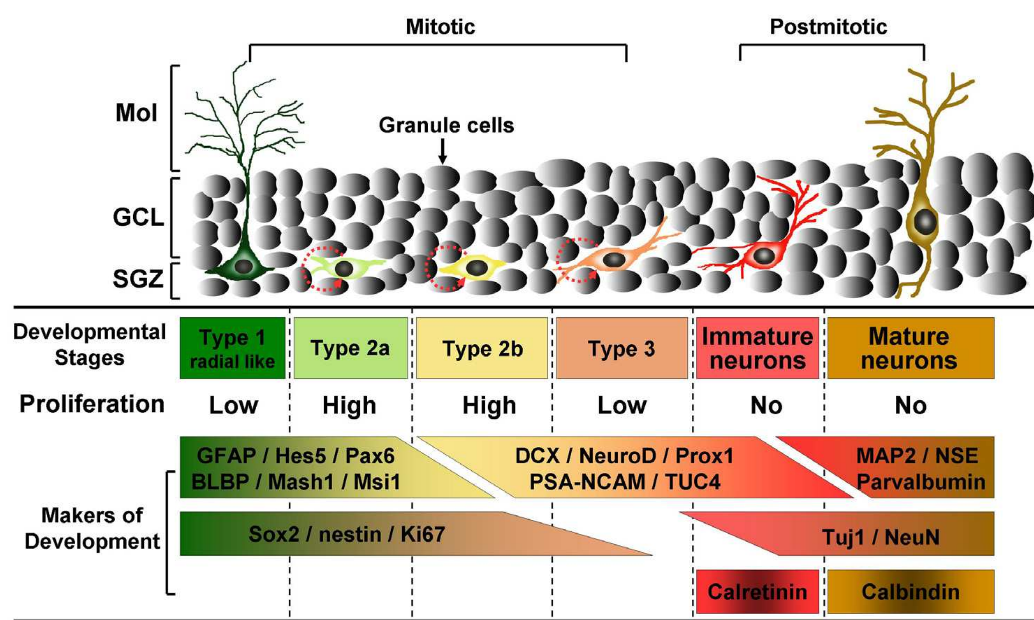


Figure 1.5 - Adult hippocampal neurogenesis in the subgranular zone (SGZ) of the dentate gyrus

Adult hippocampal neurons originate from type-1, type-2ab and type 3 cells. The neuronal determination is at stage type 2b. Type 3 cells gradually exit from the cell cycle and then form the immature and mature neurons. These newborn granule cells extend their dendrites and axons integrating into the molecular layer. Cells in different stages of neurogenesis express neural specific markers (Zhang and Jin, 2012).

1.5.4.2 Adult hippocampal neurogenesis in humans

Furthermore, studies have shown that hippocampal neurogenesis in humans can differ from hippocampal neurogenesis in rodents. Spalding and collaborators (2013), demonstrated that in humans, a larger proportion of hippocampal neurons are replaced in adulthood and the age-dependent decline in the rate of hippocampal neurogenesis is less pronounced in humans. Furthermore, hippocampal neurogenesis results in increase in the number of neurons in rodent DG, whereas neurogenesis in the human hippocampus generates a population of neurons with specific functional properties (Spalding et al., 2013). Also, recently, Boldrini and collaborators (2018), reported that healthy older subjects, with no cognitive impairment, show a preserved hippocampal neurogenesis. They demonstrated an age-related decline in the pool of quiescent NSCs in the DG, however, proliferating progenitors and immature neurons pools remain unchanged with ageing (Boldrini et al., 2018).

However, findings about neurogenesis in human are discrepant, while several studies suggested that a large number of new cells are generated in the hippocampus of humans in adulthood, other studies have found only a small number of newborn neurons (Eriksson et al., 1998, Dennis et al., 2016). Most recently, a study analysed post-mortem hippocampal tissue of humans and monkeys and strongly suggested that hippocampal neurogenesis drops from childhood to undetectable levels in adulthood in thesis species (Sorrells et al., 2018). These recent findings about the decline in hippocampal neurogenesis since the early stages of life raises some questions about the possible differences between neurogenesis in humans and other species, where adult neurogenesis has been shown to be well preserved.

1.5.4.3 Balance between and neurogenesis and gliogenesis

As mentioned before, it is important to mention that new cells other than neurons can originate from the progenitor cells. The progenitor cells can generate glia cells (astrocytes, oligodendrocytes and microglia), through the process known as gliogenesis. Studies have demonstrated that extrinsic and intrinsic factors, such as

the trigger of specific signalling and transcription factors, can drive and determine the NSCs commitment and cell fate (Wen et al., 2009, Rusznak et al., 2016). Accordingly, changes in the balance of neurogenesis and gliogenesis have been reported in a number of pathological conditions of the central nervous system (CNS), including brain injury (Burns et al., 2009, Wang et al., 2016) and neurodegenerative diseases (Winner and Winkler, 2015, Choi et al., 2016).

1.5.4.4 Adult hippocampal neurogenesis and learning and memory

Adult neurogenesis has been linked to neural plasticity and cognition in rodents, as the newborn granule cells have been shown to correlate with modifications in hippocampus-dependent memory (Shors, 2004). For instance, young-adult rats and mice housed in an enriched environment for up to 8 weeks demonstrated increase in hippocampal neurogenesis, which was associated with enhancement in spatial learning and memory performance in the MWM task (Nilsson et al., 1999, Iso et al., 2007). On the other hand, negative modulation of hippocampal neurogenesis has been demonstrated to impair spatial learning and memory. For example, lesion of the cholinergic neurons in the septohippocampal pathway was reported to decrease hippocampal neurogenesis and significantly impair spatial learning and memory performance (Mohapel et al., 2005).

Pattern separation has also been shown to be dependent of hippocampal neurogenesis. In this context, a study in mice demonstrated that reduced hippocampal neurogenesis leads to impairment in contextual discrimination and modification in the activation of neuronal population in the CA3 region, suggesting that adult hippocampal neurogenesis can facilitate population coding in the CA3, and then contribute to enhanced pattern separation (Niibori et al., 2012). In addition, most recently, studies demonstrated the role of adult neurogenesis in promoting forgetting of old memories to the acquisition of new ones (Akers et al., 2014, Goodwin, 2018).

1.5.4.5 Hippocampal neurogenesis and ageing

Adult hippocampal neurogenesis in the dentate gyrus (DG) has been shown to be impaired due to the ageing process in different mammalian species (Seki and Arai, 1995, Cameron and McKay, 1999, Gould et al., 1999, Kempermann et al., 2002, McDonald and Wojtowicz, 2005) and it has also been partially linked to age-related cognitive decline (Lazarov et al., 2010, Mu and Gage, 2011, Galea et al., 2013). In aged rats, the proliferation rate of NSCs in the SGZ of the dentate gyrus can be reduced by approximately 80%, suggesting a link between an age-related decrease in neurogenesis and a decrease in both NSCs proliferation and neural progenitor cell (NPCs) differentiation (Jin et al., 2003, Kuhn et al., 1996).

Several neurological diseases are linked with decreased rates of neurogenesis. For instance, the triple transgenic mouse model of AD (3xTg-AD) is reported to have lower hippocampal neurogenesis when compared to their wild type control, and this decrease is linked to cognitive impairment (Rodriguez et al., 2008). Furthermore, chronic stress and rodent models of anxiety and depression have also been shown to affect hippocampal neurogenesis (Surget et al., 2008). Age-associated decline in neurogenesis may be related to changes in the microenvironment of NSCs, which can interfere with proliferation, differentiation and survival of newborn cells. In this context, inflammation has been shown to play a role in this microenvironment (e.g. microglia activation and pro-inflammatory mediators infiltration from periphery), thereby influencing cell proliferation and survival of newborn neurons (Yirmiya and Goshen, 2011).

It has also been shown that expression of neurotrophins and other growth factors is altered in the aged brain and can contribute to change CNS environment (Erickson et al., 2010). For instance, exogenous addition of growth factors, such as insulin-like growth factor I (Igf-I), nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) can increase cell proliferation and differentiation (Lichtenwalner et al., 2001, Frielingsdorf et al., 2007, von Bohlen und Halbach, 2010). In addition, it has also been reported that decreased hippocampal neurogenesis in aged mice is rescued by physical exercise and enriched environment (van Praag et al., 1999, van Praag et al., 2005), supporting the

hypothesis that age-related decrease in the rate of neurogenesis may be a consequence of changes in the microenvironment of NSCs, such as decreased levels of neurotrophins and growth factors and low-grade inflammation.

1.6 Neurotrophins and growth factors

Neurotrophins belong to the family of growth factors that promote and regulate neuronal development, growth, survival or death as well as neurogenesis and brain plasticity (Blum and Konnerth, 2005) and are widely described to enhance synaptic plasticity and memory, in particular BDNF (Schinder and Poo, 2000, Gooney et al., 2004). The neurotrophin family comprises NGF, BDNF, neurotrophin 3 (NT3) and neurotrophin 4/5 (NT4/5). Neurotrophins are synthetised as precursors proteins, pro-neurotrophins, and later these precursors undergo proteolytic cleavage, to yield the mature-neurotrophins (Mowla et al., 2001).

These proteins act by binding two different membrane receptors, the tyrosine kinase receptor (Trk) family and the pan-neurotrophin (p75) receptor. It has been widely described that NGF binds preferentially to TrkA receptor, while BDNF and NT4/5 are have high affinity for the TrkB receptor and NT3 binds the TrkC receptor (Barbacid, 1995, Huang and Reichardt, 2003). Despite the fact that the p75 receptor was the first neurotrophin receptor to be isolated (Johnson et al., 1986), its ligand affinity and signalling is less understood than the Trk receptors family. It has been shown that the binding affinity of neurotrophins to the p75 receptor is higher for the pro-form of neurotrophins than the mature form (Dechant and Barde, 2002).

Binding of neurotrophins to their receptors activates signalling cascades that lead to gene upregulation, resulting in the trophic effect of neurotrophins on specific neuronal populations (Chao, 2003, Binder, 2007). However, Trk and p75 receptors initiate different downstream signal transduction pathways, leading to opposite physiological responses. While activation of Trk receptor promotes cell survival and enhancement of synaptic plasticity (Chao, 2003, Bramham and Messaoudi, 2005), activation of p75 receptor mediates apoptosis (Roux et al., 1999, Hempstead, 2002). There are also several others growth factors that do not belong

to the neurotrophin family but are also widely associated with promotion of cell growth and survival, including the Igf-1, the vascular endothelial growth factor (VEGF) and the glial cell line-derived neurotrophic factor (GDNF).

1.6.1 Brain-derived neurotrophic factor (BDNF)

Among the neurotrophins, BDNF has been most widely studied and shown to play an important role in the mature nervous system. Increased levels of BDNF have been linked to enhanced synaptogenesis, neurogenesis and learning and memory (Carvalho et al., 2008, Nagahara et al., 2009). BDNF was the second member of the neurotrophin family to be discovered, in 1982 (Barde et al., 1982). As mentioned before, BDNF exerts its trophic effects by binding TrkB receptor (Luikart et al., 2003). In the adult brain, BDNF and TrkB receptor can be found in different brain regions and cell types, most abundantly in neurons and astrocytes (Moretto et al., 1994, Barakat-Walter, 1996, Binder and Scharfman, 2004). In the CNS, BDNF is highly expressed in the hippocampus, in association with glutamatergic synapses (Bramham and Messaoudi, 2005), whereas, peripherally, BDNF is found in platelet and endothelial cells (Yamamoto and Gurney, 1990, Fujimura et al., 2002).

Similar to other neurotrophins, BDNF is synthesised as a pro-neurotrophin (proBDNF; ~30kDa), which then is cleaved, generating the mature-BDNF (mBDNF; ~14kDa). Although proBDNF was originally thought to act only intracellularly, evidence has suggested it is secreted and cleaved extracellularly (Barnes and Thomas, 2008). However, studies have shown that most BDNF found and secreted in the CNS is in its mature form (Matsumoto et al., 2008) and the amount of BDNF secreted is regulated in an activity dependent-manner via voltage-gated Ca^{2+} channels and NMDA receptor (Hartmann et al., 2001, Kolarow et al., 2007).

The well-known synaptic effects of BDNF are mediated by TrkB receptor activation. The BDNF-TrkB signalling, discussed in the following section, has been implicated in the cellular and molecular changes associated with enhanced memory.

1.6.2 BDNF-TrkB signalling cascade

When released into the synaptic cleft, BDNF binds to TrkB receptor in the post synaptic membrane and consequently, a signalling cascade is activated within the cell. Short-term effects of BDNF binding at the TrkB receptor include increase of neurotransmitter release and activation of glutamate receptors in the synapse, whereas the long-term consequences are associated with the activation of intracellular pathways and the modifications in gene expression downstream in the nucleus (Cunha et al., 2010). TrkB receptor is a transmembrane receptor with an extracellular ligand-binding site and several kinase domains.

BDNF and TrkB have a relevant role in several aspects of the hippocampus, including cognitive function and neurogenesis. For instance, higher levels of BDNF in the hippocampus, induced by physical exercise, have been implicated in long-term potentiation and synaptic plasticity (Liu and Nusslock, 2018). Moreover, in mice exposed to enriched environment, BDNF was demonstrated to stimulate hippocampal neurogenesis, as well as BDNF has been reported to have coordinated effects with antidepressants drugs on hippocampal neurogenesis (Rossi et al., 2006, Sairanen et al., 2005).

The BDNF binding, induces dimerization and autophosphorylation of TrkB receptor at multiple tyrosine residues, leading to the recruitment of different intracellular adaptor proteins and the activation of specific downstream pathways (Ohira et al., 2001, Huang and Reichardt, 2003, Reichardt, 2006). There are three major BDNF-TrkB-activation signalling pathway described in the literature, which are the phosphatidylinositol 3-kinase (PI3K) and Akt (protein kinase B) pathway, the Ras-mitogen-activated protein kinase (MAPK/ERK) pathway and the phospholipase C-gamma calcium pathway (Huang and Reichardt, 2003, Minichiello, 2009), described in detail in the following sections (Figure 1.6).

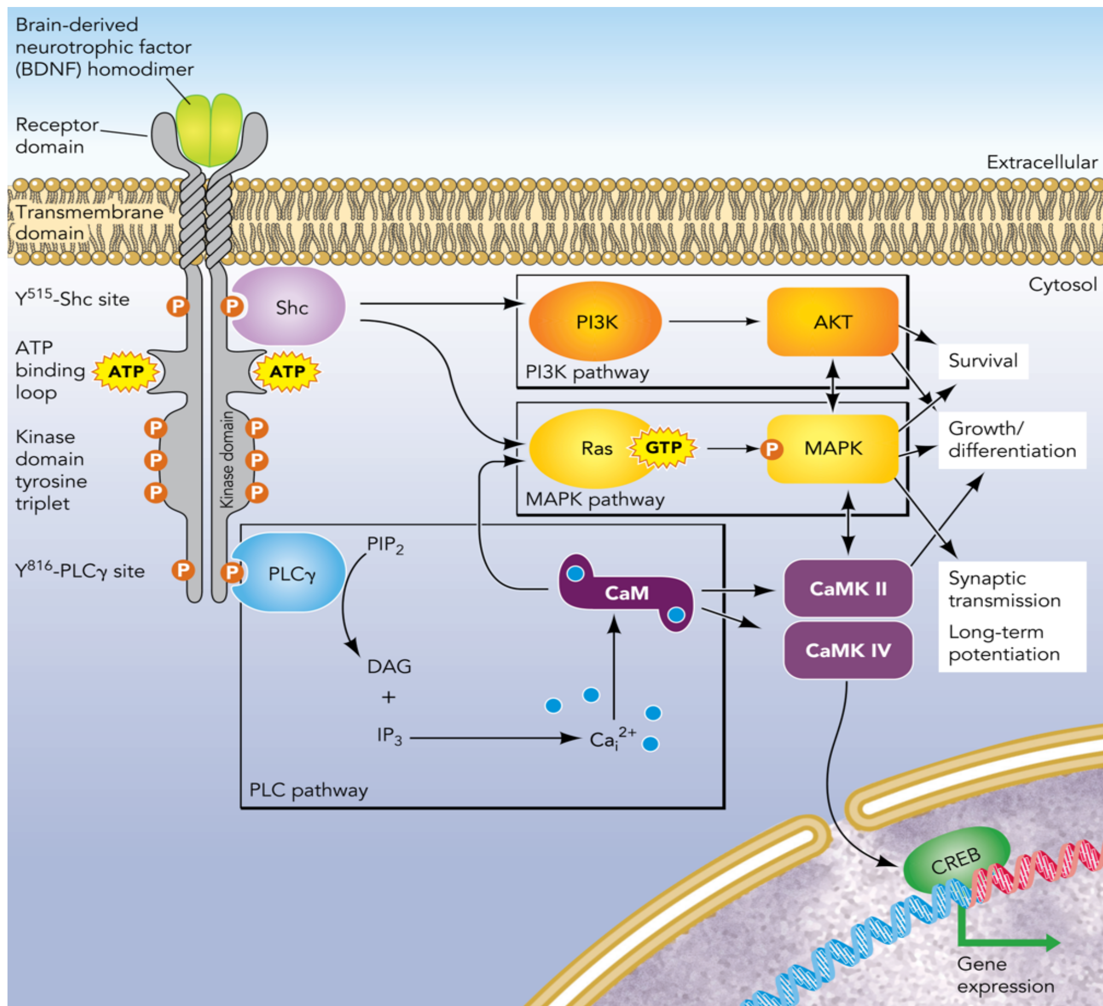


Figure 1.6 - Main BDNF-TrkB signalling cascade

BDNF binds TrkB, causing the dimerization and autophosphorylation of TrkB receptor at intracellular sites. This triggers the recruitment of adaptor proteins (including Shc), which activate the Ras-MAPK/ERK pathway and the PI3K-Akt pathway. The Ras-MAPK/ERK pathway is involved in plasticity and primarily regulating neuronal growth, differentiation and survival by activating nuclear transcription factors for genes. The PI3K-Akt pathway primarily controls neuronal survival. BDNF binding TrkB receptor can also phosphorylate different intracellular sites and activate the PLCγ-CaMKII pathway. PLCγ triggers increase in intracellular Ca^{2+} concentration and subsequent activation of Ca/Calmodulin-dependent kinase (CaMKII). The PLCγ-CaMKII pathway is involved in neuronal survival, differentiation, and gene expression as well as modulating synaptic plasticity, regulating synaptic transmission and LTP (source: Blum and Konnerth, 2005).

1.6.2.1 Ras-MAPK/ERK pathway

The activation of the Ras-MAPK/ERK pathway is initiated by BDNF binding the TrkB receptor, triggering the autophosphorilation of the tyrosine residue 515 (Y515), opening a binding site and recruiting the protein adaptor Src homology (Shc). This leads to the activation of the growth factor receptor-bound protein/sons of sevenless (Grb2/SOS), which will phosphorylate Ras. Subsequently, Ras phosphorylates and activates Ras-MAPK, which activates MEK (mitogen activated protein kinase kinase). Then, MEK activation leads to the phosphorylation of several kinase and transcription factor , such as ERK 1 and 2, calcium/calmodulin-dependent protein kinase-2 (CaMKII) and CREB (Figure 1.6).

Extracellular signal-related kinase (ERK) is a member of the MAPK family, a family of enzymes that relay signals into the cell by phosphorylation. There are two isoforms of Erk, the Erk1 (p44 MAPK) and Erk2 (p42 MAPK), and both are known to be involved in the regulation of cell proliferation, in response to growth factors, and to control cell cycle progression (Miyasaka et al., 2015). Over the years, evidence has demonstrated that the ERK pathway is essential to synaptic plasticity and learning and memory (Peng et al., 1995, Davis and Laroche, 2006). Furthermore, ERK activation has also been shown to upregulate BDNF, indicating that BDNF signalling can regulate its own transcription (Saarelainen et al., 2001).

1.6.2.2 The PI3K-Akt pathway

Another signalling pathway triggered by BDNF-TrkB binding is the PI3K-Akt pathway. The activation of Shc and Grb2 complex can also lead to the activation of PI3K-Akt pathway, through the recruitment of Grb2-associated binder-1 (GAB1). PI3K is a kinase which changes the composition of phospholipids in the membrane by phosphorylating membrane-bound phosphatidylinositol biphosphate (PIP_2), transforming it into phosphatidylinositol triphosphate (PIP_3). In the phosphorylated state, PIP_3 can activate phosphoinositide- dependent kinase-1 (PDK1), resulting in the translocation and activation of Akt (Figure 1.6).

The Pi3K-Akt pathway has been found to play an important role regulating cell proliferation, differentiation and survival (Zhao et al., 2006, Peltier et al., 2007) and a role for this pathway has been suggested in hippocampal synaptogenesis (Cuesto et al., 2011).

1.6.2.3 The PLCγ-CaMKII pathway

The third pathway activated by the biding of BDNF to TrkB receptor is the PLCγ-CaMKII pathway (Figure 1.6). Phosphorylation of TrkB at the tyrosine residue 816 (Y816) activates PLCγ, which hydrolyses phosphatidylinositol 4, 5-bisphosphate (PIP_2) to produce inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 initiates the release of intracellular stores of Ca^{2+} through IP_3-dependent channels in the endoplasmic reticulum (ER) membrane, leading to an increase of free intracellular Ca^{2+} in the cell. An increase in intracellular Ca^{2+} levels leads to the activation of Ca^{2+}-dependent enzymes, such as CaMKII. This enzyme is able to phosphorylate several proteins associated with synaptic transmission, such as NMDA receptors and the features of this kinase are crucial for its role in plasticity and memory formation (Wayman et al., 2008).

Alternatively, DAG activates protein kinase C (PKC), which also increase intracellular Ca^{2+} release. This additional Ca^{2+} also activates ERK and consequently CREB, upregulating the transcription of genes associated with cell survival. The PKC-dependent pathway is reported to enhance synaptic plasticity (Reichardt, 2003).

1.6.3 Nerve growth factor (NGF)

Similar to BDNF, NGF is known to promote cell proliferation, differentiation and survival and was the first neurotrophin to be discovered (Levi-Montalcini and Hamburger, 1951, Kaplan and Stephens, 1994). As mentioned before, NGF binds TrkA receptor with high affinity and it can also activate the ERK pathway (Wang et al., 2009). Expression of TrkA receptor is highly observed in axons of NGF-depended cholinergic neurons (Cellerino and Maffei, 1996) making NGF activity preferentially restricted to cholinergic neurons of the forebrain, which are well

documented to be involved in cognitive function (Chen et al., 1997). In this context, NGF has been demonstrated to be an important candidate in the therapy of AD, since loss of cholinergic neuros is a feature of this disease (Hefti et al., 1986, Tuszynski et al., 2002). Indeed, NGF has been a target in a gene therapy pre-clinical trial in AD patients. It has been shown in this pre-clinical trial, that NGF gene therapy administered to patients with AD results in classic trophic responses, characterized by axonal sprouting in all patients examined, with no adverse effects (Tuszynski et al., 2015).

In the hippocampus, basal NGF concentration is very low (Narisawa-Saito et al., 1996), however, activity-dependent secretion of NGF has been reported (McAllister et al., 1999) as well as its role in learning and memory (Woolf et al., 2001, Conner et al., 2009). Also, exercise has been shown to increase hippocampal levels of NGF, promoting enhancement of cognitive function (Chae et al., 2009).

1.6.4 Insulin-like growth factor 1 (Igf-1)

As mentioned before, apart from neurotrophins, other growth factors play an important role in the promotion of cell proliferation and survival, such as the Igf-1 (Kalluri and Dempsey, 2011). The Igfs are members of a family of insulin related peptides and two different subtypes, Igf-1 and Igf-2, have been described. Over the years, Igf-1 has been widely studied due to the anabolic and mitogenic effect of this growth hormone. Circulating Igf-1 is produced by the liver (Butler and LeRoith, 2001) and can cross the blood-brain-barrier using a transport system (Pan and Kastin, 2000). In the brain, Igf-1 acts by binding the Igf1R (member of tyrosine kinase receptor family) activating similar cascades to those induced by BDNF, including the mentioned above, PI3K-Akt pathway and Ras-MAPK pathway (LeRoith et al., 1995).

Although the mechanisms underlying the effects of Igf-1 on cell proliferation are still poor understood, they seem to be mediated by Akt activation. The treatment of NSCs with Igf-1 increased phosphorylation of Akt but not ERK, and in addition, the inhibition of Akt abolished Igf-1-induced cell survival, suggesting a major

involvement of Akt pathway in Igf-1-induced cell growth (Kalluri et al., 2007). Igf-1 has been demonstrated to be induced by exercise and linked to positive effects on cognition (Ding et al., 2006, Trejo et al., 2008). Decreased levels of circulating Igf-1, repress exercise-induced effects on hippocampal neurogenesis, indicating that Igf-1 uptake from the brain is necessary to mediate exercise-induced neurogenic effect (Trejo et al., 2001). Also, there is evidence showing circulating Igf-1 can upregulate central levels of BDNF (Carro et al., 2000) and Igf-1 interacting with VEGF and mediating angiogenesis (Cotman et al., 2007).

1.6.5 Vascular endothelial growth factor (VEGF)

The growth factor VEGF is a specific mitogen for vascular endothelial cells. Studies have demonstrated VEGF as a prime regulator of angiogenesis and vasculogenesis (Ferrara and Bunting, 1996, Wood et al., 2006). The VEGF family of genes contains at least 7 members, however, the binding to VEGF-1 and VEGF-2 receptors is required to initiate the major roles of VEGF in physiological and pathological angiogenesis (Muller et al., 1997).

Exercise is known to induce vascular adaptations, increase cerebral blood flow and increase angiogenesis (Leosco et al., 2007, Ainslie and Ogoh, 2010). In this context, exercise has been demonstrated to induce peripheral and central increases in VEGF levels, suggesting that these changes in vasculature induced by exercise might be associated with VEGF (Prior et al., 2004, Tang et al., 2010). Increased blood flow into the brain, enhances oxygenation, contributing to enhanced neuronal function. It has been shown that cognitive decline in age is associated with decreased brain perfusion (Brown and Thore, 2011). Recent studies have demonstrated that VEGF plays an important role stimulating neurogenesis and neuronal plasticity (Licht and Keshet, 2013, Kirby et al., 2015) and depletion of VEGFR causes impairment in neurogenesis, which is rescued by exercise (Han et al., 2015). Moreover, adult neurogenesis occurs in parallel with the generation and growth of new blood vessels, which can provide an “angiogenic niche” for the neuronal progenitors and VEGF can contribute in this process (Louissaint et al., 2002).

Taken together, these findings suggest exercise-induced changes in brain vasculature, with consequent improvements in neurogenesis and cognition, could be modulated by VEGF.

1.6.6 Glial cell line-derived neurotrophic factor (GDNF)

GDNF, a member of the transforming growth factor-β family, is another potent neurotrophic factor known to play a role in cell survival (Feng et al., 1999). GDNF was first described to promote the survival of the embryonic dopaminergic neurons of the midbrain (Lin et al., 1993) and later, it was shown to be a potent trophic factor for spinal motorneurons and central noradrenergic neurons (Henderson et al., 1994, Arenas et al., 1995). This trophic factor acts by binding the RET receptor tyrosine kinase, activating similar signalling cascades to BDNF and regulating cell growth and survival.

GDNF expression is widely described in the peripheral and central nervous system, mainly in neurons and astrocytes (Henderson et al., 1994, Moretto et al., 1996, Springer et al., 1995, Suzuki et al., 1998). In the brain, GDNF has been demonstrated to promote hippocampal neurogenesis (Chen et al., 2005), to be neuroprotective and rescue cognitive impairment after stroke (Mokhtari et al., 2017) and it has been used in pre-clinical trials for the treatment of Parkinson's disease (PD) (Patel et al., 2005), GDNF has also been reported to have anti-inflammatory effects, protecting midbrain dopaminergic neurons against lipopolysaccharide toxicity (Xing et al., 2010).

While the effects of exercise on cognition mediated by BDNF, Igf-1, NGF and VEGF have been widely investigated, the contribution of GDNF to exercise-induced enhancements in memory are still poorly studied. The majority of the studies have described an increase in GDNF expression induced by exercise in muscles and spinal cord (Wehrwein et al., 2002, McCullough et al., 2013). However, a link between exercise-induced changes in GDNF expression in the brain and the cognitive effects of this trophic factor has not been established yet.

1.6.7 Neurotrophins, growth factors and ageing

Neurotrophic factor signalling is severely affected due to ageing and AD, and usually associated with cognitive decline. Indeed, age-related change in the expression of neurotrophins such as NGF and BDNF, which are crucial to the neuroplasticity process and play an important role in the cognitive function (Mora et al., 2007, Mattson et al., 2004), have been widely reported. Studies in humans, suggest BDNF concentration as an indicator of impaired memory and general cognitive function in aging women (Komulainen et al., 2008) and have linked decreased levels of serum BDNF with age-related reduction of hippocampal volume (Erickson et al., 2010). Also, animal studies have shown age-related reduction in hippocampal BDNF concentration associated with delayed development and decreased activity of the granule cell of hippocampus (Trinchero et al., 2017). Further, chronic BDNF deficiency has been associated with age-dependent impairments in spatial learning and memory (Petzold et al., 2015).

Regarding age-related changes in NGF, studies have suggested that decline in NGF release and tyrosine receptor kinase signalling, contribute to age-related deficits in long-term potentiation in rats (Kelly and Lynch, 2000). Also, decreased hippocampal concentration of NGF is linked with poor performance in spatial learning and memory task (Henriksson et al., 1992), whereas chronic administration of NGF has been demonstrated to rescue age-related impairments in LTP and spatial memory (Bergado et al., 1997).

As mentioned before, pro-neurotrophins bind with greater affinity to the p75 receptor and evidence has suggested that negative effects of pro-NGF on cell proliferation and differentiation might be mediated by activation of p75 receptor (Wang et al., 2010). Interestingly, studies have shown an imbalance of proNGF/NGF in ageing and age-related disease. Increased levels of pro-NGF have been reported in the cerebrospinal fluid (CSF) and in the brain of MCI and AD patients (Fahnestock et al., 2001, Counts et al., 2016). Also, increase in pro-NGF concentration has been observed in the brain of transgenic models of AD, contributing to neurodegeneration and cognitive impairment (Tiveron et al., 2013),

and in the hippocampus of aged rats, associated with cell death (Al-Shawi et al., 2008).

Expression of other growth factors are also reported to be altered with ageing, including Igf-1, VEGF and GDNF. Decrease in Igf-1 expression in the aged brain has been reported as well as a reduction on the density of Igf type 1 receptors. These changes in Igf-1 expression have been linked to age-related cognitive impairment and suggested as a risk factor for a number of age-related disease (Sonntag et al., 2000). In addition, decrease in VEGF has also been associated with age-related disease, such as AD. For instance, patients with AD have lower levels of serum VEGF and it is correlated with decline in cognitive function and brain atrophy (Mateo et al., 2007, Hohman et al., 2015). VEGF administration in APP transgenic mice results in increased hippocampal angiogenesis, reduced memory impairment, and reduced β-amyloid (Aβ) deposition (Wang et al., 2011).

Finally, changes in GDNF expression have also been reported in ageing, although the exact mechanisms by which GDNF may modulate age-related cognitive deterioration have not been well documented. GDNF has been shown to rescue age-related decline in spatial learning and memory in rats and to be a potential therapeutic option for neurodegenerative disease, including PD (Slevin et al., 2005, Pertusa et al., 2008). Taken together, these data strongly support the association between age-related changes in neurotrophins and growth factors and age-related structural, metabolic, and functional brain changes.

1.7 Inflammation

Inflammation can be defined as a complex immunological response to an external stimulus, e.g. pathogen or internal signal, e.g. damaged tissue, characterised by acute and chronic adaptations. The acute inflammatory response induces changes in the local vascularity, leading to an increase in blood flow to the affected region and recruitment of the innate immune system to repair the damaged area (Majno and Joris, 2004, Soehnlein and Lindbom, 2010). However, if an acute inflammatory response persists it acquires new features and a chronic inflammatory state is initiated, characterised by activation of lymphocytes and adaptive immune

responses. Although inflammation is crucial for tissue repair, unresolved chronic inflammation can be detrimental (Chen and Nunez, 2010).

1.7.1 The immune response

The inflammatory response is coordinated by several mediators and a complex regulatory system. The first step of the inflammatory response involves the recognition of the source of infection or damage. This recognition, by the immune system, is initiated with the detection of pathogen-associated molecular patterns (PAMPs - molecules expressed in pathogens) and/or detection of damage-associated molecular patterns (DAMPs - molecules released following tissue injury or cell death) (Takeuchi and Akira, 2010). These molecules trigger, in the surface of immune cells and intracellularly, the activation of pattern-recognition receptors (PRRs), such as Toll-like receptor (TLR) and the nucleotide oligomerization domain-like receptors (NLRs), respectively (Barton, 2008). The activation of TLRs initiate intracellular signalling culminating in the activation of nuclear factor kappa b (NF-κB), MAPK and interferon regulatory factor (IRFs). Activated NF-κB is translocated to the nucleus and upregulates the transcription of inflammatory mediators, such as inactive IL-1B (pro-IL-1β) and IL-18 (pro-IL-18). NLRs are inflammasome sensor molecules connected to caspase-1 via an adaptor protein, and when activated, convert pro-inflammatory inactive cytokines into active forms (Takeuchi and Akira, 2010).

Together, these signalling pathways and the increase in gene transcription start the inflammatory cascade, which is characterised by upregulated expression of inflammatory mediators, such as cytokines (IL-1β, IL-6, TNF-α and others) and chemokines, facilitating the recruitment of more effector cells, such as neutrophils and macrophages, to the inflammation zone. While neutrophils are responsible for the production of reactive oxygen and nitrogen species (ROS and RNS) and proteases, in order to achieve resolution of inflammation, macrophages release histamine, leukotrienes, and prostaglandins, which have rapid effects on the vasculature, including vasodilation and increased vascular permeability, resulting in recruitment of more immune cells (Sherwood and Toliver-Kinsky, 2004).

As mentioned before, if the acute inflammatory response fails to restore tissue homeostasis, this causes an exacerbation of responses, initiating a chronic inflammatory response. The beginning of chronic inflammation is mainly characterized by the replacement of recruited neutrophils with macrophages and other immune cells, such as T cells (in case of infection). In this state, in a final attempt to restore the affected tissue, granulomas are typically formed and also, the persistent chronic inflammation state leads to an increase in cellular turnover (Murakami and Hirano, 2012). Chronic inflammation is associated with several cardiovascular, metabolic and neurodegenerative diseases. Also, it has been demonstrated that increased inflammation in early life can accelerate ageing. Although the pattern of transition from acute inflammation to chronic inflammation is known, the exact mechanisms underlying the chronic inflammatory states are still not well-established.

1.7.2 Inflammatory mediators

1.7.2.1 Nuclear factor-kappa B (NF-κB) signalling

The nuclear factor NF-κB pathway is considered an important regulator of inflammatory signalling pathway because of the ability of NF-κB to control the expression of pro inflammatory genes including cytokines, and chemokines (Lawrence, 2009). NF-κB is present in a inactivated form in the cell cytoplasm, associated with regulatory proteins called inhibitors of κB (IκB). An important step for NF-κB activation is the phosphorylation of IκB, which is mediated by IκB kinase domain (IKK). The release of NF-κB from IκB causes the translocation of NF-κB to the nucleus to upregulate the transcription of inflammatory gene.

Rodents and humans studies have demonstrated that activation of NF-κB increases the expression of adhesion molecules (Chen et al., 1995) and it can also initiate the induction of apoptosis in some cell types (Chen et al., 1999). Moreover, NF-κB is undoubtedly one of the central regulators of pro inflammatory gene transcription, mediating the expression of cytokines, such as IL-1β, IL-6, and TNF-α (Tak and Firestein, 2001). However, some evidence also demonstrates that NF-

κB can play a role in the control of the inflammatory response, affecting the magnitude and duration of this response (Bohuslav et al., 1998, Greten et al., 2007).

1.7.2.2 Cytokines

Cytokines are members of a large group of proteins with the ability to provide communication between cells and to orchestrate complex cellular adaptations (Kelso, 1998). This family includes the interferons, the interleukins, the chemokine family and the tumour necrosis factor family. These cells share a common characteristic, their pleiotropic effect, which means that a given cytokine can trigger different and opposite cascades dependent on the cell type the cytokine is targeting and the conjunct of signals and other cytokines acting in the cell environment (Veldhoen et al., 2006, Becher et al., 2017). Regarding the pleiotropic nature of these cells, cytokines can be divided by their main function as pro-inflammatory: IL-1β, interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ); or anti-inflammatory cytokines: interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-13 (IL-13) and interferon-α (IFN-α).

Cytokines affect several biological and pathological processes, including embryonic development, diseases, inflammatory response, changes in cognitive functions and progression of the degenerative processes of aging (Foster, 2001). The inflammatory response is characterized by an interplay between pro- and anti-inflammatory cytokines and exacerbated cytokine production may contribute to deleterious consequences of severe and persistent inflammation. IL-1β and TNF-α are potent pro-inflammatory cytokines and are crucial for responses against infection and they are also associated with exacerbated damage during chronic disease and acute tissue injury (Dinarello, 1996, Sedger and McDermott, 2014). The binding of IL-1β to its receptor IL- 1RI triggers signalling pathways such as MAPK (Ridley et al., 1997) and activation of NF-κB, which are important in cytokine induction and cell survival (Basu et al., 1998). TNF-α binds TNF receptor-1 initiating a downstream signalling cascade, which in turn also activate transcription

factors such as NF-κB and cause further upregulation in inflammation, apoptosis, and neurodegeneration (Hohmann et al., 1990).

IL-6 has well known context-dependent pro- and anti-inflammatory effects. The pleiotropic nature of IL-6 has given this cytokine a hormone-like attribution that is known to affect several mechanisms, such as insulin resistance, mitochondrial activities, and neuropsychological behaviour (Bethin et al., 2000, Hodes et al., 2014). On the other hand, Interleukin 10 (IL-10) is a cytokine with strong anti-inflammatory properties and it plays a central role in limiting the inflammatory response, thereby maintaining normal tissue homeostasis (Sabat et al., 2010). IL-4 has also been demonstrated to have a potent pleiotropic effect and studies have shown that IL-4 can bind different cell-surface receptors inducing functionally different intracellular signalling (Luzina et al., 2012). However, it has been demonstrated that IL-4 can have anti-inflammatory effects with beneficial effects in several functions of the normal brain, such as memory and learning (Derecki et al., 2011).

1.7.2.3 Chemokines

Chemokines are members of the large cytokine family and also play an important role in signalling inflammation. Chemokines are critical in controlling the recruitment and migration of immune cells. They can be categorised in four families as well as in two types: inflammatory chemokines (recruits leukocytes in response to physiological misbalance, and homeostatic chemokines (controls the basal leukocyte trafficking) (Gerard and Rollins, 2001, Moser and Loetscher, 2001).

Among the different families and types of chemokines, the Cx3cl1 (fractalkine), has unique characteristics and has been found to play an important role in inflammation. Cx3cl1 acts by binding a specific receptor, the Cx3cr1, which in the periphery is highly expressed in lymphocytes, such as natural killer cells and in monocytes during inflammatory process (Umehara et al., 2004). The presence of fractalkine (Cx3cl1) and its receptor Cx3cr1 in lymphocytes and their enhanced expression during the inflammatory response suggest that Cx3cl1-Cx3cr1 plays a critical role in the mechanism of cell adhesion in immune-related inflammatory

response. In the CNS, Cx3cl1-Cx3cr1 also has been demonstrated to be important in regulating inflammation. It has been shown that fractalkine (Cx3cl1) signalling, though Cx3cr1, determines microglial migration during development and regulates microglia neurotoxicity under inflammatory conditions (Mizutani et al., 2012, Limatola and Ransohoff, 2014).

1.7.2.4 Inducible nitric oxide synthase (iNOS)

The production of nitrite- and nitrate- generating compounds by mammalian cells was first demonstrated in the mouse macrophage, and since then, the production of nitric oxide (NO) has been show to play a role in the inflammatory response (Coleman, 2001). The main source of production of NO is the L-arginase-nitric-oxide pathway, through the action of the enzymes nitric oxide synthase (NOS). There are three NOS isoforms, the neuronal-NOS (nNOS), the endothelial-NOS (eNOS) and the inducible-NOS (iNOS). Typically, iNOS is not expressed in resting cells, this isoform is induced by certain cytokines or microbial products. For this reason, during the inflammatory response, iNOS is the enzyme primarily responsible for the production of NO.

Studies have demonstrated that pro- and anti-inflammatory cytokines can affect the expression of iNOS and consequently, regulate the production of NO. iNOS has been demonstrated to be highly expressed upon activation of NF-κB in response to several stimuli, including pro-inflammatory cytokines, such as TNF-α, IFN-γ and IL-1β, and lipopolysaccharide (LPS) (Kanno et al., 1994, Xie et al., 1994). On the other hand, anti-inflammatory cytokines, such as IL-4 and IL-10 have been shown to down-regulate the expression of iNOS in activated macrophages (Howard et al., 2010, Bogdan et al., 1994).

Production of NO is important to eliminate pathogens in the inflammatory response. However, if NO production is sustained and excessive, which is associated with increase expression of iNOS, it can contribute to the tissue damage observed in chronic inflammation (Zamora et al., 2005). Indeed, increased expression of iNOS has been reported in several chronic inflammatory disease, including arthritis,

inflammatory bowel disease as well as AD (Fletcher et al., 1998, Vodovotz et al., 1996, Perner and Rask-Madsen, 1999).

1.7.2.5 Cell surface adhesion molecule CD44

As mentioned above, one of the first steps in the classical inflammatory response involves the recruitment of immune cells to the affected zone. This process is typically mediated by the selectin and integrin molecules; however, infiltration of immune cells can also be mediated by CD44. CD44 is a widely-expressed family of adhesion receptors and its principal ligand is the hyaluronan (HA), which is a major component of the extracellular matrix and responsible for regulating tissue repair during the inflammatory response. Studies have demonstrated that HA levels are increased in the inflammation site, and CD44 has been shown to be important for the resolution of inflammation (Petrey and de la Motte, 2014).

Also, CD44 has been shown to be involved in cell adhesion, migration and signalling (Dzwonek and Wilczynski, 2015). Within the peripheral immune system, CD44 is expressed on several cells including hematopoietic stem cells, monocytes and macrophages, neutrophils and lymphocytes, whereas in the CNS CD44 is expressed in both glial and neuronal cells. Emerging evidence demonstrated CD44 to be involved in many physiological and pathological processes in the CNS, including neuronal development, synaptic plasticity, seizures, brain injuries and neurodegeneration (Kochlamazashvili et al., 2010, Roszkowska et al., 2016).

1.7.3 Apoptosis and inflammation

Apoptosis is the process of programmed cell death, regulated by cellular mechanisms and important for the removal of unnecessary, aged or damaged cells (Elmore, 2007). Apoptosis is crucial in several physiologic processes, including cell turnover, embryonic development and proper functioning of the immune system. The caspases, a group of proteases, coordinate and execute the process of apoptosis. These caspases can be activated by different pathways, including the activation of death receptor pathway and the intracellular stress signal pathway. Specifically, activation of caspases through intracellular stress pathways (initiated

by cellular and mitochondrial damage) is directly mediated by proteins of the Bcl-2 family. This family includes pro (Bax and Bak) and anti-apoptotic (Bcl-2, Bcl-xL and Bcl-w) proteins. It has been demonstrated that Bcl-2 can prevent cytochrome *c* release from damaged mitochondria by blocking the action of Bax, and consequently preventing Caspase-9 activation (Cory and Adams, 2002).

Studies have demonstrated that many signalling cascades and molecules activated by inflammation are involved in the regulation of cell apoptosis. For instance, TNF-α can activate death receptors triggering the recruitment of caspase-8 and caspase-3, in neutrophils (Takeda et al., 1993). In addition, inappropriate (too much or too little) apoptosis is associated with several conditions including cancer, stroke and neurodegenerative diseases (Kerr et al., 1994, Norbury and Hickson, 2001, Ethell and Buhler, 2003, Hochhauser et al., 2003).

1.7.4 Neuroinflammation

It is known that the brain is "protected" from infiltration of circulating substances from the peripheral vasculature by the presence of the blood-brain barrier (BBB). The BBB is a complex and tight junction of vessels lined with specialised endothelial cells, astrocytes, pericytes and neuronal terminations that controls the movement of molecules between the CNS and periphery (Abbott et al., 2006). Under normal conditions, with the exception of tiny and/or fat-soluble molecules, only a few molecules can access the brain from the periphery. However, dysfunction in BBB permeability is reported as a cause or a consequence in several conditions, including multiple sclerosis (MS), stroke, epilepsy, traumatic brain injuries and AD and in most of these cases, BBB permeability is affected as a result of the inflammation or degenerative processes specific to each pathology (Daneman and Prat, 2015).

The BBB is the first layer of protection for the CNS followed by its own resident population of immune cells. These cells are glial cells (microglia, astrocytes, and oligodendrocytes) and they work in conjunct, as a secondary barrier in the CNS, as the neutrophils and macrophages work in the periphery. In the brain, the primary response to several types of CNS insults with cellular damage (injuries and

neurodegenerative) is the activation of resident immune cells (microglia and astrocytes) and the release of inflammatory mediators, including cytokines and chemokines from these cells and limited participation of peripheral immune cells (Kreutzberg, 1996). However, in case of CNS acute inflammation and chronic inflammatory disease, increased infiltration of peripheral immune cells to the CNS is driven and leucocytes have been demonstrated to be a major source of cytokines production (Kelso, 1998).

The balance of cytokines within the CNS is important in several physiological process, however the disturbance of that system can result in tissue dysfunction and degeneration. It has been demonstrated that resident immune cells of CNS (microglia and astrocytes) release, upon activation, early pro-inflammatory mediators, such as IL-1β, IL-6, and TNF-α in response to cellular abnormality and neurodegeneration, in an effort to counteract damage (Becher et al., 2017). On the other hand, studies have shown that depletion of IL-1β and TNF-α in a mouse model of AD protected against neurodegeneration and cognitive impairment (He et al., 2007, Kitazawa et al., 2011), demonstrating the pivotal role of microglia and astrocytes in CNS inflammatory response.

1.7.4.1 Microglia

Under normal conditions, microglia cells exist primarily in a state of surveillance in the CNS and their quiescent role is the maintenance of homeostasis within brain environment (Derecki et al., 2013). In this state, microglia are known to express reduced levels of major histocompatibility complex-II (MCH-II) and cluster of differentiation molecule 11b (CD11b). Also, maintenance of microglia in a relatively quiescent state is attributed, in part, to astrocyte and neuronal activity (Ransohoff and Cardona, 2010), for example, neurons have been shown to accomplish this by secreting signal factors, including CD200, Cx3cl1 and neurotrophins (Sunnemark et al., 2005, Lyons et al., 2007).

Microglia share phenotypic characteristics with peripheral monocytes cells and, during injury to the CNS, resident microglia are polarized towards a pro-inflammatory phenotype (M1 state), induced mainly by the exposure to pro-

inflammatory cytokines, such as IFN-γ, TNF-α and cellular or microbial debris. The M1 state is characterised by production of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 and increased expression of iNOS, inducing elevated production of NO and morphological change of microglia to an amoeboid shape (Liu and Hong, 2003, Bernardino et al., 2005). In addition, the presence of ionized calcium-binding adapter molecule-1 (Iba1) has been found to distinguish between the morphological changes of microglia (Deininger et al., 2002).

However, in an effort at neuroprotection and repair, microglia can assume an 'alternative' activation, featured by an anti-inflammatory phenotype (M2 state). The M2 activation can be driven by anti-inflammatory cytokines, such as IL-4, IL-13 and IL-10 (Stein et al., 1992) and is characterised by increased production of anti-inflammatory cytokines, including IL-4, IL-10, IL-13, as well as arginase-1 (Arg-1), chitinase-3-like-3 (Ym1, in rodents) and mannose receptor C (Mrc1) (Ajmone-Cat et al., 2016). Microglia activated towards the M2 state can also trigger inflammation resolution through the release of other anti-inflammatory factors, such as neurotrophins (BDNF) and growth factors (Igf-1 and transforming growth factor beta -TGF-β) (Ryu et al., 2012, Gomes et al., 2013). Although in macrophages these states are termed M1 and M2 respectively, and while this nomenclature has been applied extensively in the literature to microglia, it is likely that a variety of microglial phenotypes exist, especially in vivo (Ransohoff, 2016). Studies have demonstrated the involvement of microglial activation in the pathophysiology of several neurodegenerative diseases, such as AD and PD, mainly by increasing neurotoxicity and cellular damage, thereby contributing to the degenerative process (Figure 1.7) (Liu and Hong, 2003).

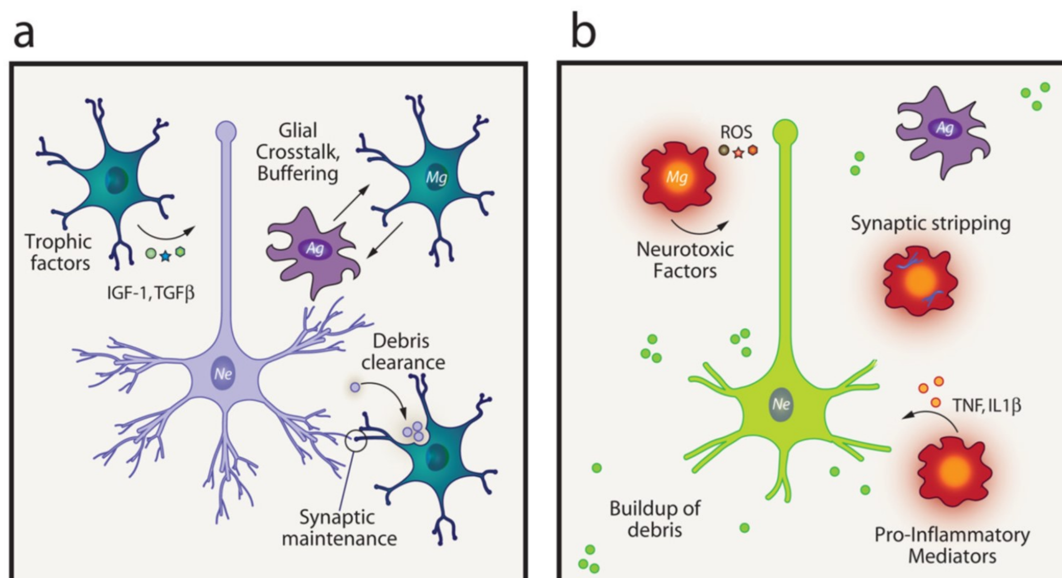


Figure 1.7 - Microglia phenotype

(a) Microglia in quiescent state maintain homeostasis in the CNS, performing functions including phagocytosis of neuronal debris, secretion of trophic factors and synaptic maintenance. (b) In response to injury or infection. Microglia can be activated towards a M1 state, assuming an amoeboid shape, and secrete pro-inflammatory factors and reactive oxygen species (Derecki et al., 2013).

1.7.4.2 Astrocytes

In the CNS, astrocytes have important roles in providing structural and functional support to neurons, constituting BBB formation and participating in synaptic formation (Perea et al., 2009). Among these astrocyte functions, it also has been demonstrated that they are involved in the inflammatory response in the CNS (Sofroniew and Vinters, 2010). Similar to microglia cells, astrocytes can be activated and release pro- and anti-inflammatory mediators, contributing to the inflammatory response. Most recently, it became accepted that astrocytes can also assume the classical and alternative phenotype activation (A1 and A2 states) (Jang et al., 2013, Roybon et al., 2013). However, in this study the markers used to assess astrocytic activation are not the typical A1 and A2 markers reported previously (Liddelow et al., 2017).

It has been shown that astrocytes can become reactive (reactive astrogliosis) in the presence of pro-inflammatory factors, such as TNF-α, IL-1β and IFN-γ in response to insults and pathologies. Astrocyte reactivity leads to production of several pro- and anti-inflammatory factors, including chemokines, cytokines and growth factors and is characterised by increased expression of GFAP (Allaman et al., 2011). Indeed, astrocytes have been shown to be an important source of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 as well as iNOS, when activated in the inflammatory response (Jang et al., 2013, Rothhammer and Quintana, 2015).

On the other hand, when activated, astrocytes can also help in tissue repair by releasing IL-10, which has been reported to supress neuronal apoptosis through TLR/NF-κB pathway activation in a rat model of hypoxic-ischemic brain damage (Park et al., 2006a, He et al., 2017a). Also, astrocytes exposed to IL-4 and IL-10, show typical “alternative” activation, increasing expression of Arg-1, Mrc1 and Ym1 (Jang et al., 2013). In addition, astrocyte reactivity has been associated with several neurodegenerative diseases, including HD, PD and AD (Bradford et al., 2009, Allaman et al., 2011, Halliday and Stevens, 2011) and most recently, it was suggested that the normal process of ageing induces astrocytes to an A1-like astrocyte reactivity, with pro-inflammatory features (Clarke et al., 2018).

1.7.5 Peripheral inflammation and neuroinflammation

During an inflammatory response, pro-inflammatory cytokines, such as IL-1β and IL-6, produced in the periphery, can enter the circulation and be involved in signalling a systemic inflammatory response. It has been shown that systemic inflammation increases hepatic production and release of acute-phase proteins, including C-reactive protein (CRP) (Heinrich et al., 1990), which has been linked with an increased risk of cardiovascular diseases and cognitive impairment conditions, including dementia (Rosenson and Koenig, 2002, Mancinella et al., 2009)

Although the CNS has been considered an “immunologically privileged site”, over the past years, a growing body of evidences supports the existence of a

bidirectional crosstalk between the CNS and peripheral immune system. For example, studies have shown that systemic administration of LPS, IL-1β and TNF-α induce "sickness behaviour", which is accompanied by decrease in social behaviour and impairment in memory consolidation (Bluthe et al., 1994, Pugh et al., 1998). Cytokines have been shown to be important effectors in this crosstalk between CNS and periphery. It has been demonstrated that peripheral cytokines can alter activity in the vagus nerve, activating CNS inflammatory response (Tracey, 2002) as well as stimulate vascular endothelial cells to produce prostaglandin E_2 and consequently increase production of pro-inflammatory cytokines in the CNS (Ek et al., 2001). In addition, most recently, many studies suggested an association between alterations of gut microbiome and peripheral inflammation with neurodegenerative and neuropsychiatric disorders (Rhee et al., 2009, Clapp et al., 2017).

1.7.6 Models of neuroinflammation

Neuroinflammation is an important feature of several neurodegenerative diseases, including AD, Parkinson disease (PD), dementia and amyotrophic lateral sclerosis (ALS). For instance, studies in post-mortem brain samples of patients with neurodegenerative diseases, such as AD and dementia, have demonstrated increased activation of microglia, astrocytes and elevated mRNA levels of pro-inflammatory cytokines (Luterman et al., 2000). Based on these findings, targeting inflammation might be an important approach to the development of strategies to counteract neurodegenerative diseases. Therefore, models of neuroinflammation have been widely used to investigate the mechanism of inflammation.

One of the most used animal models of peripheral inflammation-induced neuroinflammation and neurodegeneration is the LPS-challenge (Hoshino et al., 1999, Saban et al., 2001). LPS is a gram-negative endotoxin, found as a component of bacterial cell walls and it is a potent trigger of inflammation (Poltorak et al., 1998). Peripheral administration of LPS in mice has been investigated in different strains and doses (Cunningham et al., 2005, Godbout et al., 2005) and it results in induction of astrocytes and microglia activation, increase in peripheral and central levels of pro-inflammatory cytokines, iNOS expression, increased

apoptosis and impairment in cognitive function in several learning and memory tasks (Cunningham et al., 2005, Qin et al., 2007, Park et al., 2012).

1.7.7 Inflammation and ageing

Ageing is characterised by a chronic and low-grade inflammation usually referred to by the term “inflammaging” (Cevenini et al., 2010, Franceschi and Campisi, 2014). With ageing, there is a dysfunction and decline in several physiological functions and this could contribute to the features of inflammaging. It has been described that one important source of inflammaging could be the debris from damaged cells and macromolecules, which accumulate with age due to an imbalance between cell production and inadequate cell elimination. This debris can trigger an inflammatory response in order to repair and restore physiological homeostasis, however if the inflammatory response is sustained and persists, a chronic and unresolved inflammation is established, characterising inflammaging (Franceschi and Campisi, 2014).

Other factors associated with inflammaging could be the leakage of microbiota products from the gut and the changes in the microbiota due to ageing, cellular senescence (cellular response to damage and stress), immunosenescence and mitochondrial dysfunction (Franceschi and Campisi, 2014, Lynch et al., 2015). These factors together, contribute to trigger and sustain a low-grade inflammatory environment, which is associated with several age-related dysfunctions and impairments. As mentioned before, although the inflammation response is intended to be protective, an uncontrolled and unresolved response can contribute to tissue damage and pathologies (Lyman et al., 2014). Indeed, many age-related conditions share this common feature, the presence of low-grade inflammation, including arthritis, atherosclerosis, type-2 diabetes, PD, and AD (Frnceschi and Campisi, 2014).

In addition, an exacerbated immune response has been reported in the aged brain of rodent models in association with decline in hippocampal neurogenesis and cognitive impairment (Kohman and Rhodes, 2013). An exaggerated inflammatory response has also been shown to impair cognitive function in mice overexpressing

IL1β, and in LPS-injected rats (Moore et al., 2009, Rosi et al., 2006), suggesting that inflammaging plays a central role in the age-induced cognitive decline. Therefore, it can be noted that several brain structures and mechanisms seem to be vulnerable and affected during the ageing process, and inflammation makes an important contribution to this process. For this reason, understanding age-related changes and how these changes contribute to cognitive impairments in normal ageing is imperative for the development of strategies to promote healthier ageing.

1.8 Reserve Hypothesis

1.8.1 Reserve concept

Sometimes, even though morphological and physiological changes could be observed in the brain of aged individuals, they will not experience the cognitive decline associated with ageing. This divergence between cognitive performance and the level of brain changes in age and pathologies, such as Alzheimer's disease (AD), has been discussed and investigated. For instance, in 1989, a study reported that some people who had normal cognitive performance were discovered to present advanced AD pathology markers, in a post-mortem examination (Katzman et al., 1988). To explain this, the reserve hypothesis was proposed with the intention of explaining the frequent discrepancy between an individual's measured level of brain pathology (or age-related changes) and the functional and/or cognitive deficits that are expected to result from that pathology or from normal ageing (Stern, 2002, Barulli and Stern, 2013). This hypothesis suggests that the gap between the brain insult or age-related changes and behavioural manifestations is associated with individual differences in brain capability of reserve (Stern, 2002).

According to Stem (2002), capability reserve includes two types of reserves, which are cognitive reserve (CR) and brain reserve (BR) (Stern, 2002, Steffener and Stern, 2012). Brain reserve can be defined as the differences in brain size and other quantitative aspects of the brain, such as number of neurons, synapses and dendritic branching. These differences can explain the distinct susceptibility to functional impairment in the presence of neurological insult and/or normal ageing

(Stern, 2009). CR is referred as the individual variability to processing tasks. CR includes neural reserve and neural compensation (Figure 1.8).

The neural reserve can be considered as the ability to more efficiently use the pre-existing brain network in normal conditions. The neural compensation can be understood as the ability to recruit more resources to maintain the normal functioning in the presence of brain insult or normal ageing (Stern, 2009, Stern, 2006, Barulli and Stern, 2013). In this context, Stem (2002) suggests that CR refers to the ability to make a more efficient use of available BR when performing tasks (Stern, 2002). Therefore, as many of the factors associated with increased CR, such as stimulating experiences, have a direct effect on the brain, it has become clear that both BR and CR contribute to maintaining functionality and creating brain resilience in the presence of insults and neurodegeneration.

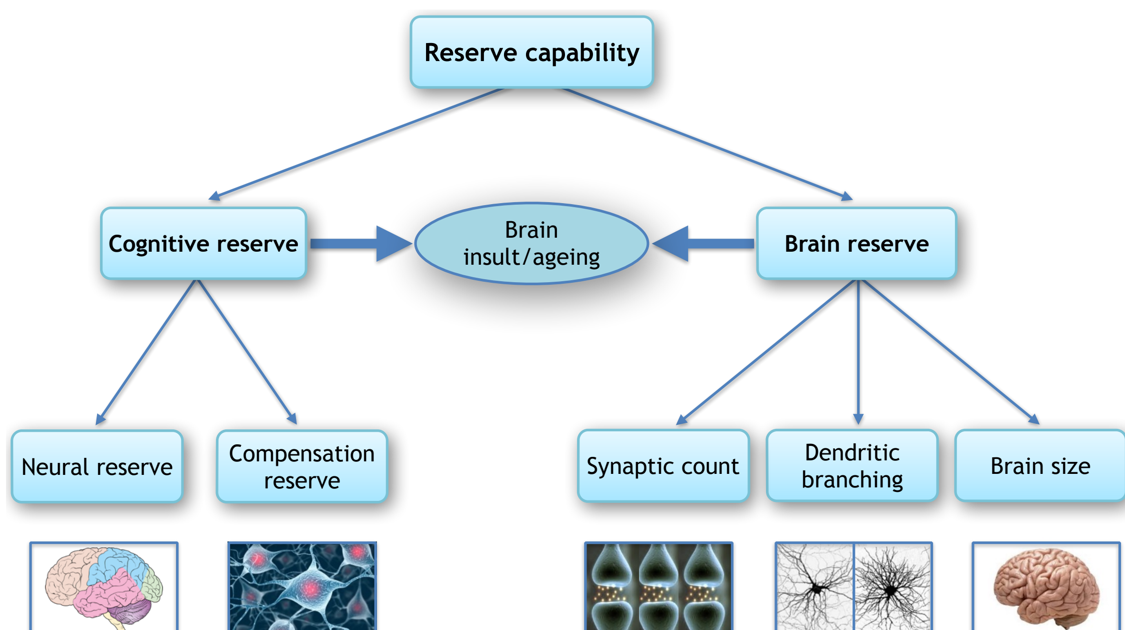


Figure 1.8 - Reserve hypotheses.
The reserve capability is composed of Cognitive reserve and Brain reserve, which have been shown to present an imperative role in maintaining cognitive deficits associated with ageing and brain insults (Adapted from (Xu et al., 2015).

1.8.2 Evidence for the reserve hypothesis

There is some evidence for the existence of this capability reserve and its role in maintaining cognition in normal ageing and in the presence of pathologies. The BR

can be estimated by anatomical measurements, such as, the counting of neurons and synapses, brain volume and dendritic branching (Satz et al., 2011). On the other hand, the CR cannot be estimated by quantitative measurement as the BR. However, CR can be estimated considering the lifetime experiences and cognitive activities, such as, years and level of education, measurement of IQ, types of leisure activities and socioeconomic status (Stern, 2002). For instance, bilingualism has been suggested to stimulate CR and contribute to delayed onset of AD (Gold et al., 2013). Moreover, other studies reported slower cognitive decline associated with normal ageing in individuals with higher educational level (Albert and Teresi, 1999, Chodosh et al., 2002). In addition, engagement in leisure activities has been associated with decreased risk of developing dementia, more successful aging, and reduced clinical manifestation in several other conditions, including traumatic brain injuries, PD and AD (Suchy et al., 2011, Fay et al., 2010, Poletti et al., 2011, Xu et al., 2015).

In this context, studies have suggested that BR and CR are overlapped by demonstrating the plasticity of the brain in response to life experience (van Praag et al., 1999, Barulli and Stern, 2013). Indeed, it has been shown that the quantitative brain substrates associated with BR can be modulated by external factors and life exposure, such as engagement in physical and social activities. For instance, a study in a mouse model of HD exposed to an enriched environment has shown that this exposure can delay the onset of disease manifestation, by delaying degenerative loss of cerebral volume (van Dellen et al., 2000). Also, previous studies have shown that an enriched environment is associated with positive brain effects in rodents, such as, increase of dendritic length and branching, increase in the number and size of synapses, enhancement of neuronal stem cells proliferation and maturation of new neurons. These changes seem to enhance brain plasticity, improving the reserve capability and making the brain more resilient in the case of brain disorders, insults and degeneration (Nithianantharajah and Hannan, 2006).

One important component of the enriched environment is physical exercise, which also has been shown to stimulate neurogenesis, up-regulate BDNF levels and enhance cognitive performance in normal ageing and in the presence of brain insult

(van Praag et al., 2005, van Praag, 2009, Wojtowicz et al., 2008, Creer et al., 2010). Further, animal and human studies have suggested that physical exercise counteracts molecular modifications, related to AD, such as deposition of beta-amyloid plaque and tau protein phosphorylation (Liang et al., 2010, Intlekofer and Cotman, 2013). Therefore, the effects of life exposure and experiences have been shown to stimulate greater brain reserve capability, which could make the brain more resistant and resilient to changes associated with normal ageing and pathology. However, when during the lifespan, those life exposures and experiences contribute to an enhanced brain capability remains unclear.

1.9 Exercise

1.9.1 Exercise and memory

The effects of exercise on overall health are well described, particularly on the cardiovascular system and metabolism (Powell and Paffenbarger, 1985, Booth et al., 2002) and over the years, accumulating evidence has also demonstrated the beneficial effects of physical exercise on the CNS and in the process of learning and memory (Vaynman and Gomez-Pinilla, 2005). For instance, aerobic exercise has been reported to enhance performance of young healthy rats in the MWM task, as well as improved performance in passive avoidance learning (Allard, 2004, Liu et al., 2009) and in the novel recognition object task (Griffin et al., 2009).

Although growing evidence supports the link between exercise and cognitive enhancement, the underlying mechanisms are a constant field of study. This effect is linked to several mechanisms modulated by exercise, such as induction of neurotrophins and growth factors, facilitation of synaptic plasticity, stimulation of neurogenesis and anti-inflammatory roles. Also, the pro-cognitive effect of exercise could be affected by several factors, including the type of exercise (aerobic or resistance), the paradigm of exercise (voluntary running or forced running), the intensity of exercise (mild, moderate, intense, intermittent) and the duration of exercise paradigm (Liu-Ambrose et al., 2012).

1.9.2 Exercise and synaptic plasticity

Several studies have demonstrated the role of regular physical exercise on synaptic plasticity. In the past years, a growing number of studies have been investigating the underlying mechanisms of exercise on cognition. It has been suggested that exercise increases expression of neurotrophic factors in the brain, such as, BDNF and NGF (Neeper et al., 1995, Radak et al., 2001, Ang et al., 2006, Ding et al., 2006, Dietrich et al., 2008, van Praag, 2009, Bechara et al., 2014), stimulating cell proliferation, differentiation and survival, maintenance of LTP and consequent enhancement in learning and memory (Bramham and Messaoudi, 2005, Guo and Mattson, 2000, Leeds et al., 2005).

Several studies have shown long-term exercise to induce LTP facilitation. For instance, one month of voluntary wheel running facilitated hippocampal LTP in young rodents (van Praag et al., 1999, Patten et al., 2013). Also, previous work from our laboratory reported that one week of forced exercise facilitate LTP in the DG of young rats (O'Callaghan et al., 2007). In addition, long-term exercise has been shown to prevent LTP and learning and memory impairment in a model of AD-like pathology (Dao et al., 2016).
Furthermore, exercise as well as enriched environment have been demonstrated to modulate morphological changes in the hippocampus, stimulating synaptogenesis (Birch et al., 2013, Dietrich et al., 2008), characterised by increase in dendritic spine density (Stranahan et al., 2009), elevated expression of PSD-95 and Synapsin-1 (Shih et al., 2013) and improved synaptic plasticity (van Praag et al., 1999).

1.9.3 Exercise and neurogenesis

Exercise has been demonstrated to play an important role in the promotion and maintenance of adult hippocampal neurogenesis, being considered one of the most potent neurogenic interventions (van Praag et al., 1999, Fabel and Kempermann, 2008). Effects of exercise on cell proliferation are reported as early as 3 days after voluntary running and remain elevated for up to one month (Kronenberg et al., 2006). For instance, a recent study using mice lacking the anti-

proliferative gene Btg1 has shown that running provides an increment of proliferation, differentiation, and maturation of newborn neurons. They also demonstrated that running increases the pool of neural stem cells and positively affects the cell cycle kinetics of stem and precursor cells (Farioli-Vecchioli et al., 2014).

Moreover, exercise seems to affect not only NSCs proliferation, but also differentiation and survival. For example, one study has shown that five weeks of forced exercise enhanced NSCs proliferation and increased the number of immature neurons in hippocampus in middle age mice (Wu et al., 2008). Also, van Praag and colleagues (2012), showed that eight months of voluntary running improved hippocampal neurogenesis in aged rodents and it was associated with an increase in the levels of mature BNDF and enhancements in learning and memory (Marlatt et al., 2012, van Praag et al., 2005). However, most recently, a study using a contrasting rat model system for low and high response to aerobic exercise suggested that exercise promotes neurogenesis most effectively due to aerobic and sustained exercise, and especially when it is accompanied by a genetic predisposition for response to exercise (Nokia et al., 2016). In addition, Sah and co-authors also provided evidence that morphology, physiology and early network of new neurons can be reorganized by physical activity (Sah et al., 2017).

Hippocampal volume has been demonstrated to be increased by exercise. Aerobic exercise can induce an increase in hippocampal volume in older adults, associated with increase in BDNF levels and improvement in memory (Erickson et al., 2010). Fuss and co-workers, translated human data into a mouse model and confirmed exercise increases hippocampal volume in mice (Fuss et al., 2014), suggesting changes in hippocampal size may be associated with increased neurogenesis.

1.9.4 Exercise and neurotrophins

Of all neurotrophins and growth factors, exercise has been described to have the most profound and consistent impact on BDNF expression in the brain. Indeed, many studies, including various types of exercise ranging in intensity and length of experimental intervention, have shown BDNF protein increases in different regions

of the brain of exercised rodents (O'Callaghan et al., 2007, Griffin et al., 2009, Sleiman et al., 2016). In addition, increases in BDNF levels have also been described in blood (serum and plasm) of human and rodents (Zoladz and Pilc, 2010). Furthermore, BDNF has been shown to be one of the strongest candidates to link exercise effects to neurogenesis and cognition, as many studies have found an association between pro-neurogenic and pro-cognitive effects of exercise and increased concentration of BDNF (Liu and Nusslock, 2018).

Although BNDF concentrations have been widely reported to be increased after exercise, studies have also shown no changes in brain BDNF expression in rodents or a transient change in serum BDNF levels in humans following exercise (Ferreira et al., 2011, Griffin et al., 2011), suggesting the efficacy of exercise in upregulating peripheral and central BDNF expression may be dependent on the type, duration and intensity of the exercise paradigm. Also, exercise in aging animals is apparently less effective at increasing hippocampal BDNF levels than in young rodents (Adlard et al., 2005).

There is strong evidence linking exercise-induced increase in BDNF levels and enhancement in several learning and memory tasks. For instance, forced exercise has been shown to enhance NOR and OD memory associated with increased BDNF protein expression in DG (O'Callaghan et al., 2007, Griffin et al., 2009). Interestingly, BDNF administration mimics the effect of exercise-induced signalling and improvement in spatial memory in rats (Bechara et al., 2014). On the other hand, exercise-induced improvement in MWM learning and memory was blocked by infusion of a specific TrkB-IgG, that mimics BDNF receptor (Vaynman et al., 2004), supporting the hypothesis that exercise-induced increase in BDNF signalling is an important mechanism associated with pro-cognitive effect of exercise.

1.9.5 Exercise and inflammation

Accumulating evidence has strongly supported the anti-inflammatory role of exercise, however, the mechanisms underlying the exercise anti-inflammatory effects are still not entirely clear (Beavers et al., 2010). In part, this anti-

inflammatory effect seems to be related to the direct action of exercise on immune adaptations that occur locally in the exercised skeletal muscle. Interestingly, acute exercise is a stressor and consequently it triggers locally an acute inflammatory response.

Over the years, it has become widely accepted that an exercise-induced acute inflammatory response plays a major role in the adaptations observed in exercised muscles. Studies have shown that skeletal muscle produces and secretes several cytokines, called myokines, which mediate the metabolic changes induced by exercise (Febbraio et al., 2002). The best known myokine is IL-6, which has been shown to be largely released from muscle during exercise and its increase is reported to upregulate systemic production of anti-inflammatory cytokines, mainly IL-10, and to downregulate systemic levels of pro-inflammatory cytokines, such as TNF-α and IL-1β (Ostrowski et al., 1999), suggesting that IL-6 produced by skeletal muscle is a primarily anti-inflammatory effector.

Exercise anti-inflammatory effects may also be a result of adaptations in intracellular balance between generation of ROS and antioxidant defence mechanisms. Indeed, exercise increases mitochondrial respiration, with consequent elevation in the production of ROS and increased transcription for compensatory antioxidant machinery (Scheele et al., 2009). Moreover, human studies with exercise intervention demonstrated that ROS can stimulate the production of myokines in skeletal muscle in response to exercise, suggesting that IL-6 production in skeletal muscle could be dependent on ROS (Vassilakopoulos et al., 2003).

Moreover, exercise can modulate human macrophage polarisation towards an anti-inflammatory phenotype, contributing to increased systemic production of anti-inflammatory mediators. In one study in adults with elevated risk for cardiovascular diseases, long-term aerobic exercise was shown to decrease expression of pro-inflammatory cytokines, such as INF-γ, TNF-α and IL-1β and to increase the expression of IL-10, IL-4, TGF-β in mononuclear cells (Smith et al., 1999). In animal studies, exercise upregulated IL-10 and attenuated the increase in pro-inflammatory cytokine expression in the brain associated with age (Gomes da Silva et al., 2013), and LPS-induced inflammation (Littlefield et al., 2015).

In addition, exercise has been shown to prime microglia and astrocyte activation by decreasing the number of positive Iba-1 and GFAP cells in the hippocampus and cortex, accompanied by enhancement in spatial memory (He et al., 2017b), while it has also been shown to increase the number of microglial cells co-localized with BDNF and to protect against cognitive decline in aged mice (Littlefield et al., 2015). Together, these data suggest that exercise-induced anti-inflammation effects might be associated with the modulatory effect of exercise in systemic and central inflammatory profiles.

In contrast, a transgenic mouse model of AD (APP/PS1) exposed to wheel running showed no changes in the numbers of GFAP positive astrocytes and Iba-1 positive microglia cells. Also, a recent study suggested that voluntary wheel running had minimal effect in M2 markers, such as Arg-1, IL-1ra, TGF-β, and CD206, in the hippocampus of adults and aged mice following IL-4/IL-13 administration (Littlefield and Kohman, 2017). This discrepancy in some findings regarding the anti-inflammatory effects of exercise may be related to some exercise variables, such as type and duration of exercise.

1.9.6 Exercise and ageing

Over the past years, studies have suggested exercise to be beneficial in a variety of neurological disorders such as, MS, HD, PD and AD (Laurin et al., 2001, Tillerson et al., 2003, Kohl et al., 2007). Clinical and epidemiological studies indicated that exercise can enhance learning capacity (Erickson 2011), protect against cognitive decline associated with aging (van Praag, 2009, Laurin et al., 2001, McAuley et al., 2004) and also reduce the risk of dementia (Fratiglioni et al., 2004, Larson, 2008).

In addition, physical exercise has been shown to enhance hippocampal-dependent tasks, such as spatial memory in adult and aged rodents (van Praag et al., 1999, Van der Borght et al., 2007) and context fear conditioning (Greenwood et al., 2013). For instance, one study provided aged mice with access to running wheels for 45 days and found increased neurogenesis in the dentate gyrus accompanied by improved acquisition and retention of spatial memory in the MWM task (van Praag

et al., 2005). Furthermore, a study reported the ability of exercise to enhance spatial memory and restore presynaptic density in the dentate gyrus and CA3 hippocampal region of aged rats, to levels beyond those observed in younger animals (Siette et al., 2013).

Most recently, it has been shown that voluntary running reduced anxiety and depression-like behaviour in middle-aged mice, accompanied by enhanced recognition memory (Morgan et al., 2018). In addition, a study reported early-age running to enhance activity of newborn neurons after learning in adulthood, even 4 months after exercise cessation (Shevtsova et al., 2017), suggesting that regular aerobic exercise in early adulthood and the maintenance of exercise throughout the lifespan might contribute to brain resilience against age-related changes, supporting the cognitive reserve hypothesis (van Praag, 2009).

1.10 Hypothesis

Taken together, the literature reviewed above supports the important role of exercise in protecting the brain against age-related cognitive decline. However, despite the wide body of evidence about the beneficial effects of exercise on cognition, not all studies have demonstrated improvements in learning and memory after exercise. It suggests that the underlying effects of exercise on cognition could be associated with different variables, such as, duration of exercise paradigm, the type of exercise (aerobic or resistance, voluntary or forced) and the intensity of the exercise. Further, it is still unclear for how long these effects on cognition persist after cessation of exercise.

According to the capacity reserve hypothesis, age-related cognitive deficit in humans is influenced by the level of early age exposures and engagement in physical exercise and social activities (Aberg et al., 2009, Mortimer, 1997, Puccioni and Vallesi, 2012). Also, Kempermann (2008) has demonstrated an activity-dependent maintenance of neurogenesis by increasing the pool of recruitable cells, which persists into old age and could be associated with a greater capacity for sustained plasticity and brain resilience in ageing (Kempermann, 2008). Furthermore, previous studies in our laboratory have shown that both physical and mental activity enhance learning and memory in animal models and in human

subjects and have linked these improvements to specific cellular changes in hippocampal neurons.

Therefore, as it is well documented that physical exercise can modulate synaptic plasticity, neurogenesis and inflammation, we hypothesised that exposure to physical exercise, from youth until adulthood, could stimulate brain plasticity in certain stages of life, thereby contributing to a greater brain reserve capability and brain resilience, which could protect against age-related cognitive deficit.

1.11 Objectives

The overall objective of this study is to investigate the effects of forced exercise on memory and learning throughout the mouse lifespan, investigating the cellular mechanisms involved and the reserve hypothesis. We wish to investigate, specifically:

- The effect of long-term forced exercise, during youth and middle-age, on learning and memory throughout the lifespan of the mouse, in parallel with its effects on age-related anxiety and depression like behaviour;

- The mechanisms underlying the possible effects of long-term forced exercise, during youth and middle-age, on learning and memory throughout the lifespan of the mouse, with a focus on neurogenesis, inflammation and the cognitive reserve hypothesis;

- The effects of short-term forced exercise on memory and its effects on inflammation and polarization of isolated population of microglia and astrocytes cells, in a mouse model of neuroinflammation.

Chapter 2

Material and Methods

Chapter 2: Material and Methods

2.1 Materials

2.1.1 Animals

Mice C57BL/6 (male 3 month old)	Charles River Laboratories UK, Ltda.
Mice C57BL/6 (male 6 month old)	BioResources Unit, TCD
Irradiated animal pellets	LabDiet, St. Louis, MO, USA

2.1.2 Animal treatments and interventions

5-bromo-2'-deoxyuridine (BrdU)	Sigma Aldrich, Wicklow, Ireland
Lipopolysaccharide from *E. Coli*	Sigma Aldrich, Wicklow, Ireland
Treadmill, Exercise 3/6	Columbus Instruments Int, Ohio, USA

2.1.3 General laboratory chemicals

Acrylamide	Sigma Aldrich, Wicklow, Ireland
Ammonium persulfate	Sigma Aldrich, Wicklow, Ireland
Aprotinin	Sigma Aldrich, Wicklow, Ireland
BCA Protein Assay Kit	Thermo Fisher Scientific Inc.
Bovine Serum Albumin (BSA)	Sigma Aldrich, Wicklow, Ireland
Bromophenol blue	Sigma Aldrich, Wicklow, Ireland
Buffer, reference standard; pH 4, 7, 10	Sigma Aldrich, Wicklow, Ireland
EDTA	Sigma Aldrich, Wicklow, Ireland
Ethanol	Sigma Aldrich, Wicklow, Ireland
Gelatin	Sigma Aldrich, Wicklow, Ireland
Glucose solution	Sigma Aldrich, Wicklow, Ireland
Glycerol	Sigma Aldrich, Wicklow, Ireland
Glycine	Sigma Aldrich, Wicklow, Ireland
Hanks' Balanced Salt Solution (HBSS)	Invitrogen, Dublin, Ireland
HEPES Buffer Solution 1M	Bio-sciences, Dublin, Ireland
Hydrochloric acid (HCl)	Sigma Aldrich, Wicklow, Ireland
Isofluorane	Comparative Medicine, TCD
Leupeptin	Sigma Aldrich, Wicklow, Ireland
Magnesium sulfate ($MgSO_4$)	Sigma Aldrich, Wicklow, Ireland
β-Mercaptoethanol	Sigma Aldrich, Wicklow, Ireland
Methanol	Sigma Aldrich, Wicklow, Ireland

2-Methylbutane (isopentane)	Sigma Aldrich, Wicklow, Ireland
N, N'-Methylenebisacrylamide	Sigma Aldrich, Wicklow, Ireland
NP-40	Sigma Aldrich, Wicklow, Ireland
OptiPrep™ Density Gradient Medium	Sigma Aldrich, Wicklow, Ireland
Paraformaldehyde	Sigma Aldrich, Wicklow, Ireland
PBS pH7.4	Invitrogen, Dublin, Ireland
Pentobarbital sodium (Euthatal)	Comparative Medicine, TCD
Percoll	Sigma Aldrich, Wicklow, Ireland
Potassium chloride (KCl)	Sigma Aldrich, Wicklow, Ireland
Potassium chromium (III) sulfate dodecahydrate	Sigma Aldrich, Wicklow, Ireland
Potassium phosphate, dibasic (KH_2PO_4)	Sigma Aldrich, Wicklow, Ireland
2-Propanol	Sigma Aldrich, Wicklow, Ireland
Sodium azide (NaN_3)	Sigma Aldrich, Wicklow, Ireland
Sodium chloride (NaCl)	Sigma Aldrich, Wicklow, Ireland
Sodium dodecylsulfate (SDS)	Sigma Aldrich, Wicklow, Ireland
Sodium hydroxide (NaOH)	Lennox, Dublin, Ireland
Sodium orthovanadate (Na_3VO_4)	Sigma Aldrich, Wicklow, Ireland
Sodium phosphate, dibasic (Na_2HPO_4)	Sigma Aldrich, Wicklow, Ireland
Sodium phosphate, monobasic (NaH_2PO_4)	Sigma Aldrich, Wicklow, Ireland
Sucrose	Sigma Aldrich, Wicklow, Ireland
Sulfuric acid (H_2SO_4)	Sigma Aldrich, Wicklow, Ireland
Tetramethylbenzidine (TMB)	Sigma Aldrich, Wicklow, Ireland
N, N, N', N'-Tetramethylethylene-diamine	Sigma Aldrich, Wicklow, Ireland
Tris-Base	Sigma Aldrich, Wicklow, Ireland
Tris-HCl	Sigma Aldrich, Wicklow, Ireland
TritonTM X-100	Sigma Aldrich, Wicklow, Ireland
Tween-20	Sigma Aldrich, Wicklow, Ireland

2.1.4 General laboratory products and plastics

BioPointe filter/sterilised pipette tip -10µl, 20µl	Medical Supply Co. Ltd, Dublin, Ireland
Biosphere filter pipette tips - 100µl, 200ul	Sarstedt, Nümbrecht, Germany
Cell strainer, 70µm	VWR International Ltd., Dublin, Ireland
Cork discs	Fisher Scientific Ltd, Leicestershire, UK
Cover Glass 22 x 50mm	VWR International Ltd., Dublin, Ireland
Cryostat blades c35	PFM Medical UK, Cheshire, England
Eppendorfs tubes - 0.5ml, 1.5ml, 2ml	Sarstedt, Nümbrecht, Germany

Falcon Tubes - 15ml, 50ml	Sarstedt, Nümbrecht, Germany
Liquid Blocker Super PAP pen	Sigma Aldrich, Wicklow, Ireland
Microscope slide single frosted 1-1.2mm thick	Fisher Scientific Ltd, Leicestershire, UK
Microlance needles (25g 5/8 inch)	BD Plastipak, Oxford, UK
Nitrocellulose Membrane (Hybond-c Extra)	GE Healthcare, Buckinghamshire, UK
Optical Adhesive Film	Applied Biosystems, Dublin, Ireland
Petri dish	VWR International Ltd., Dublin, Ireland
Parafilm	Lennox, Dublin, Ireland
Pipette tips - 200µl, 1000µl	Sarstedt, Nümbrecht, Germany
Plastic transfer pipettes	Sarstedt, Nümbrecht, Germany
Pipette serological sterile - 10ml, 25ml	VWR International Ltd., Dublin, Ireland
RNase-free microfuge tubes – 0,5ml, 2ml	Ambion, Bio-sciences, Dublin, Ireland
Scalpels (disposable)	Fisher Scientific Ltd, Leicestershire, UK
Scienceware® Disposable Labmat	Sigma Aldrich, Wicklow, Ireland
Standard grade no.3 filter paper	Whatman Ltd., UK
Standard grade no.1 filter paper	Whatman Ltd., UK
Sterile syringes – 1ml, 20ml	BD Plastipak, Oxford, UK
Top Line filter/sterilised pipette tips - 1000µl	Lennox, Dublin, Ireland
96-microwell Nunc ELISA plates	Nunc, Roskilde, Denmark
96-well plates	Sarstedt, Nümbrecht, Germany
96-well MicroAmp Plates (Fast PCR)	Applied Biosystems, Dublin, Ireland

2.1.5 Enzyme-linked immunosorbent assay (ELISA) kits

Human BDNF Duoset® ELISA kit	R&D Systems Europe, Oxon, UK
Mouse TNF-α Duoset® ELISA kit	R&D Systems Europe, Oxon, UK
Mouse IL-1β Duoset® ELISA kit	R&D Systems Europe, Oxon, UK
Mouse IL-10 Duoset® ELISA kit	R&D Systems Europe, Oxon, UK

2.1.6 Immunohistochemistry reagents and antibodies

Alexa Fluor 633 goat anti-chicken IgG	Invitrogen, Bio-sciences, Dublin, Ireland
Alexa Fluor 488 goat anti-rabbit IgG	Invitrogen, Bio-sciences, Dublin, Ireland
Chicken anti-BrdU monoclonal IgG	Abcam, Cambridge, UK
DAPI dihydrochloride	Sigma Aldrich, Wicklow, Ireland
Normal goat serum	Vector Labs Ltd, Peterborough, UK
Rabbit anti-GFAP monoclonal IgG	Agilent Technologies Ltd., Cheshire, UK

Rabbit anti-Iba1 Polyclonal IgG	Wako Chemicals, Neuss, Germany
Rabbit anti-NeuN Polyclonal IgG	Millipore, Cork, ireland
Tissue Tek OCT compound	VWR International Ltd., Dublin, Ireland
VectaShield mounting medium	Vector Labs Ltd, Peterborough, UK

2.1.7 Polymerase chain-reaction (PCR) reagents

Absolute ethanol	Sigma Aldrich, Wicklow, Ireland
High Capacity cDNA reverse transcription kit	Applied Biosystems, Dublin, Ireland
Molecular-grade water	Sigma Aldrich, Wicklow, Ireland
Nucleospin RNA II isolation kit	Macherey-Nagel, Dublin, Ireland
RNA*later*	Ambion, Bio-sciences, Dublin, Ireland
RNase away	Ambion, Bio-sciences, Dublin, Ireland
RNaseZap wipes	Ambion, Bio-sciences, Dublin, Ireland
TaqMan Fast Advanced Master Mix	Applied Biosystems, Dublin, Ireland
TaqMan Gene Expression Assays	Applied Biosystems, Dublin, Ireland

2.1.8 Western immunoblotting reagents and antibodies

Anti-mouse (goat) IgG peroxidase conjugate	Sigma Aldrich, Wicklow, Ireland
Anti-rabbit (goat) IgG peroxidase conjugate	Sigma Aldrich, Wicklow, Ireland
Magic Mark^{TM} XP Western Protein Standard	Invitrogen, Bio-sciences, Dublin, Ireland
Mouse anti-β-Actin monoclonal IgG	Sigma Aldrich, Wicklow, Ireland
Rabbit anti-Akt IgG	Cell Signalling Technology, Mass., USA
Rabbit anti-Ceb1 IgG	Millipore, Cork, ireland
Rabbit anti-NF-Kβ p65 IgG	Cell Signalling Technology, Mass., USA
Rabbit anti-PSD95 IgG	Cell Signalling Technology, Mass., USA
Rabbit anti-p44/42 MAPK (ERK1/2) IgG	Cell Signalling Technology, Mass., USA
Rabbit anti-pAkt IgG	Cell Signalling Technology, Mass., USA
Rabbit anti-pCreb IgG	Millipore, Cork, ireland
Rabbit anti-pNF-Kβ p65 IgG	Cell Signalling Technology, Mass., USA
Rabbit anti-phospho-p44/42 MAPK (ERK1/2)	Cell Signalling Technology, Mass., USA
Rabbit anti-pTrkB IgG	Abcam, Cambridge, UK
Rabbit anti-TrkB IgG	Millipore, Cork, ireland
Rabbit anti-Synapsin1 IgG	Cell Signalling Technology, Mass., USA
PrecisionPlus Protein (Dual Colour) Standards	Bio-Rad, Laboratories, California, USA
ReBlot Plus Strong Antibody Stripping Solution	Millipore, Cork, ireland
SuperSignal West Dura (Luminol)	Fisher Scientific Ltd, Leicestershire, UK

2.2 Animals

Three and six month old male C57BL/6 mice obtained from Charles River Laboratories U.K. Ltd. were used in all experiments. At the beginning of all experiments, mice weighed between 20 and 30g. Mice were given one week to acclimatize to the Bioresources Unit and were handled daily for five minutes during this period. All experiments were performed in accordance with National and European directives on the protection of animals (European Union (Protection of Animals used for Scientific Purposes) Regulations 2012 (SI 543/2012), and European Union (Protection of Animals used for Scientific Purposes) (Amendment) Regulations 2013 (SI 434/2013), under a license issued by the Health Products Regulatory Authority and were approved by the Animal Research Ethics Committee, Trinity College Dublin.

2.3 Housing conditions

Mice were group-housed four to five per cage (for the entire period), in Tecniplast 1285LN individually ventilated cages (396mm Width x 215mm Depth x 172mm Height) with food and water available *ad libitum*. All mice were maintained in a facility with controlled ambient temperature (22°C ± 2°C) and humidity (55% ± 5%) under a 12:12-hour light-dark cycle.

2.4 Experimental design I

Three-month-old mice (n=38) were randomly assigned to either sedentary control group (SED) or exercise group (EX). Exercise group animals (n=19) underwent one hour of forced moderate exercise in a treadmill, three times per week, for a period of eight months. Sedentary control animals (n=19) for this study were placed in a stationary treadmill for an equivalent time period. At the end of this period of exercise, the adult-young mice were middle-aged (11 months). At this point, animals ceased exercise and were maintained in the facility for a period of approximately 10 months, until they achieved old age (21 months old). During the experimental period, mice were tested for learning and memory every two months to assay spatial and non-spatial memory. To assay spatial memory, we used the

Object Displacement Task (OD) and to evaluate non-spatial memory, Novel Object Recognition Task (NOR) was employed. Before the last NOR and OD tasks, when mice were 19 months old, motor activity was measured in the Open Field. Also, at the end of experiments, mice were tested in the Morris Water Maze to assess space reference memory and in the Elevated Plus Maze and tail suspension tasks, to evaluate anxiety and depression-like behaviors, respectively. Hippocampal volume was assessed in the Magnetic Resonance Image Scanner (MRI) at old age (21 months), immediately following the behavior tests. Furthermore, during the 18 months of the experiment, mice were injected with the thymidine analogue 5'bromo-2-deoxyuridine (BrdU - 50mg/kg) at different time points to allow later assessment of neurogenesis. At 21 months of age, mice were sacrificed; the brain was removed for assessment of BrdU labelling and cell phenotype by immunohistochemistry, assay of mRNA expression and protein expression of different targets of interest (Figure 2.1). Also, peripheral tissues, such as liver, muscle, blood, bone marrow, caecum and faeces samples were collected for analyses of peripheral changes induced by exercise.

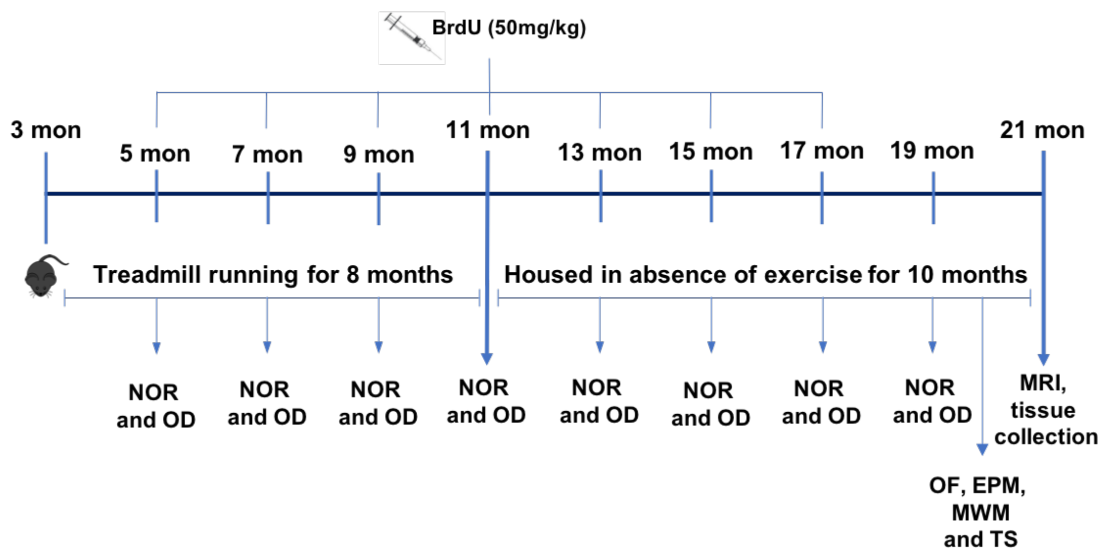


Figure 2.1 - Timeline experimental design I

Timeline showing physical exercise protocol, behaviour assessment, MRI and tissue collection time points

2.5 Experimental design II

Six-month-old male mice (n=47) were randomly assigned to either sedentary (SED, n=23) or exercise (EX, n=24) group. EX mice experienced one hour per day of moderate exercise on a motorised treadmill for a period of nine consecutive days (days 1 to 9, Figure 2.1). Twenty-four hours after the last session of exercise (day 10, Figure 2.2), EX and SED mice received a single intraperitoneal (i.p.) injection of saline (0.9% NaCl (v/v)) or a sub-septic dose of lipopolysaccharide (LPS) *from Escherichia coli* (100µg/kg). Four hours later, mice were trained in the Object Displacement (OD) task and tested thirty minutes after training. Mice were euthanised immediately following behavioural testing by sodium pentobarbital overdose and transcardial perfusion with sterile phosphate-buffered saline (PBS).

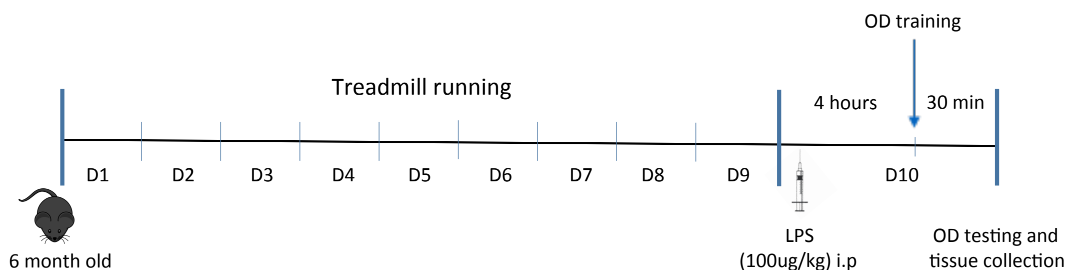


Figure 2.2 - Timeline experimental design II

Timeline showing the physical exercise protocol, LPS injection, and Object Displacement (OD) task employed.

2.6 Treadmill exercise

Mice were randomly assigned to either a sedentary control group or exercise group. All mice were familiarized to motorized rodent treadmills (Exercise 3/6 treadmill, Columbus Instruments, Figure 2.3) for at least five days prior to the commencement of the exercise protocol. Following familiarization, mice were exercised for either nine days or eight months, depending on the experiment. The exercise protocol consisted of one hour of running per day (different belt speeds, at zero inclination). Sedentary control mice were placed on stationary treadmills for the same period as exercise group, to control for possible effects of handling, stress or novelty. The treadmills are equipped with wire loops at one end of the belt

through which a mild electric shock can be delivered to encourage animals to run. In our experiment, mice ran in the absence of the electric shock, just a gentle hand prodding was used to motivate animals to run.

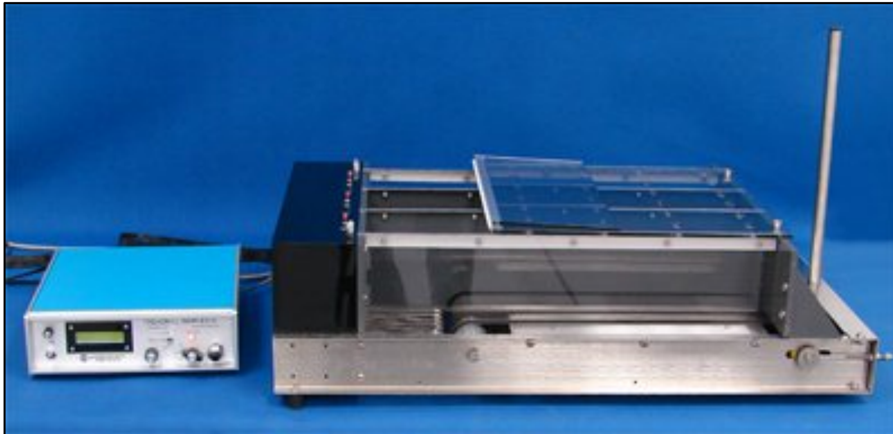


Figure 2.3 - Exercise 3/6 animal treadmill
Image of the rodent treadmill used in the forced exercise protocol (Columbus Instruments).

2.7 BrdU administration

Mice were injected with the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU), which has been shown to be stably and specifically incorporated into the DNA in place of thymidine during the DNA synthesis phase of the cell cycle (Pan et al., 2013). A variety of injection protocols, with different doses and injection timings, have been published. Based on previous experiments from our group and an assessment of the literature, we decided to perform daily intraperitoneal (i.p.) injections of BrdU (50mg/kg in 0.89% w/v saline solution) for five consecutive days every 2 months, following the behavioral tasks. The last BrdU injection was performed three months before the end of the experiment.

2.8 LPS administration

A single injection of LPS was administered via intraperitoneal injection four hours prior to behavioural assessment. Mice were assigned to receive either saline (0.89% NaCl) or a subseptic dose (Hennigan et al., 2007) of *Escherichia coli* LPS (100 µg/kg) in saline. Approximately 200-300µl, determined by body mass, was administered to each animal.

2.9 Behavioral assessment

To assess cognitive function mice underwent different behavioral tasks. The novel object recognition (NOR) and the object displacement (OD) tasks were used to assay non-spatial and spatial memory, respectively. The Morris water maze task was used to assess spatial referential memory and the elevated plus maze and the tail suspension task were used to evaluate anxiety and depression-like behavior, respectively (Figure 2.1). For all behavioral assessment, mice were brought in their home cages into the testing room 30 min prior to testing and were allow to acclimate to the testing room before starting the tasks.

2.9.1 Novel object recognition and object displacement tasks

For both the NOR and OD tasks, the apparatus consisted of a black circular open field 50cm in diameter, placed in a dimly lit room. Objects were constructed from standard plastic Lego® blocks and they were replaced with new objects every two months of testing, to avoid the confound of memories of previously-used objects. The objects were fixed to the floor of the open field 15cm from the wall and spatial cues were fixed to the walls of the open field. The test arena and the objects were cleaned with 70% alcohol after each trial to ensure the absence of olfactory cues.

2.9.1.1 Novel object recognition (NOR) task

For this experiment, the two-object variant of NOR task was chosen to assay non-spatial memory in sedentary and exercise mice. The task consisted of two days of habituation, one session of acquisition (training) and one session of testing (Figure 2.4). Two days prior to the task, animals were placed into the arena in groups (cage-by-cage) in the absence of objects for a 10 min period. On the second day of habituation, mice were habituated individually in the same empty open field for a 5 min period. The acquisition (training) day occurred 24 hours after the second day of habituation, whereupon two different objects constructed from standard Lego blocks were placed in the open field. Mice were placed in the arena and allowed to freely explore the two objects for 5 min. The criteria for exploration were

based on active examination of the object by the animal, with the mouse touching the objects with at least its nose. Measurement of the time spent exploring each object was recorded during the 5 min trial and on the completion of the task, animals were returned to their home cages. 24 hours following acquisition, one object was replaced by a novel and different object. Animals were allowed to explore the objects for a 5 min period and the amount of time spent exploring the objects was recorded. Mice that have learned the task should spend significantly more time exploring the novel object.

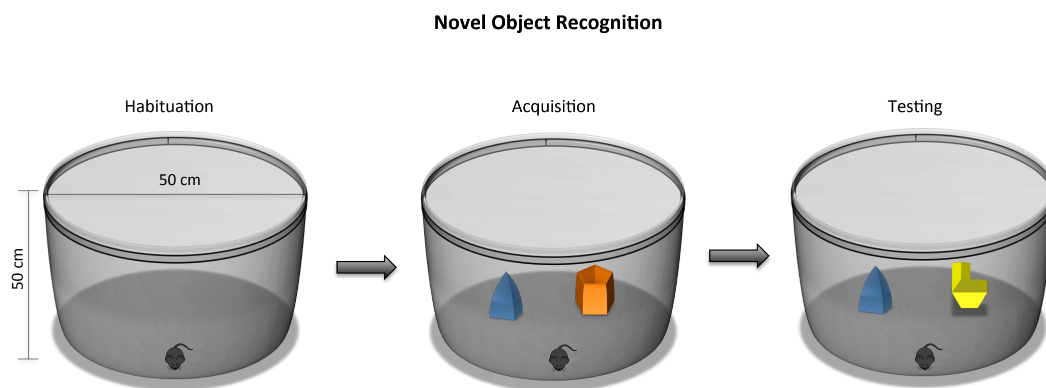


Figure 2.4 - Novel object recognition task (NOR)
Representative image of the arena, objects and the timeline of the Novel object recognition task.

2.9.1.2 Object displacement (OD) task

We used the OD task to assess spatial memory in sedentary and exercise mice. The task consisted of two days of habituation, one session of acquisition and one session of testing (Figure 2.5). The habituation phase of the OD task was identical to the habituation for the NOR. Two days prior to the task, mice were placed into the arena in groups in the absence of objects for a 10 min period and they were allowed to explore the open field. On the second day of habituation, mice were placed individually in the same empty open field for a 5 min period. 24 hours following the habituation phase three different objects constructed from standard Lego blocks were placed in the open field. Mice were allowed to explore the objects for 5 min. Criteria for exploration were the same as in section 2.9.1.1 Time spent exploring each object was recorded during the 5 min. 24 hours or 30 min following acquisition, one of the three objects were displaced to a different quadrant of the

open field. Mice were allowed to explore the three objects for 5 min. The time spent exploring the objects was recorded following the same criteria used in the acquisition day. Mice that have learned the task should spend more time exploring the displaced object.

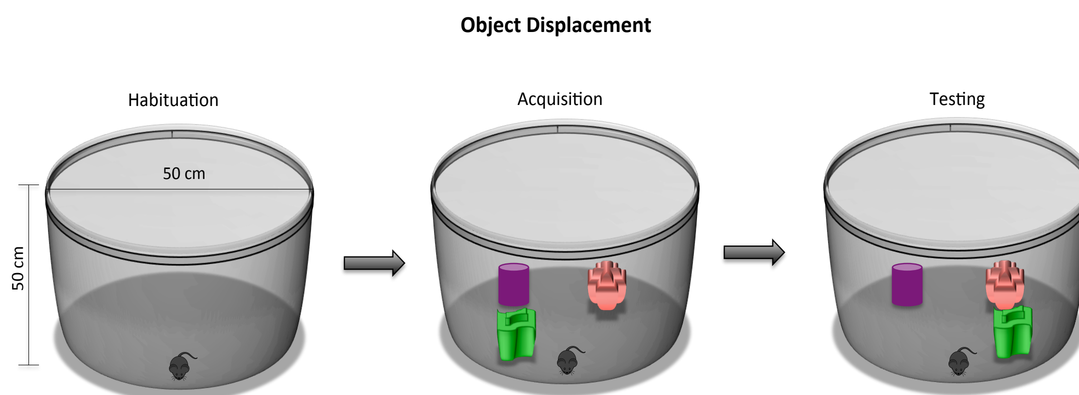


Figure 2.5 - Object displacement task (OD)
Representative image of the arena, the objects and the timeline of the Object displacement task.

2.9.2 Morris water maze (MWM) task

MWM apparatus consisted of a white circular tank (120cm diameter and 60cm depth) filled with water at 20 - 22°C. The water was made opaque by the addition of white tempura paint powder (Reeves Tempura Powder paint; Crafty Devils, UK). The maze was set up in a soundproof room surrounded by visual cues on the walls to help mice navigate in the maze. A camera was fixed to the ceiling above the water maze and was connected to the computer-based tracking programme Ethovision 3.1 (Noldus, Nottingham, UK). Imaginary lines were drawn in the maze and the end of each line demarcated four cardinal points: north (N), South (S), East (E) and West (W). These imaginary lines divided the maze into four quadrants namely northeast (NE), northwest (NW), southeast (SE) and southwest (SW). A platform (15cm in diameter and 23cm in height) was submerged 15cm bellow the water surface and 13cm from the edge of the maze wall. The hidden platform was placed in one of the four quadrants and the groups were counterbalanced so that

some mice went to each of the four quadrants (Figure 2.6, A and B). The task consisted of 5 days of training followed by one day of probe trial (testing).

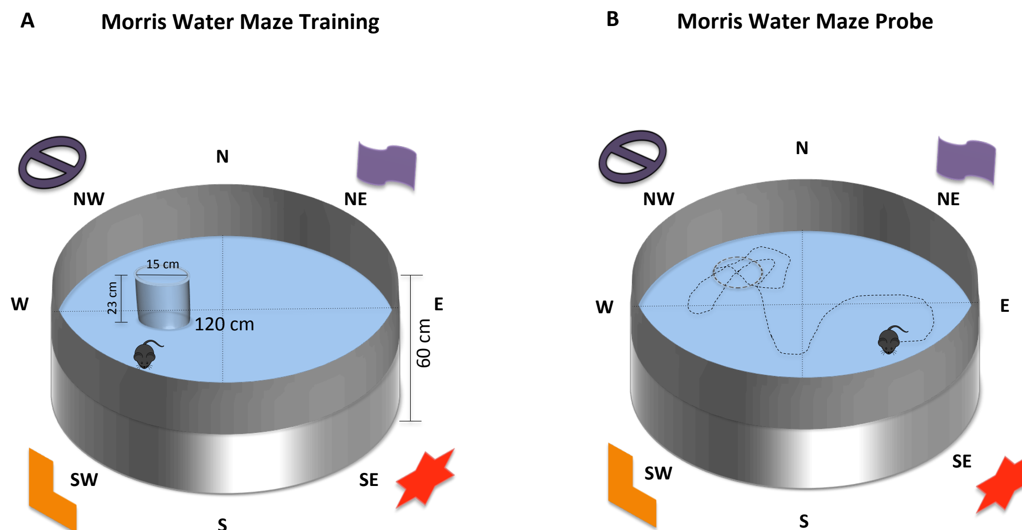


Figure 2.6 - Morris water maze task (MWM)

Representative image of the maze, platform and the timeline of the Morris water maze task.

2.9.2.1 Morris water maze training

Training was carried out for 5 consecutive days, at the same time each day. Each training day consisted of 4 one-minute trials with an inter-trial-interval of 30 seconds during which the animals were left on the platform. Each animal was placed in a holding cage and then gently lowered by the tail into the maze facing the tank wall. In each trial, animals were randomly released from different start locations (N, NE, E, SE, S, SW, W or NW) with each start location being used once per day and balanced each day. During each trial, animals were allowed one minute to find the hidden platform. The computer-tracking program starts the moment the animal is released, and it stops after one minute or when the animal reaches the platform. If the animal found the platform within one minute, the animal was left on the platform for 30 seconds (inter-trial-interval) to become familiar with the platform location. If mice failed to find the platform within the one-minute trial, they were guided and placed on the platform by the experimenter and left there for 30 seconds (inter-trial-interval). After 4 one-minute trials mice were removed from the maze, placed into a heat holding cage to dry and then returned to their home

cages. The latency to find the platform, speed of swimming, time in the quadrants and distance travelled by each mouse were the parameters recorded.

2.9.2.2 Morris water maze probe trial

The probe trial was carried out 24 hours after the final training day. The probe consisted of a single one-minute trial in which the hidden platform was removed from the maze. All animals were placed in a novel start position (180° from the original platform position), facing the tank and allowed to one minute to explore the maze. The computer-tracking program measured the time animals spent in each quadrant, how many times they swan across the annulus area (area previously containing the platform) and the speed and path length of swimming during the probe trial. After the one-minute probe trial, animals were removed from the tank, placed into a heat holding cage to dry and then returned to their home cages.

2.9.3 Open field task

The open field apparatus consisted of a square chamber made from white high-density plastic (50cm length x 35cm width x 30cm height). The floor of the cage was divided in a series of 5cm x 5cm square zones and centre (inner zone) and corners (outer zone) were also delimited (Figure 2.7, A). The test consisted in one single trial of 5 min, where mice were allowed to explore the open field freely. Animals were placed in the centre of the open field and the number of crossings between the 5cm x 5cm square zones and the number of rearings were recorded. Also, the number of entries and the total time mice spent in the inner and outer zones were measured. Entries and crossings were defined as mice moving their four limbs between the inner and outer zones or between the small squares, respectively. Rearing was considered to be when mice were standing without touching the forelimbs to the floor. The open field arena was cleaned with 70% alcohol between each animal to ensure the absence of olfactory cues.

2.9.4 Elevated plus maze (EPM) task

The EPM apparatus used was made from high density plastic in the form of a cross (+); in which two of the arms were open (25cm length x 5cm width) and the other two were closed (25cm length x 5cm width x 16cm height walls to enclose the arms) with a centre platform (Figure 2.7, B). The EPM apparatus was placed in the centre of the testing room, 50cm above the floor. The test consisted of one single trial of 5 min when mice were placed individually in the centre platform, facing one of the open arms, and they were allowed to move freely in the apparatus. During the 5 min of testing, the entries (defined as mice crossing the four limbs into the arms) in the open and closed arm and the time spent in the open and closed arms were recorded and these parameters were used to calculate the index of anxiety-like behavior. After each mouse all arms and the centre platform were cleaned with 70% alcohol to avoid olfactory cues.

2.9.5 Tail suspension test (TST)

For the TST, the apparatus used consisted of a clear box made from plastic and divided in three compartments (20cm length x 15cm width x 50cm height), to avoid observation and interaction between the mice; also, each compartment was equipped with a small aluminum suspension bar (Figure 2.7, C). The apparatus was placed over a table, approximately 1m above the floor and a camera was placed in front of the apparatus to record the test. The apparatus structure allowed the assessment of three animals at the same time (one in each compartment). For the test, mice were suspended by the tail (in the centre of the compartment), the width and depth of compartments were sufficient to avoid contact with the walls and the floor. A small piece of tape was used to attach the animal’s tail to the aluminum suspension bars. To avoid mice climbing their tails, a “climb stopper” (small piece of plastic) was placed around the tail prior to applying the tape. When all mice were suspended in the bars, the recording was started. The test consisted of a single 5 min trial, in which the immobilization time of each animal was analysed (Lad et al., 2007). After the trial, mice were returned to their home cages and the apparatus was cleaned with 70% alcohol to avoid olfactory cues.

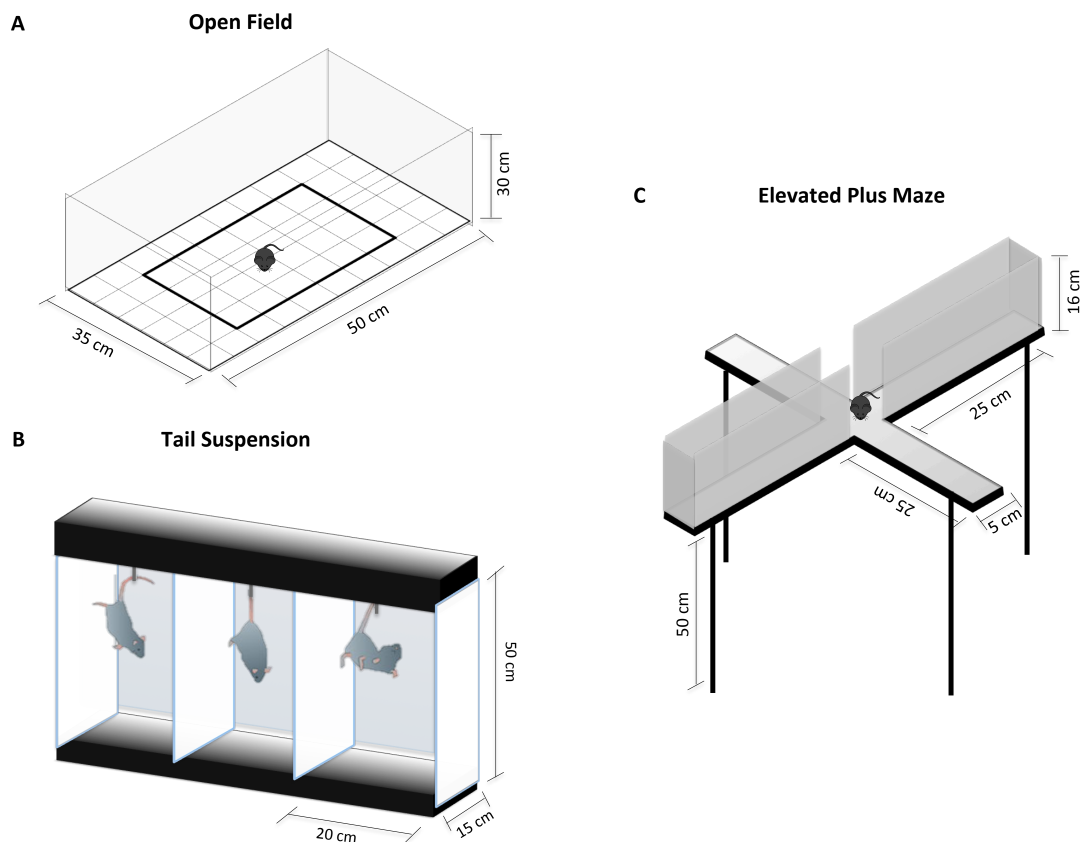


Figure 2.7 - Open field (OF), tail suspension (TS) and elevated plus maze (EPM) tasks
Representative image of the apparatus employed for the OF (A), TS (B) and EPM (C) task.

2.10 Magnetic resonance image (MRI)

2.10.1 MRI acquisition

Structural MRI was conducted using a small animal Bruker Biospin MRI system with & Tesla magnet and 30cm bore, set up to a workstation running ParaVision 4.0 software (Bruker BioSpin, Germany) for data reconstruction and analysis. Animals were anaesthetised (isoflurane 5% induction, 1-2% maintenance) and placed on a cradle using ear bars to stabilise the head. A built-in water pump-driven temperature regulator (SA Instruments Inc., Stony Brook, NY, USA) was used to maintain rectal temperature at 37°C ± 1°C. A mechanical ventilator (Ugo Basile, Comerio, VA, Italy) was used to deliver adequate inflowing gas to the facemask and the respiration signal was monitored using custom hardware and

software (SA Instruments Inc., Stony Brook, NY, USA). Respiratory rate was maintained at 60-70 breathes per minute throughout the session. To ensure accurate positioning an initial, rapid pilot image was recorded followed by a T2-weighted axial anatomical scan [spatial resolution, 0.0417 x 0.0417 x 0.3 mm3; matrix, 256x256; Field of view (FOV), 16x16; Rare factor, 8; flip angle, 90°; Repetition time (TR), 4008ms; Echo time (TE), 12ms, repetitions, 1; averages, 12; scan time, 28m 51s]. Animals were placed on a heated pad to recover from the anaesthetic before being returned to their home cage.

Figure 2.8 - Magnetic resonance imaging (MRI)
Representative image of the 7 Tesla MRI Scanner (Bruker BioSpec 7T system)

2.10.2 MRI data analysis

Manual volume measurements were performed blinded using MIPAV software (NHI, USA) to manually trace regions of interest slice by slice along the coronal plane (Vernon et al., 2010). Regions of interest analysed included both hemispheres of hippocampus and total brain volume. The regions were defined with reference to anatomical landmarks set in the mouse brain atlas. Manual tracing of these regions along the coronal plane formed a three-dimensional mask which were then quantified using MIPAV tools. Briefly, these ROI masks were summed up voxel-wise and accounted for the actual measured voxel size resulting

in volume estimates, expressed in mm^3. This was then normalised for brain size by calculating the relative percentage volume of each region in relation to total brain volume.

2.11 Tissue collection

For animals from the first experiment, after behavioral assessment and MRI assay, one set of sedentary (n=10) and exercise (n=9) animals was anaesthetised by intraperitoneal injection of pentobarbital sodium (1g/kg) and sacrificed via transcardial perfusion with PBS. Hippocampus and prefrontal cortex was dissected free from the brain, while samples of liver, gastrocnemius muscle, blood, bone marrow, caecum and faeces were removed and stored for later analysis. Microglia and astrocytes cells were purified and isolated from the remainder of the brain tissue, except for the cerebellum. Another set of sedentary (n=9) and exercise (n=10) animals was also sacrificed by transcardial perfusion with PBS, followed by paraformaldehyde (PFA, 4% (w/v)) fixation; brains were removed and analysed by immunohistochemistry.
For the second experiment, after the behaviour assessment, one set of animals (n=23) was sacrificed by cervical dislocation, the brain was removed and hippocampus and a portion of prefrontal cortex were dissected free. Another set of mice from the first experiment (n=24) was anaesthetised by intraperitoneal injection of pentobarbital sodium (1g/kg), a blood sample was removed via cardiac puncture and animals were transcardially perfused with phosphate buffered saline (PBS) [162mM NaCl; 16.2mM Na_2HPO_4; 3.8mM NaH_2PO_4; pH 7.4]. The brain was removed, hippocampus and prefrontal cortex samples were dissected free and microglia and astrocyte enriched populations of cells were isolated from the remaining brain, except for the cerebellum. All samples were stored at -80°C until required for further analysis.

2.11.1 Tissue preparation

Tissue samples (hippocampus, prefrontal cortex, liver and muscle) were homogenized for western blot and ELISA in lysis buffer [500μl; NP-40 (1%), Tris-Base (50mM, pH 8.0), NaCl (150mM), glycerol (10% (w/v)), EDTA (2mM), Na_3VO_4

(1mM), aprotinin, leupeptin]. For RNA analysis, samples were placed in RNase-free tubes containing RNA*later*™ buffer, which preserves tissue RNA. These samples were stored at -80° C until RNA extraction.

2.11.2 Transcardial perfusion and tissue fixation

To remove intravascular blood, animals were deeply anesthetized with sodium pentobarbital and perfused with an intracardiac injection of PBS (≃20mL per mouse) followed by an intracardiac injection of PFA (4%(w/v); ≃30ml per mouse). Whole brain was removed and post-fixed overnight in PFA (4% (w/v)) at 4°C. Then, brains were cryoprotected in sucrose solution (20% (w/v) sucrose in phosphate buffer), where they were kept, at 4°C, until they were frozen. Before freezing, brains were coated in an optimum cutting temperature (OCT) medium (TissueTek, VWR International). A cylinder of aluminium foil (molded around a tube) was used to hold the brain and coat it in OCT, and these brain cylinders (with OCT) were frozen in isopentane on dry ice and stored at -80°C for later sectioning.

2.11.3 Microglia and astrocyte isolation

After hippocampus and a sample of prefrontal cortex were dissected, the remaining brain tissue (except cerebellum) was placed in 1× Hank's Balanced Salt Solution (HBSS; Invitrogen, UK), cross-chopped and homogenised for the isolation of an enriched population of microglia and astrocytes. Microglia and astrocytes were isolated from adult mice with the help of Dr. Aedin Minogue. using previously described methods (Minogue et al, 2014). The cell suspensions were filtered through a cell strainer (70µm) and were centrifuged for 10min at 200 x g at 4°C. Supernatants were discarded and pellets were re-suspended in 75% Percoll (10mL; Sigma Aldrich, Ireland), overlaid with 25% Percoll (10mL; Sigma Aldrich, Ireland) and 1× PBS (6mL; Invitrogen, Ireland; composition: 155mM NaCl; 1.5mM KH_2PO_4; 2.7mM Na_2HPO_4; pH 7.2), and centrifuged at 800 x *g* for 30 min at 4°C. After centrifugation, the myelin layer was carefully removed and an enriched microglial population was collected from the 25%–75% interface (de Hass et al, 2007).

In order to obtain an enriched astrocyte population, after the microglia layer was removed, cell suspensions were re-suspended in OptiPrep™ Density Gradient Medium (10mL; Sigma Aldrich, Ireland) and centrifuged again at 800 x *g* for 30min at 4°C. Remaining myelin cells were discarded and the enriched astrocyte population was collected. Enriched cell populations of microglia and astrocytes were washed with PBS (30ml) and centrifuged for 10 min at 200 x g at 4°C. Supernatants were discarded and pellets were re-suspended in 1ml of PBS for cell counting. Viability and the number of microglia and astrocytes were counted in the microscope using a hemocytometer, samples were then centrifuged for 10 min at 200 x g at 4°C, supernatant was removed and the pellets containing microglia or astrocyte enriched populations were stored at -80°C.

2.12 Protein quantification

After tissue preparation, the protein content of samples was measured using the BCA protein assay method (Pierce®, Thermo Scientific). Samples were diluted 1:10 with lysis buffer for BCA assay. A standard curve was prepared from a serial dilution of a 200µg.ml^{-1} stock solution of bovine serum albumin (BSA). All samples and standards were analysed in triplicate (25µl per well) on a 96-well plate (microtest plate; Starstedt, Ireland). BCA working reagent was prepared by mixing BCA Reagent A and BCA Reagent B (50:1, reagent A:B), and 200µl of this working reagent was pipetted in each well. Plate was covered and incubated for 30 min at 37°C. After incubation, the plate was cooled at room temperature and the absorbance was quantified using a plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek) at a wavelength of 562nm. Regression analysis was used to calculate the protein concentration of samples. Samples were then equalised in lysis buffer to ensure equal protein content.

2.13 Enzyme linked immunosorbent assay (ELISA)

This technique was used to examine the concentration of cytokines in samples of hippocampus (IL-1β, TNF-α and IL-10 and BDNF), liver (IL-1β and TNF-α) and muscle (IL-1β and TNF-α). Samples and standards were analyzed using commercially available IL-1β, TNF-α, IL-10 and BDNF Duoset® ELISA

development systems kit (R&D Systems Europe, Oxon, United Kingdom). Standard curves were constructed and concentrations of cytokines in samples were calculated by extrapolation from these curves. Protein content of the samples was analyzed by the BCA method (see section 2.12 above) and results were expressed as concentration of the protein of interest per mg total protein.

For each assay, Nunc Immuno MaxiSorp 96 well plates were coated with capture antibody (see table 2.1, diluted in PBS, 100μl per well), covered and incubated overnight at room temperature with constant agitation. The following day, the plate was washed three times with wash buffer (PBS-T; 0.05% (w/v) of Tween®20 in PBS; 400μl per well; PBS composition: 137mM NaCl; 2.7mM KCl; 8.1mM Na_2HPO_4; 1.5mM KH_2PO_4; pH 7.4) and incubated with reagent diluent (BSA 1% (w/v) in PBS; 100μl per well) for at least 1 hour at room temperature with constant agitation, to block non-specific binding.

After the blocking, the plate was washed three times with wash buffer (400μl per well). Standards (diluted with reagent diluent, according to the manufacturer's guidelines; see table 2.1) and samples, in the prior determined dilution (diluted with lysis buffer), were added in triplicate (100μl per well) and incubated for two hours at room temperature with constant agitation. Plates were washed three times with wash buffer (400μl per well) and incubated with detection antibody (see table 2.1, diluted in reagent diluent, 100μl per well) for two hours at room temperature in constant agitation. The plate was washed three times with wash buffer (400μl per well) and incubated for twenty min at room temperature with Streptavidin-HRP (diluted with reagent diluent; dilution of 1:200 for BNDF assay and 1:40 for IL-10, IL-1β and TNF-α; 100μl per well). Plates were washed three times with wash buffer (400μl per well) and incubated with the substrate solution (Tetramethylbenzidine - TMB; Sigma Aldrich, Ireland; 100μl per well) in the dark for 20 min at room temperature. The colour reaction was stopped using the stop solution (1M H_2SO_4; 100μl per well). The absorbance of standards and samples was read at 450nm and 540nm on a plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek). The regression equations of the standard curves were constructed for each assay, and concentrations of BDNF, IL-10, IL-1β and TNF-α were extrapolated from these curves and results were expressed in pg.mg $protein^{-1}$.

ELISA kit	Capture Antibody	Standard (top standard conc.)	Detection Antibody
Human BDNF	Mouse anti-human BDNF (2μg.ml^{-1})	Recombinant human BDNF (1500pg.ml^{-1})	Biotinylated mouse anti-human BDNF (25ng. ml^{-1})
Mouse IL-1β	Rat anti-mouse IL-1β (4μg.ml^{-1})	Recombinant mouse IL-1β (1000pg.ml^{-1})	Biotinylated goat anti-mouse IL-1β (500ng.ml^{-1})
Mouse IL-10	Rat anti-mouse IL-10 (4μg.ml^{-1})	Recombinant mouse IL-10 (2000pg.ml^{-1})	Biotinylated goat anti-mouse IL-10 (300ng.ml^{-1})
Mouse TNF-α	Goat anti-mouse TNF-α (800ng.ml^{-1})	Recombinant mouse TNF-α (2000pg.ml^{-1})	Biotinylated goat anti-mouse TNF-α (50ng.ml^{-1})

Table 2.1 – ELISA kits

List of ELISA kits with standards and antibodies concentrations used in the experiments.

2.14 SDS-PAGE and Western immunoblotting

2.14.1 Tissue preparation for SDS polyacrylamaide gel electrophoresis (SDS-PAGE)

Protein content of homogenized samples was measured and equalized according to the BCA protein assay method (as described above in item 2.12). Sample buffer [100μl; d.H_20, Tris-HCl (0.5M, pH 6.8), glycerol (10% (w/v)), SDS (10% (w/v)), β-mercaptoethanol, bromophenol blue (0.05% (w/v))] was added to the equalized samples (100μl) and boiled in a heating block for 10 min at 100°C. After 10 min, samples were removed from the heating block and allowed to stand at room temperature. Samples were stored at -20°C until required.

2.14.2 SDS-PAGE

Separating gels [7.5%, 10% or 12%, composition: bisacrylamide, 1.5M Tris-HCl, (pH 8.8), SDS, APS, TEMED] were prepared and set between two Bio Rad Electrophoresis plates (1mm spacing) and allowed to polymerase. Following this, 4% stacking gel [Bisacrylamide, 0.5M Tris-HCl (pH 6.8), SDS, APS, TEMED] was prepared. Bio Rad gel combs were inserted into the stacking gel for well formation and gels were allowed to set. Gel cassettes were inserted into the electrophoresis unit (BioRad Mini-PROTEON 3, BioRad Laboratories, Hertfordshire, England) and

electrode running buffer [Tris base (25mM), Glycine (200mM), SDS (17mM)] was added to the inner and outer reservoirs. Samples prepared in sample buffer (10µl/well – approximately 25-30µg of protein per well) or dual colour Precision Plus Protein™ Standards (5µl/well; BioRad Laboratories Ltd.) and Chemiluminescent protein marker (5µl/well; Invitrogen, Ireland) were loaded into the wells and proteins were separated using gel electrophoresis (30mA per gel for approximately 45 min).

2.14.3 Western immunoblotting

After protein separation, the separating gel was removed from the cassette and immersed in running buffer [Tris base (25mM), glycine (192mM), SDS (0,5%)]. The plates were pried apart and stacking gel removed from the separating gel. Then, a 'sandwich' was made using nitrocellulose membrane (Amersham Bioscience) placed on top of one sheet of filter paper (Whatman no. 3 grade), followed by the gel and finally the second sheet of filter paper. All components were pre-soaked in transfer buffer [Tris Base (25mM), glycine (192mM), d.H_2O, metanol (20%), SDS (0.05%)].

The 'sandwiches' were covered in enough transfer buffer to keep them moist and placed on a semi-dry blotter (Apollo Instruments, Alpha Technologies, Dublin, Ireland). The unit was connected to a power source activating both anode (base) and cathode (lid) and allowed to transfer for 75 to 120 min at 225 mA, depending on the molecular weight of the target protein. Membranes were removed from the semi-dry blotter and incubated for non-specific binding of primary antibody in a blocking solution of BSA (5% (w/v)) made up in TBS-T [Tris-HCl (20mM, pH 7.5), NaCl (150mM), Tween (0.05%)] for two hours at room temperature. Membranes were incubated overnight at 4°C with primary antibody made up in BSA (2% (w/v)) in TBS-T (see table 2.2).

Following overnight incubation, membranes were washed four times for 10 min in TBS-T and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody made up in BSA (2% (w/v)) in TBS-T for 1 hour at room temperature (see table 2.2). Immunoreactive bands were detected with HRP-conjugated secondary

antibody using Supersignal® Chemiluminescence reagent (Pierce®, Rockford, U.S.A.). Membranes were developed using a Fuji-film LAS-3000 intelligent dark box (FujiFilm, USA) in conjunct with image reader LAS-3000 (FujiFilm, USA). Immediately after exposure, membranes were washed with TBS-T and stripped using ReBlot plus strong antibody stripping solution (1:10 dilution in dH_2O, 10ml; Millipore, Chemicon) and re-probed for a different protein following the same steps described above.

1° Antibody	Dilution	Incubation	2° Antibody	Dilution	Incubation
Mouse anti-ß-actin Monoclonal IgG (Sigma)	1:5000 in 2% BSA in 1x TST-T	4°C, over night	Goat anti-Mouse IgG (Sigma)	1:2000 in 2% BSA in 1x TST-T	1 hour, room temperature
Rabbit anti-Akt Monoclonal IgG (Cell Signalling Tech.)	1:1000 in 2% BSA in 1x TST-T	4°C, over night	Goat anti-Rabbit (Sigma)	1:2000 in 2% BSA in 1x TST-T	1 hour, room temperature
Rabbit anti-phospho-Akt (Ser473) Polyclonal IgG (Cell Signalling Tech.)	1:1000 in 2% BSA in 1x TST-T	4°C, over night	Goat anti-Rabbit (Sigma)	1:2000 in 2% BSA in 1x TST-T	1 hour, room temperature
Rabbit anti-Creb-1 (P43) Polyclonal IgG (Millipore)	1:1000 in 2% BSA in 1x TST-T	4°C, over night	Goat anti-Rabbit (Sigma)	1:2000 in 2% BSA in 1x TST-T	1 hour, room temperature
Rabbit anti-phospho Creb (Ser133) Polyclonal IgG (Millipore)	1:1000 in 2% BSA in 1x TST-T	4°C, over night	Goat anti-Rabbit (Sigma)	1:2000 in 2% BSA in 1x TST-T	1 hour, room temperature
Rabbit anti-NF-κB p65 Polyclonal IgG (Cell Signalling Tech.)	1:1000 in 2% BSA in 1x TST-T	4°C, over night	Goat anti-Rabbit (Sigma)	1:2000 in 2% BSA in 1x TST-T	1 hour, room temperature
Rabbit anti-phospho-NF-κB p65 (Ser536) Polyclonal IgG (Cell Signalling Tech.)	1:1000 in 2% BSA in 1x TST-T	4°C, over night	Goat anti-Rabbit (Sigma)	1:2000 in 2% BSA in 1x TST-T	1 hour, room temperature
Rabbit anti-p44/p42 MAPK [ERK1/2] Polyclonal IgG (Cell Signalling Tech.)	1:1000 in 2% BSA in 1x TST-T	4°C, over night	Goat anti-Rabbit (Sigma)	1:2000 in 2% BSA in 1x TST-T	1 hour, room temperature
Rabbit anti-phospho-p44/p42 MAPK [ERK1/2] (Thr202/Tyr204) Monoclonal IgG (Cell Signalling Tech.)	1:1000 in 2% BSA in 1x TST-T	4°C, over night	Goat anti-Rabbit (Sigma)	1:2000 in 2% BSA in 1x TST-T	1 hour, room temperature
Rabbit anti-PSD 95 Polyclonal IgG (Cell Signalling Tech.)	1:1000 in 2% BSA in 1x TST-T	4°C, over night	Goat anti-Rabbit (Sigma)	1:2000 in 2% BSA in 1x TST-T	1 hour, room temperature
Rabbit anti-Synapsin 1 Polyclonal IgG (Cell Signalling Tech.)	1:1000 in 2% BSA in 1x TST-T	4°C, over night	Goat anti-Rabbit (Sigma)	1:2000 in 2% BSA in 1x TST-T	1 hour, room temperature
Rabbit anti-TrkB Polyclonal IgG(Millipore)	1:1000 in 2% BSA in 1x TST-T	4°C, over night	Goat anti-Rabbit (Sigma)	1:2000 in 2% BSA in 1x TST-T	1 hour, room temperature
Rabbit anti-phospho-TrkB (Y515) Polyclonal IgG (Abcam)	1:1000 in 2% BSA in 1x TST-T	4°C, over night	Goat anti-Rabbit (Sigma)	1:2000 in 2% BSA in 1x TST-T	1 hour, room temperature

Table 2.2 – List of antibodies for Western Blotting

List of primary and secondary antibodies used in protein detection by Western Blotting

2.14.4 Western immunoblotting analysis

Protein bands were quantified by densitometric analysis, using the FujiFilm LAS-3000 intelligent dark box (FujFilm, USA) in conjunction with the software programs Image reader LAS-3000 (FujiFilm, USA) and the ImageJ (NHI, USA). B-actin was used as a loading control for all assays and results were expressed as percentage of control.

2.15 Polymerase chain reaction (PCR) analysis

PCR was used in order to establish possible changes in mRNA levels in the hippocampus, cortex, liver and in the enriched population of microglia and astrocytes of the animals. As described in section 2.11.1, fresh samples were placed in RNase-free tubes containing RNA*later*™ buffer and stored at -80° C until RNA extraction.

2.15.1 Total RNA extraction

RNA was extracted from samples using the commercially available Nucleospin® RNA II isolation kit (Macherey-Nagel). In an RNase-free working area (cleaned with RNaseZap wipes and RNAse away (Ambion)), tissue samples were homogenized in lysis buffer (350µl RA1 buffer, 3.5µl β-mercaptoethanol) using a polytron tissue grinder. The samples homogenates were added to Nucleospin® filters and were centrifuge for 1 min at 11.000 x g. The filtrates were collected, mixed with 350µl of ethanol (70%) and added to Nucleospin® RNA II columns. To bind the RNA to the silica membranes of the Nucleospin® RNA II columns, the columns were centrifuged for 30 sec at 11.000 x g. Bound RNA was de-salted using 350µl membrane desalting buffer (MDB, provided in kit), and centrifuged to dry the membrane at 11.000 x g for 1 min. DNA was digested using rDNase (1:10 dilution of reconstitute rDNase in rDNase reaction buffer, both provided in kit). 95µl of this mixture was added into the centre of the columns and incubated at room temperature for 15 min.

To stop the digestion reaction, 200µl of RA2 buffer (provided in the kit) was added to the columns to wash the silica membrane and columns were centrifuged at 11.000 x g for 30 sec. Next, 600µl of RA3 buffer (provided in the kit) was added to the columns which were centrifuged at 11.000 x g for 30 seconds. A further 200µl of RA3 buffer was added to the columns and the columns were centrifuged for 11.000 x g for 2 min. Finally, RNase-free water was used to remove and elute the RNA from the membrane. The columns were placed in a nuclease-free collection tube, 50µl of RNase-free water was added and the column was centrifuged at 11.000 x g for 1 min. The eluted RNA was aliquoted into PCR tubes and stored at -80°C.

2.15.2 RNA quantification and reverse transcription

A Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, UK) was used to determine the purity and concentration of RNA in each sample. 1µl of each sample was placed on the platform and a reading was produced. Optical densitometry (OD) of each of the samples was measured at 260nm and from this the RNA concentration was calculated. OD of 1.0 at 260nm represents an RNA concentration of 40µg.mg^{-1}. Thus, the RNA concentration of each sample can be calculated and quantified using the following equation:

$$RNA = OD_{260} \times \text{dilution factor} \times 40\mu g.mg^{-1}$$

In addition, the absorbance of the samples is also measured in the spectrophotometer at a wavelength of 280nm, to assess the purity of the samples. A ratio of $OD_{260nm:280nm}$ of approximately 1.8-2.0 indicates that the RNA is pure. Then, RNA concentrations were equalized using RNase-free water to ensure all the samples would be within a similar working range. Equalized samples were stored at -80°C until required for cDNA synthesis.

The ABI High Capacity cDNA archive kit (Applied Biosystems, Ireland) was used to reverse transcribe the equalized RNA samples. This kit provides the necessary components for the process of reverse transcription of total RNA to single-stranded complementary DNA (cDNA). Equalized RNA samples (10µl) were mixed with 10µl

Reverse Transcriptase master mix (2µl 10x reverse transcriptase buffer, 0.8µl 25x deoxyribonucleotide triphosphates (dNTPs), 2µl random primers, 1µl multiscribe reverse transcriptase, 4.2µl nuclease-free H_2O, per sample) in a PCR mini-tubes. Samples were placed in the thermal cycler (PTC-200 Peltier Thermal Cycler, Biosciences, Dublin, Ireland) and incubated at 25^oC for 10 min, at 37^oC for 120 min, and at 85^oC for 5 min. The cDNA was diluted with RNase-free H_2O (1:5 dilution) and stored at -20^oC for real-time polymerase chain reaction (RT-PCR) analysis.

2.15.4 RT-PCR

RT-PCR was performed using TaqMan gene expression assays (Applied Biosystems, see table 2.3), which contains target primers and FAM-labelled MGB target probes. VIC-labelled MGB target probes quantified β-Actin (see table 2.3), which was used as the endogenous control for all target genes. For multi-target (multiplex) Q-PCR, 4µl of cDNA was pipetted onto a PCR plate. Next, a mixture of target primer/probe for the gene of interest (0.5µl, see table 2.3), β-Actin primer/probe (0.5µl) and Taqman master mix was added to each well (6µl per well). The plate was sealed and centrifuged for 1 minute at 800rpm.

RT-PCR measurements were performed using Step One Plus TM Software (Applied Biosystems, Ireland). Samples were placed in a thermocycler and entered a holding stage of two min at 50^oC, 20 sec at 95^oC, and then 40 successive cycles of one sec at 95^oC followed by 20 sec were completed at 60^oC. Temperatures of 95^oC cause denaturation of the cDNA and ensure that the cDNA is all single stranded. Temperatures of 60^oC cause primers to attach to single strands, and as the temperature rises the dNTPs align to elongate the complimentary strand so that the DNA becomes double-stranded. Then, the temperature returns to 95^oC to split this new double strand, and the process repeats, resulting in exponential amplification of the target gene.

Target Gene	TaqMan Assay
Mouse ACTB (ß-actin) Endogenous Control	Mm00607939_s1
Arginase 1	Mm00475988_m1
Bax	Mm00432051_m1
Bcl-2	Mm00477631_m1
BDNF	Mm04230607_s1
CD11b (Itgam)	Mm00434455_m1
CD44	Mm01277163_m1
Creb1	Mm00501607_m1
CRP	Mm00432680_g1
Cx3cl1	Mm00436454_m1
Cx3cr1	Mm02620111_m1
GDNF	Mm00599849_m1
GFAP	Mm01253033_m1
Iba-1 (Aif1)	Mm00479862_g1
Igf-1	Mm00439560_m1
IL-1β	Mm00434228_m1
IL-4	Mm00445259_m1
IL-6	Mm00446190_m1
IL-10	Mm01288386_m1
iNOS (Nos2)	Mm00440502_m1
Ki67	Mm01278617_m1
Mrc1	Mm00475988_m1
NGF	Mm00443039_m1
p75 (NGFR)	Mm00446296_m1
TNF-α	Mm00443258_m1
TrkB (NtrK2)	Mm00435422_m1
VEGFA	Mm00437306_m1
Ym1 (Chil3)	Mm00657889_mH

Table 2.3 - Primer used in RT-PCR

List of gene expression assays used in RT-PCR assays.

2.15.5 RT-PCR analysis

The ΔΔCT method was used to quantify gene expression. This is determined from the CT readings produced during the reaction. For analysis of the ΔCT, the difference of the CT for the target gene and the endogenous control, in the same well, was calculated. The difference between the ΔCT of the sample compared to the mean of the ΔCT from the control samples was calculated, giving the ΔΔ CT. To determine the relative quantity (RQ) of target gene of each sample the follow equation was used: 2- ΔΔ CT, as a difference in one cycle is equal to a two-fold

difference in levels of amplified target sequence (due to the exponential nature of the process). Then, RQ values were plotted and compared for statistically significant differences.

2.16 Immunohistochemistry

This technique, in conjunction with fluorescent microscopy, was used to assess neurogenesis by immunolabelling of BrdU in the dentate gyrus of hippocampus of animals. Cells were double-stained with BrdU and Neuronal nuclear antigen (NeuN), Glial fibrillary acidic protein (GFAP) or Ionized calcium-binding adapter molecule 1 (Iba-1) to establish whether BrdU-labelled cells were differentiated into mature neuronal or glial cell phenotypes, respectively. This technique was also used to establish the number of positive GFAP and Iba-1 cells in the CA1 and CA3 regions of hippocampus. Quantification of staining was conducted blind using stereological sampling.

2.16.1 Tissue preparation for immunohistochemistry

Before the immunohistochemistry procedure, samples were cryoprotected and prepared as described above (Section 2.11.2) and sections were sliced using the cryostat (Leica CM1900). Brains in OCT were taken from the -80°C freezer and allowed to come to -20°C in the cryostat. They were fixed with OCT onto a cork disc, which was mounted onto a chuck, and brain tissue was sectioned in coronal slices of 40µm thickness. Once the cutting had begun, care was taken to detect the beginning of the hippocampus. Slices containing the hippocampal structure were transferred onto prepared slides. The ‘one-in-six’ series counting protocol was utilized, so once the first slice of tissue was collected, the following slices were collected on five different slides, and so on. Thus, each slide had four slices that are each six slices (240µm) apart (see figure 2.9). Once all slices were collected they were stored in slide boxes and kept at -20°C until the immunohistochemistry assay.

Slide 1	Slide 2	Slide 3	Slide 4	Slide 5	Slide 6
1	2	3	4	5	6
7	8	9	10	11	12
13	14	15	16	17	18
19	20	21	22	23	24

Slide 7	Slide 8	Slide 9	Slide 10	Slide 11	Slide 12
25	26	27	28	29	30
31	32	33	34	35	36
37	38	39	40	41	42
43	44	45	46	47	48

Slide 13	Slide 14	Slide 15	Slide 16	Slide 17	Slide 18
49	50	51	52	53	54
55	56	57	58	59	60
61	62	63	64	65	66
67	68	69	70	71	72

Figure 2.9 - Coronal brain slice

Schematic of the order in which slices were collected.

2.16.2 Fluorescent double staining

Double staining was used to detect whether BrdU-labelled cells were differentiated into mature neuronal (NeuN), or glial cell phenotypes (GFAP and Iba-1) in the dentate gyrus of the hippocampus. Different sets of slides were double stained with BrdU/NeuN, BrdU/GFAP or BrdU/Iba-1. Sections were allowed to acclimatize to room temperature for 5 min. Slides were washed three times x 5 min with PBS [162mM NaCl; 16.2mM Na_2HPO_4; 3.8mM NaH_2PO_4; pH 7.3]. Then, since DNA must be denatured to allow binding of the primary antibody to BrdU, the sections were incubated with HCl (2N) for 45 min at room temperature. Slides were washed three times x 5 min with PBS and blocked for non-specific binding using 3% goat serum (1x PBS, 0.5% (w/v) Tween®20, 3% normal goat serum) for 1 hour at room temperature. Primary antibody was prepared in 3% goat serum (1x PBS, 0.5% (w/v) Tween®20, 3% normal goat serum) and chicken anti-BrdU (1:100 dilution, see table 2.4) was added mixed with rabbit anti-NeuN (1:400 dilution, see table

2.4), or rabbit anti-GFAP (1:200 dilution, see table 2.4), or rabbit anti-Iba1 (1:400 dilution, see table 2.4) and slides were incubated overnight at 4ºC.

24 hours later, slides were washed three times x 5 min with PBS and once x 5 min with deionized H_2O and incubated for 2 hours at room temperature with secondary antibody prepared in PBS (see table 2.4) Slides were washed three times x 5 min with PBS and one time x 5 min with deionized H_2O. Slides were incubated with the nuclear marker Hoescht (DAPI; 1:500 dilution in 1x PBS) for 10 min at room temperature. Slides were washed three times x 10 min with PBS and once x 10 minutes with deionized H_2O and allowed to dry. Coverslips were placed onto the slides with hard-set mounting medium (Vectashield, Vector Labs, UK) and slides were stored in the dark at 4ºC until images were taken in the microscope.

1º Antibody	Dilution	Incubation	2º Antibody	Dilution	Incubation
Chicken anti-BrdU Monoclonal IgG (Abcam)	1:100 in 3% goat serum	4ºC, over night	Alexa Fluor 633 goat anti-chicken IgG (Invitrogen)	1:200 in PBS	2 hours, room temperature
Rabbit anti-GFAP monoclonal IgG (Agilent)	1:200 in 3% goat serum	4ºC, over night	Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen)	1:200 in PBS	2 hours, room temperature
Rabbit anti-Iba1 monoclonal IgG (Wako)	1:400 in 3% goat serum	4ºC, over night	Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen)	1:400 in PBS	2 hours, room temperature
Rabbit anti-NeuN monoclonal IgG (Millipore)	1:400 in 3% goat serum	4ºC, over night	Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen)	1:200 in PBS	2 hours, room temperature

Table 2.4 - List of antibodies for immunohistochemistry

List of primary and secondary antibodies with its dilutions and time of incubation used in Immunohistochemistry assays.

2.16.3 Microscope imaging and image analysis

Olympus Upright BX51 and Leica SP8 Scanning Confocal microscopes were used for fluorescent imaging in the SBI Microscopy Facility, TBSI, at Trinity College Dublin. Photomicrographs of the dentate gyrus, CA1 and C3 regions of

hippocampus were taken and saved for further analysis. At a x4 magnification, DAPI stained images of the whole dentate gyrus were obtained, from both hemispheres of the brain, and in all four slices on each slide and these were used to measure dentate gyrus volume. At x20 magnification, DAPI, BrdU, NeuN, GFAP and Iba-1 stained images were obtained from the whole dentate gyrus, CA1 and CA3 regions of hippocampus. Cell counts were performed using image manipulation in Image J program. This program enables grid structures to be imposed over the images to assist in unbiased stereological volume estimation. Then the Cavalieri method was applied to obtain an unbiased estimation of the volume. Each image was taken at the same resolution and magnification, and the entire dentate gyrus structure was imaged. Then, a point grid, with crosses 2000 pixels apart, was imposed with random offset over each image and an estimate of the volume of the dentate gyrus is determined with the following equation:

$$V = T. (a/p). \Sigma P$$

In the equation, T is the distance between sections (240μm). The length of the scale bar and length from the centre of one cross to the centre of the next cross were measure on screen at the same magnification and converted to μm. The actual length of the scale bar on screen was divided by the length stated on the scale bar and is called magnification factor (M). This value was then squared (M2) to obtain an absolute value. The distance in μm between the centres of two crosses (Δx) was also squared (Δx2). This value was divided by the magnification (Δx2/M2) to obtain the area per point (a/p) in μm^2. This value was constant through all volumetric analysis. The only variable factor in this equation is the sum of the points (ΣP), that is counted in all sections of dentate gyrus measured. These variables were used in the above equation and the volume of the dentate was calculated for each sample, expressed in μm^3.

After the volume was determined for each sample, the numbers of $BrdU^+$ cells, $GFAP^+$ cells and $Iba\text{-}1^+$ cells were counted in all dentate gyrus images. The total number of positive cells was expressed per μm^3. Image J allowed the separation of fluorescent images into colour channels, and then combined (merged) them for positive double staining. These merged images of Brdu positive cells and NeuN,

GFAP or Iba-1 positive cells were counted as mature neurons, astrocytes and microglia cells, respectively, that were generated and incorporated BrdU into their DNA when BrdU was injected into the mouse; these also were expressed per μm^3.

For analysis of $GFAP^+$ cells and $Iba\text{-}1^+$ cells in the CA1 and CA3 regions of hippocampus, x20 magnification images of GFAP and Iba-1 were merged with the x20 magnification images of DAPI staining and positive GFAP and Iba-1 cells were counted in CA1 and CA3 regions, from all four slices on each slide. Results were expressed as total number of positive cells.

2.17 Statistical analysis

All data were analysed and grouped using Microsoft Excel, and then imported into Graphpad Prism 5. All data are represented and expressed as mean ± standard error of the mean (SEM). Analysis was carried out using both one-way ANOVA (Analysis of Variance) when one factor was compared among three or more groups, and two-way ANOVA or two-way repeated measure ANOVA (RM ANOVA) when two or more factors were compared in two or more groups. In addition to this, data were further analysed using Bonferroni *post hoc* tests. When data did not follow a normal distribution, non-parametric test was used (Kruskal-Wallis), in conjunct with Dunn's multiple comparisons test. Statistical significance was accepted at $p<0.05$.

Chapter 3

The persistent effect of exercise on learning and memory, anxiety and depression-like behaviour throughout the lifespan

Chapter 3: The persistent effect of exercise on learning and memory, anxiety and depression-like behaviour throughout the lifespan

3.1 Introduction

Well-functioning memory is crucial to normal life, and it can be affected in different ways. Impairments in learning and memory can be a consequence of brain insults, normal ageing and age-related diseases, which can be investigated in both rodents (Latimer et al., 2014) and humans (Atkinson et al., 2008) using a variety of learning and memory tasks. The NOR task is the most common behavioural task employed to examine recognition memory in rodents (Murai et al., 2007, Leger et al., 2013) and it is known to recruit the hippocampus and the perirhinal cortex (Broadbent et al., 2004). Information about memory gained from the NOR task is based on the premise that animals have a tendency to explore novel objects in preference to familiar objects (Rutten et al., 2008b).

The OD task has been used to assess spatial memory in rodents (Murai et al., 2007, Ennaceur and Delacour, 1988), because this task also takes advantage of the natural tendency of rodents to investigate novelty (Ricceri et al., 2000). Also, the MWM has been widely employed to measure hippocampal dependent spatial learning and memory in animals (Vorhees and Williams, 2006). This type of learning and memory task is dependent on the hippocampus for encoding, consolidation, and retrieval (Mumby et al., 2002, Haettig et al., 2011)

Both recognition and spatial learning and memory tasks have been used to investigate cognition in several disease models and in normal ageing. These tasks have also been employed to evaluate the effects of pharmacological and non-pharmacological interventions on cognitive impairments. In this context, exercise is well known to enhance and improve learning and memory in both humans and animals (Griffin et al., 2011, O'Callaghan et al., 2009). Corroborating to many studies, previous work from our laboratory has shown exercise to enhance novel object recognition memory and object displacement in young rodents (O'Callaghan

et al., 2007, Griffin et al., 2009) and to protect against age-related learning impairment in the MWM (O'Callaghan et al., 2009).

Furthermore, age has been associated with mood disorders, such as depression and anxiety. The prevalence of depression is high in older age and it can be overlapped with anxiety disorder and cognitive impairment (Gorman and Kent, 1999). In rodents, depression and anxiety-like behaviours have been linked to age-related disease, such as Alzheimer´s disease (Nie et al., 2017a, Nie et al., 2017b). In mice, the TST has been widely used to assess depression-like behaviour. This test is based on the fact that mice exposed to the stress of being suspended by the tail, will present an immobile posture; antidepressant drugs can prevent this immobile behaviour towards an escape-related behaviour (Steru et al., 1985, Cryan et al., 2005). The most common task used to measure anxiety-like behaviour in rodents is the EPM. The EPM task relies on the preference of rodents for dark and enclosed spaces and an unconditioned fear of heights and open spaces (Pellow et al., 1985, Walf and Frye, 2007).

Physical exercise can impact mood as well as cognition. Studies have demonstrated physical exercise to result in an anti-depressive and anxiolytic effect in rodents tested in TS task and EPM task, respectively (Duman et al., 2001a, Crema et al., 2010). The benefits of exercise in anxiety and depression-like behaviours are consistent with the effects of exercise on cognitive functioning. Voluntary exercise has been described to enhance learning and memory in middle-aged and old rodents and it has been linked to decreased anxiety-like behaviour (Pietrelli et al., 2012, Morgan et al., 2018). Also, forced exercise has been shown to alleviate associated anxiety and depression-like behaviour, stress and cognitive impairment in rats with post-traumatic stress disorder (Patki et al., 2014).

Therefore, exercise has been widely described to enhance and improve learning and memory and to ameliorate anxiety and depression-like behaviours. However, the majority of the studies investigated the effects of chronic voluntary running on these behaviours in young-adult or aged rodents, raising a question about the effects of exercise during youth and middle-age. Furthermore, the persistence of these effects after exercise cessation have not been studied yet. For this reason, this aim of this study was to investigate the effect of long-term forced exercise on

learning and memory throughout the lifespan of the mouse, in parallel with its effects on age-related anxiety and depression like behaviour.

3.2 Methods

3.2.1 Animals

Three month old male C57BL/6 mice obtained from Charles River Laboratories U.K. Ltda. were used in all experiments. Animals were given one week to acclimatize to the Comparative Medicines Unit and were handled daily for five minutes during this period. All experiments were performed in accordance with National and European directives on the protection of animals (European Union (Protection of Animals used for Scientific Purposes) Regulations 2012 (SI 543/2012), and European Union (Protection of Animals used for Scientific Purposes) (Amendment) Regulations 2013 (SI 434/2013), under a license issued by the Health Products Regulatory Authority and were approved by the Animal Ethics Committee, Trinity College Dublin. Animals were group-housed, four/five per cage, in Tecniplast 1285LN individually ventilated cages with food and water available *ad libitum*. All animals were maintained in a facility with controlled ambient temperature (22°C ± 2°C) and humidity (55% ± 5%) under a 12:12-hour light-dark cycle.

3.2.2 Experimental design

Three month old male C57BL/6 mice (n=38) were randomly assigned to either sedentary control group (SED) or exercise group (EX). Exercise group animals (n=19) underwent one hour of forced moderate exercise in a treadmill, three times per week, for a period of eight months. Sedentary control mice (n=19) for this study were placed in a stationary treadmill for an equivalent time period. At the end of this period of exercise, the adult-young mice were middle-aged (11 months). At this point, mice ceased exercise and were maintained in the facility for a period of approximately 10 months, until they achieved old age (21 months old). During the experimental period, mice were tested for learning and memory every two months to assay spatial and non-spatial memory. To assay spatial memory, we used the

Object Displacement Task (OD) and to evaluate non-spatial memory, Novel Object Recognition Task (NOR) was employed. Before the last NOR and OD tasks, when animals were 19 months old, motor activity was measured in the Open Field (OF). Also, at the end of experiments, the animals were tested in the Elevated Plus Maze (EPM) to evaluate anxiety-like behaviour and then in the Morris Water Maze (MWM) to assess space referential memory. After MWM, the tail suspension test was employed, to evaluate depression-like behaviour (Figure 3.1).

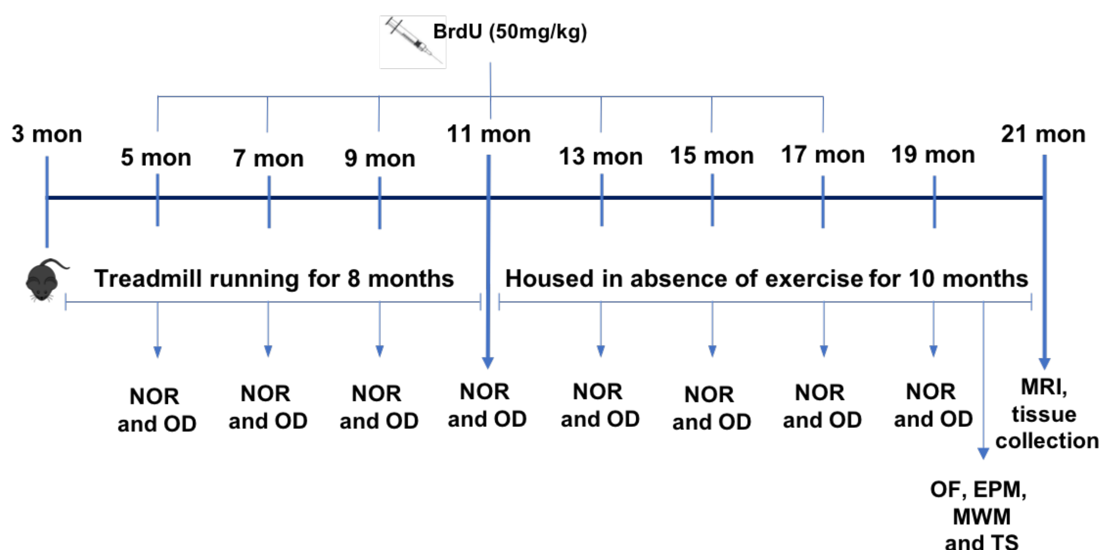


Figure 3.1 - Experimental timeline

Timeline showing physical exercise protocol and behaviour assessment time points.

3.2.3 Treadmill exercise protocol

Mice were randomly assigned to either sedentary control group (n=19) or exercise group (n=19). All animals were familiarized on motorized rodent treadmills for one week prior to the commencement of the exercise protocol. Following familiarization, mice were exercised three times per week for a period of eight months. The exercise protocol consisted of one hour of running (belt speed 6-12 m/min, at zero inclination). Sedentary control mice were placed in stationary treadmills for the same period as exercise group, to control possible effects of handling, stress or novelty. The treadmills are equipped with wire loops at one end of the belt through which a mild electric shock can be delivered to encourage

animals to run. In our experiment, animals ran in the absence of the electric shock, just a gentle hand prodding was used to motivate animals to run.

3.2.4 Behavioural assessment

To assess cognitive function, mice underwent different behavioural tasks, the OD and the NOR to assay spatial and non-spatial memory, respectively, were performed every two months. First NOR and OD behavioural testing were performed after following two months of exercise, when mice were 5 months old. Then, every two months, mice were submitted to the NOR and OD, until the end of the experiment (Figure 3.1). Also, at the end of the experiments (mice were 20 months old), the Morris Water Maze task was employed to assess spatial referential memory. Finally, the Elevated Plus Maze and the Tail Suspension tasks were used to evaluate anxiety and depression-like behaviour, respectively. For all behaviour assessment, animals were brought in their home cages into the testing room 30 min prior to the testing and mice were allowed to acclimate to the environment room before starting the tasks.

3.2.4.1 Novel Object Recognition (NOR) task

The apparatus consisted of a black circular open field 50 cm in diameter, as described in detail in section 2.9.1.1. Objects were constructed from standard plastic Lego® blocks and they were replaced with new objects every two months of testing. For this experiment, the two-objects variant of NOR task has been chosen to assay non-spatial memory. The task consisted of two days of habituation, one day of acquisition (training) and one day of testing, as described in detail in section 2.9.1.1. Briefly, for the habituation, the first day, mice were placed into the arena in groups (cage-by-cage) in the absence of objects for a 10 min period. On the second day of habituation, mice were habituated individually for a 5 min period. Twenty-four hours after the last habituation, two different objects were placed in the open field and mice were allowed to explore the two objects for 5 min. Twenty-four hours following acquisition, one object was replaced by a novel and different object and mice were allowed to explore the objects for a 5 min

(Figure 3.2). The amount of time spent exploring the objects was recorded. Mice that have learned the task should spend significantly more time exploring the novel object.

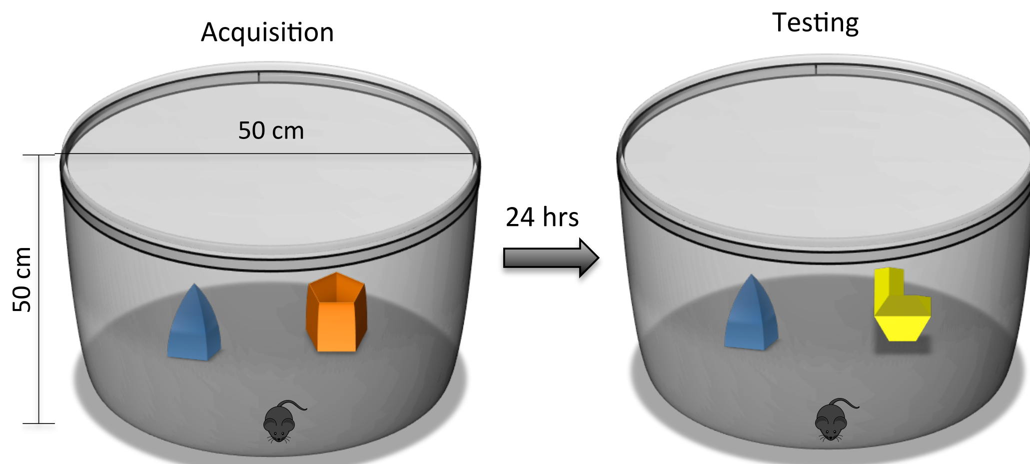


Figure 3.2 - Novel Object Recognition (NOR) Task

Representative image of the arena, objects and the delay between acquisition and testing of the NOR.

3.2.4.2 Object Displacement (OD) task

The same apparatus used for NOR was used for the OD task. The task consisted of four days of experimentation, that were two days of habituation, one day of acquisition and one day of testing, as described in section 2.9.1.2.

Habituation was identical to the habituation for the NOR, as described above. Twenty-four hours following the habituation, three different objects constructed from standard Lego blocks were displayed in the open field and mice were allowed to explore the three objects for 5 min. Twenty-four hours after acquisition, one of the three objects, was displaced to a different location in the open field and mice were allowed to explore the three objects for 5 min (Figure 3.3). The time spent exploring the objects was recorded following the same criteria used in the NOR. Mice that have learned the task should spend more time exploring the displaced object.

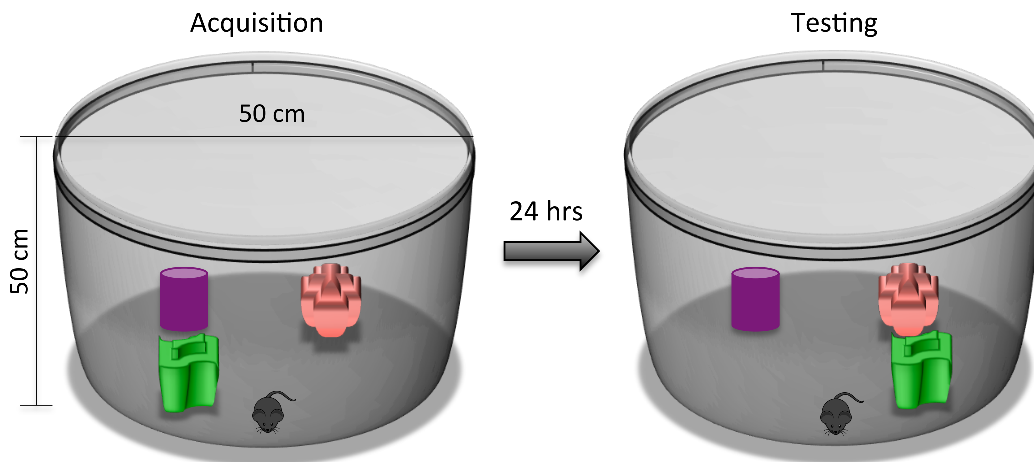


Figure 3.3 - Object Displacement (OD) Task

Representative image of the arena, the objects and the delay between acquisition and testing of the OD.

3.2.4.3 Open Field (OF) task

Four days after the last NOR and OD tasks, mice were tested in the open field to measure locomotor and exploratory activity. The open field consisted in a square chamber made from white high-density plastic, with the floor divided in a series of 5 cm x 5 cm square zones and centre (inner zone) and corners (outer zone) were also delimited as described in detail in section 2.9.3. Briefly, mice were allowed to explore freely the open field maze for 5 min and the number of crosses between the 5 cm x 5 cm square zones and rearing were recorded. Also, the number of entries and the total time animals spent in the inner and outer zones were measured, as described in section 2.9.3.

3.2.4.4 Elevated Plus Maze (EPM) test

A day after the Open Field task, mice were submitted to the Elevated Plus Maze (EPM) task to assess anxiety-like behaviour. The apparatus used for the EPM task was made from high density plastic and in form of cross (+); in which two of the arms were open and the other two were closed arms, with a centre area, as described in section 2.9.4. Briefly, mice were placed individually in the centre platform and allowed to move in the maze for 5 min. Entries in the open and close

arm and the time spent in the open and close arms were recorded and used to calculate the index of anxiety-like behaviour, as described in section 2.9.4.

3.2.4.5 Morris Water Maze (MWM) task

After the Open Field and Elevated Plus Maze, mice were allowed to rest for a week and then were trained in the MWM task. MWM apparatus consisted of a white circular tank (120cm diameter and 60cm depth) filled with water at 20 - 22° C. A hidden platform was placed in one of the four quadrants, as described in detail in section 2.9.2. The entire task consisted of 5 days of training followed by one day of probe trial (testing). Briefly, training was carried out for 5 consecutive days, which consisted of daily 4 one-minute trials with an inter-trial-interval of 30 seconds. During each trial, animals were allowed one minute to find the hidden platform, as described in detail in section 2.9.2.1. Latency to find the platform, speed of swimming, time in the quadrants and distance travelled by each mouse were the parameters recorded during training. A probe trial was carried out twenty-four hours after the final training day and consisted of a single one-minute trial in which the hidden platform was removed from the maze (Figure 3.4). All animals were placed in a novel start position and allowed to one minute to explore the maze, as described in detail in section 2.9.2.2. The parameters measured were, the time animals spent in each quadrant, how many times they swan across the annulus area and the speed and path length of swimming during the probe trial.

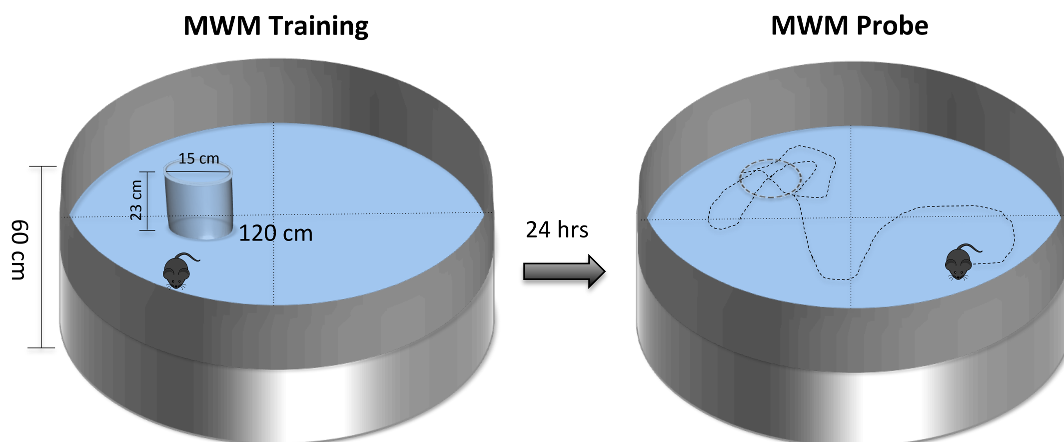


Figure 3.4 - Morris Water Maze (MWM) task

Representative image of the maze, platform and the delay between training and probe trials of the MWM.

3.2.4.6 Tail suspension test (TST)

One week after the MWM task, mice were submitted to the TST to evaluate of depressive-like behaviour. The TST apparatus consisted in a box made from plastic and divided in three compartments, which were equipped with a small aluminium suspension bar, as described in section 2.9.5. Briefly, mice were suspended by the tail and the immobilization time of each animal was recorded during 5 min, as described in detail in section 2.9.5.

3.2.5 Statistical analysis

Data were analysed and grouped using Microsoft Excel, and then imported into Graphpad Prism 5. All data are expressed as mean ± standard error of the mean (SEM). Analysis was carried out using Unpaired Student's *t*-Test, when one factor was compared among two groups; two-way ANOVA (Analysis of Variance) and two-way repeated measure ANOVA (RM ANOVA), when two or more factors were compared in two or more groups. Where significance was found, data were further analysed using Bonferroni *post hoc* tests. Statistical significance was accepted at $p < 0.05$.

3.3 Results

3.3.1 Persistent effects of long-term exercise on non-spatial memory throughout the lifespan: The Novel Object Recognition (NOR) Task

Every 2 months, from the beginning of the experiment, non-spatial memory was assessed using Novel Object Recognition (NOR) task (Figure 3.5 A). The first NOR task was performed after 2 months undergoing exercise and at this stage mice were 5 months old. At 5 months old, statistical analysis showed a slight preference for object A compared with object B in exercise group, but not in sedentary group ($p=0.0156$, two-way ANOVA Object A vs. Object B, SED: Obj. A: 49.89±0.87% and Obj. B: 50.10±0.87%; EX: Obj. A: 52.63±1.15% and Obj. B: 47.37±1.15%, Bonferroni *post-hoc*, Figure 3.5 B). However, this preference vanished, in the exercise group, when object B was replaced by object C twenty-four hours later, on the testing day (Figure 3.5 C). Twenty-four hours after the acquisition day of the first NOR, the first testing was performed. There was a significant difference between exploration of the new object (object C) compared to the exploration of the familiar object (object A) in both sedentary and exercise groups ($p<0.001$, two-way ANOVA Object A vs. Object C, SED: Obj. A: 46.37±1.63% and Obj. C: 53.63±1.63%; EX: Obj. A: 43.26±1.85% and Obj. C:56.84±1.86%, Figure 3.5 C).

The second NOR task was performed 4 months after the beginning of exercise protocol and at this point mice were 7 months old. During acquisition of the second NOR task, no difference was found between the exploration of the two objects (object A and B) in both sedentary and exercise groups ($p=0.1590$, two-way ANOVA Object A vs. Object B, SED: Obj. A: 49.31±0.84% and Obj. B: 50.68±0.84%; EX: Obj. A: 49.42±0.93% and Obj. B: 50.58±0.93%, Bonferroni *post-hoc*, Figure 3.6 A). However, in the testing phase of the second NOR task, statistical analysis showed a significant difference between exploration of the familiar object (Object A) and the novel object (Object C) in sedentary and exercise group ($p<0.001$, two-way ANOVA Object A vs. Object C, SED: Obj. A - 42.05±1.47% and Obj. C: 57.95±1.47%; EX: Obj. A: 34.42±1.70% and Obj. C: 65.58±1.70%, Bonferroni *post-hoc*, Figure 3.6 B). Also, there was a significant

interaction between the group and the exploration of objects. *Post-hoc* analysis revealed that exercise group explored the novel object significantly more than the familiar object ($p<0.001$, two-way ANOVA Object C exercise group vs. Object C sedentary group, Figure 3.6 B).

The third (Figure 3.7 A and B) and fourth (Figure 3.8 A and B) NOR task were performed after 6 and 8 months of exercise commencement, when mice were 9 and 11 months old, respectively. In both, similar to the second NOR task, there was no difference between exploration of objects in the acquisition phase of NOR (3rd NOR training, $p=0.3703$, SED: Obj. A: 48.74±1.05% and Obj. B: 51.26±1.05%; EX: Obj. A: 50.47±0.65% and Obj. B: 49.53±0.66, Figure 3.7 A; and 4th NOR training, $p=0.6780$, SED: Obj. A: 50.67±1.10% and Object B: 49.33±1.10%; EX: Obj. A: 50.76±1.23% and Obj. B: 49.23±1.22%, two-way ANOVA Object A vs. Object B, Bonferroni *post-hoc*, Figure 3.8 A). On the other hand, in the testing for both time points, mice from sedentary and exercise groups explored the novel object (Object C) more than the familiar object (3rd NOR testing, $p<0.001$, SED: Obj. A: 44.58±1.30% and Obj. C: 55.42±1.35%; EX: Obj. A: 33.53±1.06% and Obj. C: 66.47±1.05%, Figure 3.7 B; and 4th NOR testing, $p<0.001$, SED: Obj. A: 45.22±1.54%, Obj. C: 54.78±1.54%; EX: Obj. A: 31.59±1.70%, Obj. C: 68.41±1.70%, two-way ANOVA Object A vs. Object C, Bonferroni *post-hoc*, Figure 3.8 B). Furthermore, 6 and 8 months after exercise commencement, exercised mice explored the novel object significantly more than the sedentary ($p<0.001$, two-way ANOVA, Object C exercise group vs. Object C sedentary group, Figure 3.7 B and Figure 3.8 B). At this point, mice stopped exercise and were maintained in the absence of exercise for 10 more months and memory assessment, using the NOR, continued each 2 months, until mice were 19 months old.

Next NOR task (fifth assessment) was performed 2 months after cessation of exercise, when mice were 13 months old (Figure 3.9 A and B). At this time, similarly to the results observed before, there was no difference in the exploration of objects in the acquisition phase ($p=0.9246$, two-way ANOVA Object A vs. Object B, SED: Obj. A: 48.83±1.14% and Obj. B: 51.17±0.14%; EX: Obj. A: 51.30±1.53 and Obj. B: 48.70±1.53%, Bonferroni *post-hoc*, Figure 3.9 A). In the testing of the fifth NOR task, 2 months after exercise cessation, sedentary and exercise mice explored

more the novel object than ($p<0.001$, two-way ANOVA Object A vs. Object C, SED: Obj. A: 45.56±1.69% and Obj. C: 54.44±1.69%; EX: Obj. A: 35.94±1.41% and Obj. C: 64.06±1.41%, Bonferroni *post-hoc*, Figure 3.9 B) and again, mice from exercise group explored more the novel object compared to sedentary mice ($p<0.001$, two-way ANOVA Object C exercise group vs. Object C sedentary group, Figure 3.9 B).

Continuing the assessment after exercise cessation, the sixth, seventh and eighth NOR task were performed 4, 6 and 8 months after exercise cessation, when mice were 15 (Figure 3.10 A and B), 17 (Figure 3.11 A and B) and 19 months old (Figure 3.12 A and B), respectively. In all this time points, during the acquisition phase, there was no difference between the exploration of objects in both groups (6th NOR training - $p=0.0961$, SED: Obj. A: 48.28±1.02% and Object B: 51.72±1.01%; EX: Obj. A: 49.82±1.23% and Obj. B: 50.17±1.23, Figure 3.10 A; 7th NOR training - $p=0.0846$, SED: Obj. A: 51.83±1.43% and Obj. B: 48.17±1.43%; EX: Obj. A: 50.53±1.25% and Obj. B: 49.47±1.25%, Figure 3.11 A; and 8th NOR training - $p=0.1416$, SED: Obj. A: 51.44±1.61% and Obj. B: 48.55±.61%; EX: Obj. A: 50.65±1.12% and Obj. B: 49.35±1.12%, two-way ANOVA Object A vs. Object C, Bonferroni *post-hoc*, Figure 3.12 A). Interestingly, in all the time points NOR was assessed, at 15, 17 and 19 months old, only exercise mice explored the novel object more than the familiar object in the testing phase (6th NOR testing - $p<0.001$, SED: Obj. A: 47.72±2.44% and Obj. C: 52.28±2.44%; EX: Obj. A: 37.06±2.64% and Obj. C: 62.94±2.64%, Figure 3.10 B; 7th NOR testing - $p<0.001$, SED: Obj. A: 48.06±1.83% and Obj. C: 51.94±1.83%; EX: Obj. A: 40.06±1.70% and Obj. C: 59.94±1.70%, Figure 3.11 B; and 8th NOR testing: SED: Obj. A: 50.44±1.45% and Obj. C: 49.55±1.45%; EX: Obj. A: 40.41±2.01 and Obj. C: 59.59±2.01%, two-way ANOVA Object A vs. Object C, Bonferroni *post-hoc*, Figure 3.12 B). Furthermore, at all time points, statistical analysis showed significant differences in the exploration of the novel object between sedentary and exercise mice ($p<0.001$, two-way ANOVA Object C exercise group vs. Object C sedentary group, Bonferroni *post-hoc*, Figure 3.10 B, Figure 3.11 B and Figure 3.12 B).

In order to have an overview of performance of mice in the NOR throughout the lifespan, the discrimination ratio (DR, how much mice prefer the novel object

compared to the familiar) was calculated for each NOR assessment and the results were plotted over time (Figure 3.13). After mice were assigned to sedentary or exercise group (at 3 months old), a baseline performance in the NOR task was assessed, and it was used to compare the NOR results over time (Figure 3.13). When the DR was analysed over time, from 7 months old the exercise group showed a higher DR compared to the sedentary group at the same age ($p<0.05$, DR - 3 months old SED:0.09±0.05 and EX: 0.07±0.04; 5 month old SED: 0.08±0.03 and EX: 0.13±0.04; 7 months old SED: 0.16±0.03 and EX:0.28±0.04; 9 months old SED: 0.10±0.27 and EX: 0.32±0.03; 11 months old: SED: 0.09±.03 and EX: 0.35±0.04; 13 months old SED: 0.09±0.03 and EX: 0.26±0.03; 15 months old SED: 0.05±0.04 and EX: 0.24±0.05; 17 months old SED: 0.04±0.03 and EX: 0.19±0.03; and 19 months old SED: -0.008±0.03 and EX: 0.18±0.04, two-way RM ANOVA, DR sedentary vs. DR exercise from the same age, Figure 3.13). Furthermore, this difference can be observed even after exercise cessation until the last NOR assessment. Finally, statistical analysis showed significant difference between DR of exercise mice, from all the ages after 7 months old, compared to exercise in the baseline (3 months old) and 5 months NOR assessment ($p<0.05$, two-way RM ANOVA, DR exercise group compared to DR exercise baseline and 5 months old, Bonferroni *post-hoc*, Figure 3.13).

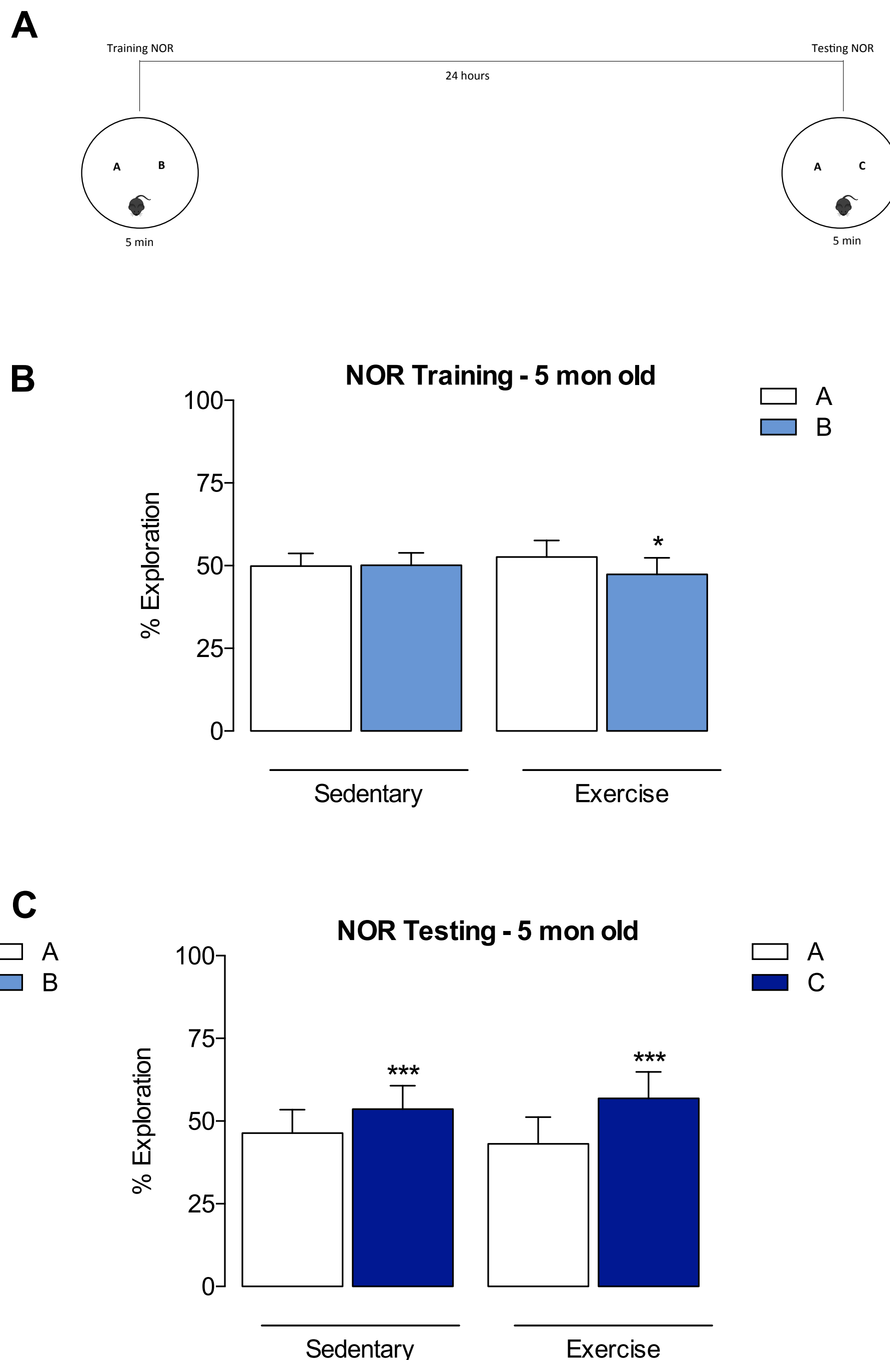


Figure 3.5 - Performance in NOR task after 2 months of exercise

A schematic of the timeline utilized every 2 months to test mice in the NOR task. This involved one 5 min acquisition trial followed by a 5 min testing trial, 24 h later **(A)**. In the acquisition trial, 2 months after of exercise commencement, when mice were 5 months old, mice displayed a slight preference for object B **(B)**. In the test, both sedentary and exercise mice explored the novel object more (Object C) **(C)**. All data are expressed as mean ± SEM (n=19). *$p<0.05$ Object A vs. Object B, ***$p<0.001$, Object A vs Object C two-way ANOVA with Bonferroni *post hoc* analysis.

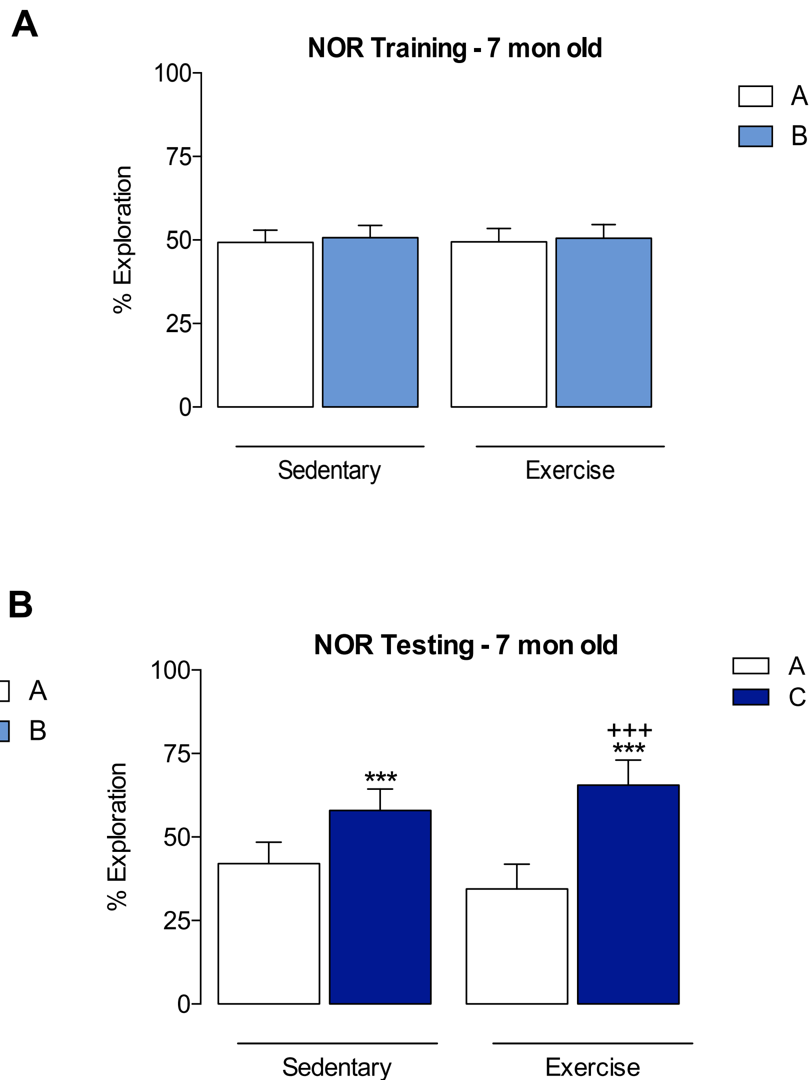


Figure 3.6 - Performance in NOR task after 4 months of exercise

In the acquisition trial, 4 months after of exercise commencement, when mice were 7 months old, there was no difference in the exploration of the 2 objects **(A)**. In the test, both sedentary and exercise mice explored the novel object more (Object C) and exercise mice explored the novel object significantly more when compared to sedentary group **(B)**. All data are expressed as mean ± SEM (n=19). ***p<0.001, Object A vs Object C, $^{+++}$*p*<0.001, Object C exercise group vs Object C sedentary group, two-way ANOVA with Bonferroni *post hoc* analysis.

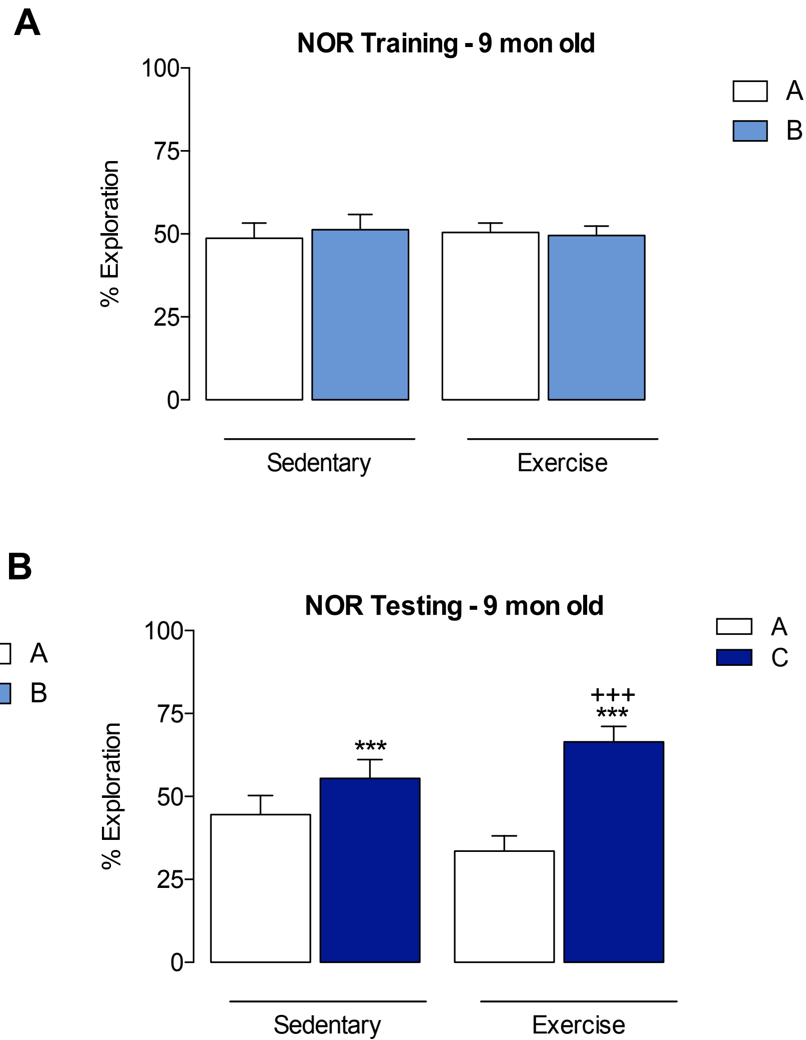


Figure 3.7 - Performance in NOR task after 6 months of exercise

In the acquisition trial, 6 months after of exercise commencement, when mice were 9 months old, there was no difference in the exploration of the 2 objects **(A)**. In the test, both sedentary and exercise mice explored the novel object more (Object C) and exercise mice explored the novel object significantly more when compared to sedentary group **(B)**. All data are expressed as mean ± SEM (n=19). ***p<0.001, Object A vs. Object C, $^{+++}$*p*<0.001, Object C exercise group vs Object C sedentary group, two-way ANOVA with Bonferroni *post hoc* analysis.

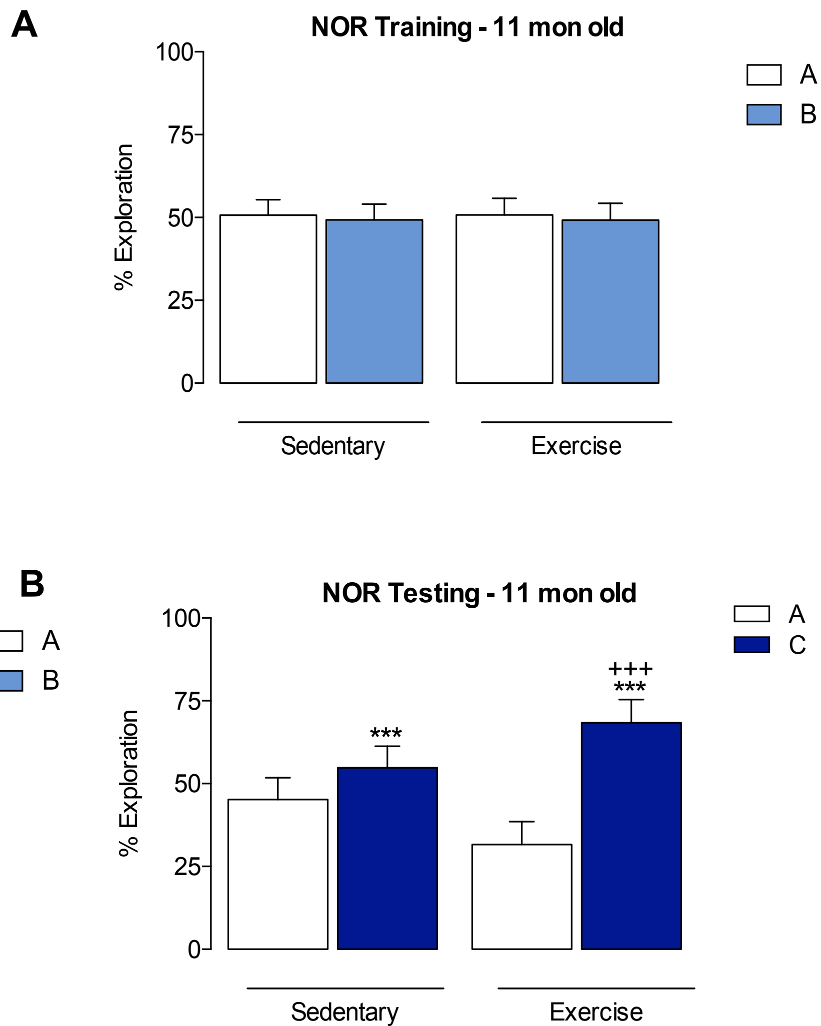


Figure 3.8 - Performance in NOR task after 8 months of exercise

In the acquisition trial, 8 months after of exercise commencement, when mice were 11 months old, there was no difference in the exploration of the 2 objects **(A)**. In the test, both sedentary and exercise mice explored the novel object more (Object C) and exercise mice explored the novel object significantly more when compared to sedentary group **(B)**. All data are expressed as mean ± SEM (n=18). ***p<0.001, Object A vs. Object C, $^{+++}$*p*<0.001, Object C exercise group vs Object C sedentary group, two-way ANOVA with Bonferroni *post hoc* analysis.

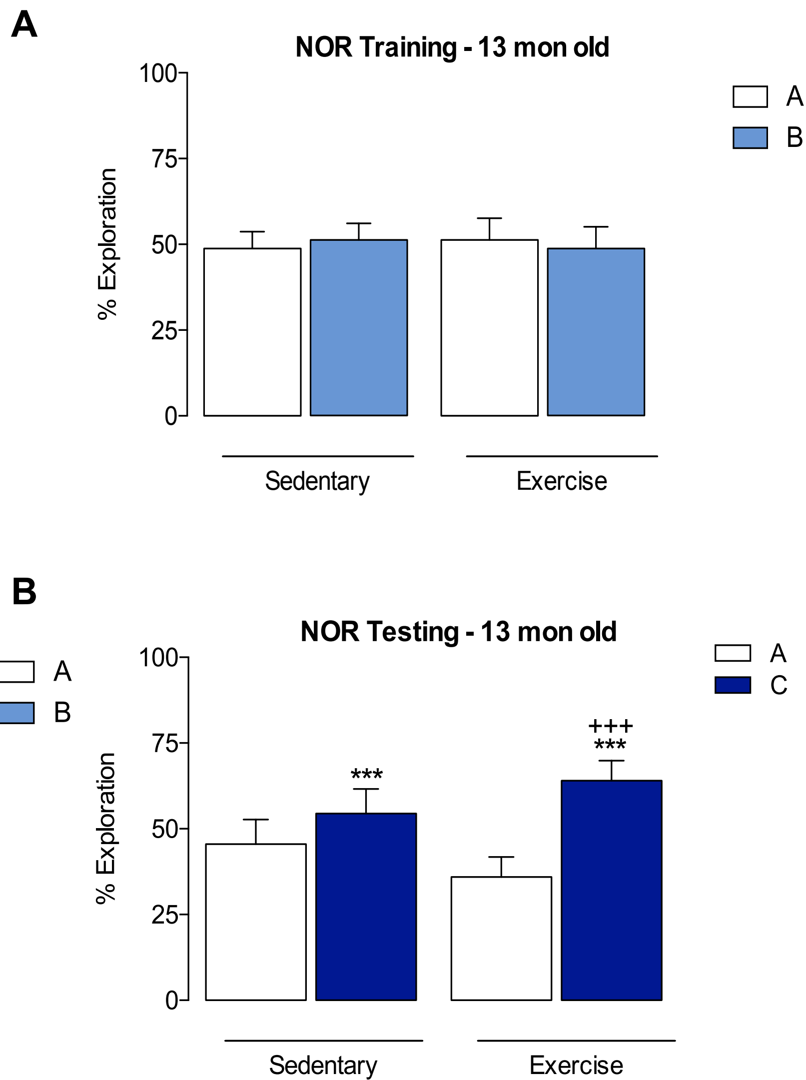


Figure 3.9- Performance in NOR task 2 months after exercise cessation

At this point, mice had stopped exercise 2 months previously, and were 13 months old. In the acquisition trial of NOR, mice explored the 2 objects equally **(A)**. During testing, both groups still displayed a significant preference for the novel object (Object C) and exercise group explored the novel object more when compared to sedentary group **(B)**. All data are expressed as mean ± SEM (n=18). ***p<0.001, Object A vs Object C, $^{+++}$*p*<0.001, Object C exercise group vs. Object C sedentary group, two-way ANOVA with Bonferroni *post hoc* analysis.

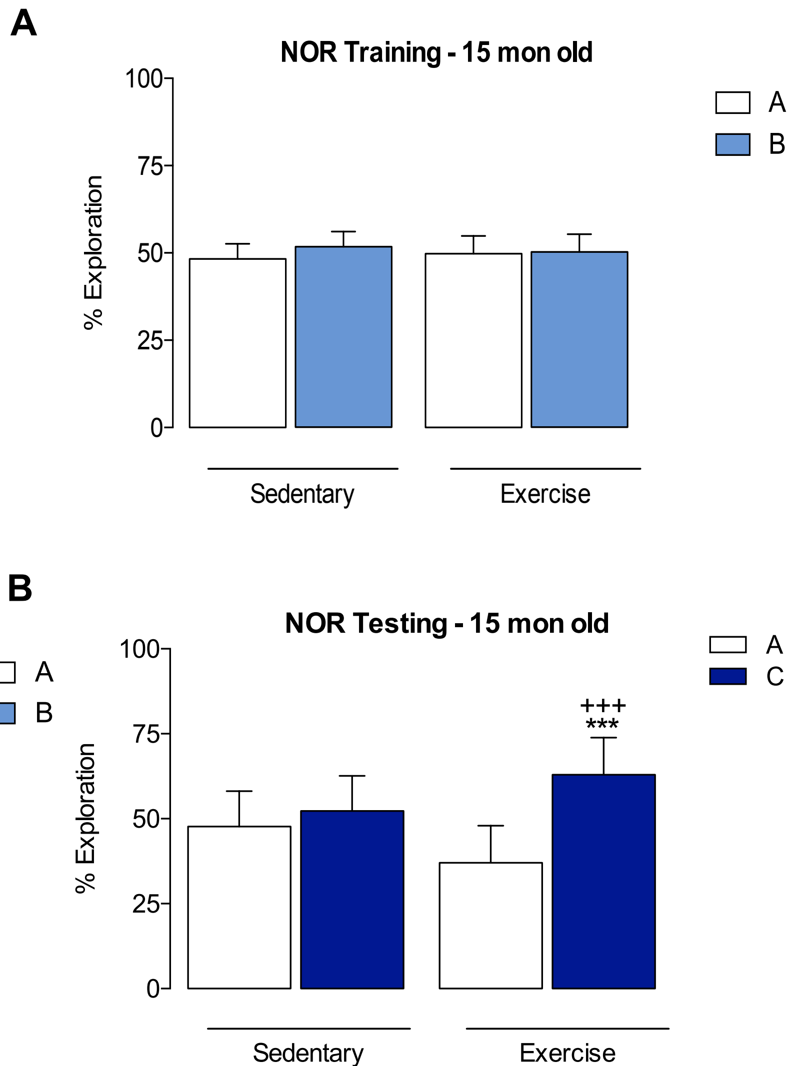


Figure 3.10 - Performance in NOR task 4 months after exercise cessation

At this point, mice had stopped exercising 4 months previously, and were 15 months old. In the acquisition trial of NOR, mice explored the 2 objects equally **(A)**. During testing, only exercise mice still displayed a significant preference for the novel object (Object C) and exercise group explored the novel object more when compared to sedentary group **(B)**. All data are expressed as mean ± SEM (n=18). ***p<0.001, Object A vs. Object C, $^{+++}p<0.001$, Object C exercise group vs Object C sedentary group, two-way ANOVA with Bonferroni *post hoc* analysis.

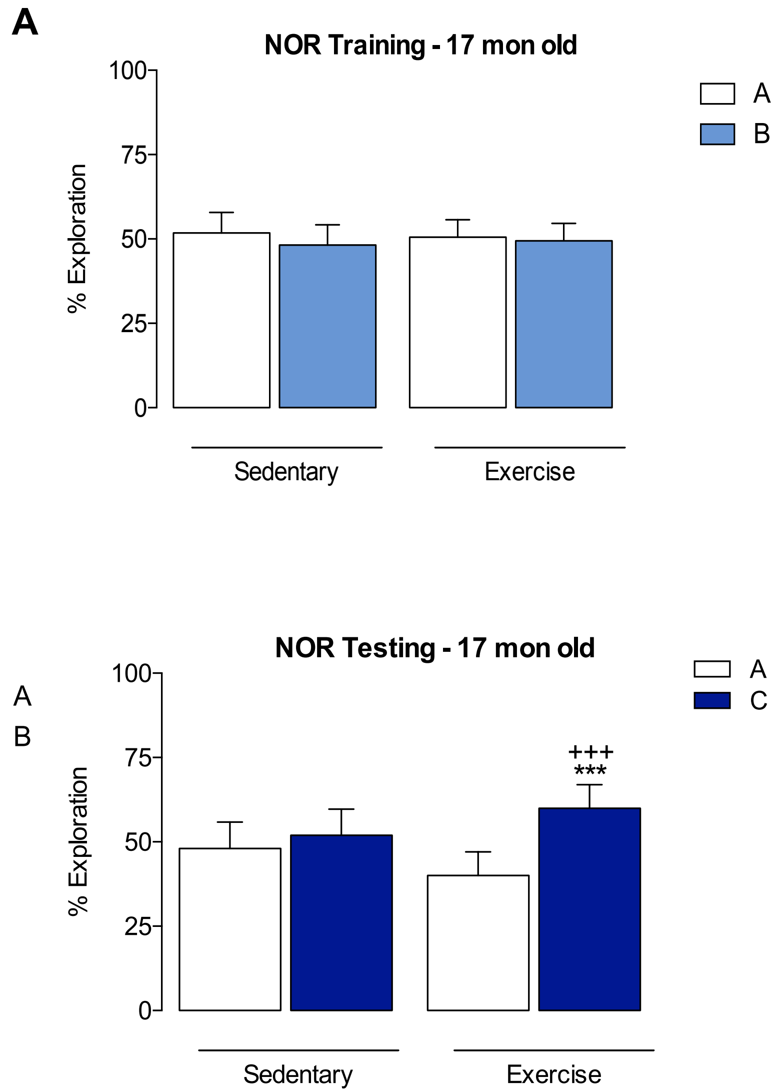


Figure 3.11 - Performance in NOR task 6 months after exercise cessation

At this point, mice had stopped exercising 6 months previously, and were 17 months old. In the acquisition trial of NOR, mice explored the 2 objects equally **(A)**. During testing, still only exercise mice displayed a significant preference for the novel object (Object C) and exercise mice still explored the novel object more when compared to sedentary group **(B)**. All data are expressed as mean ± SEM (n=18). ***p<0.001, Object A vs. Object C, +++*p*<0.001, Object C exercise group vs Object C sedentary group, two-way ANOVA with Bonferroni *post hoc* analysis.

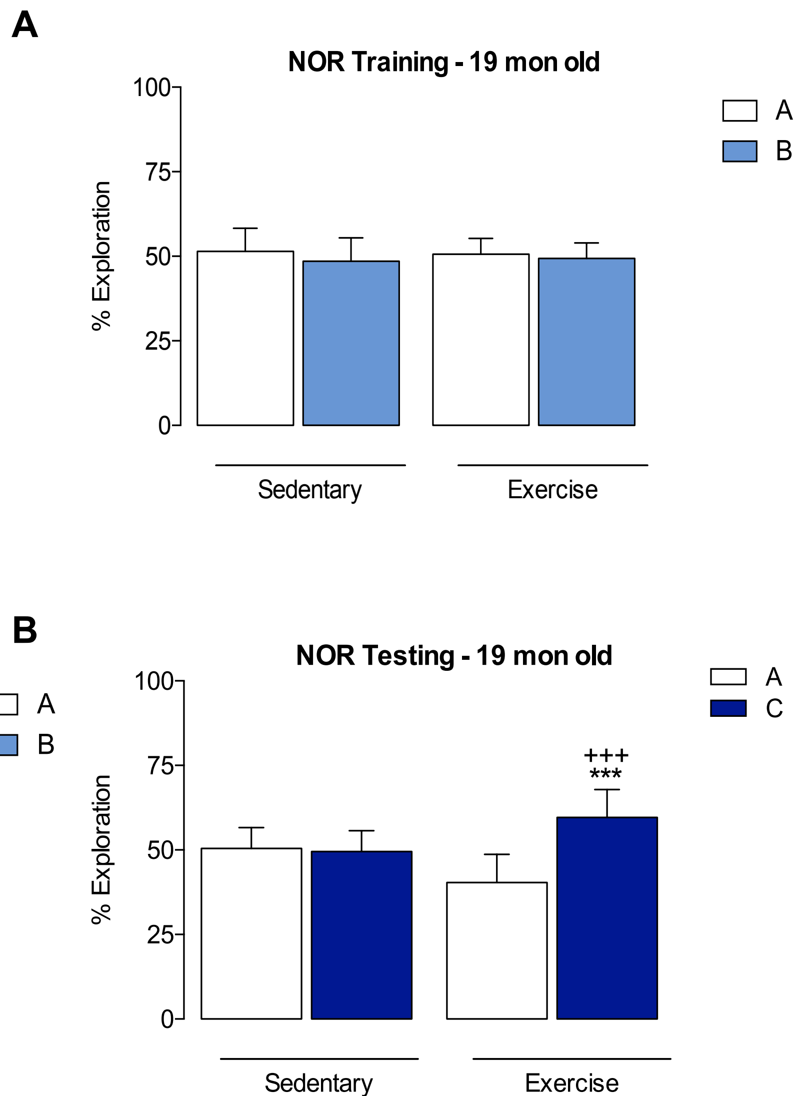
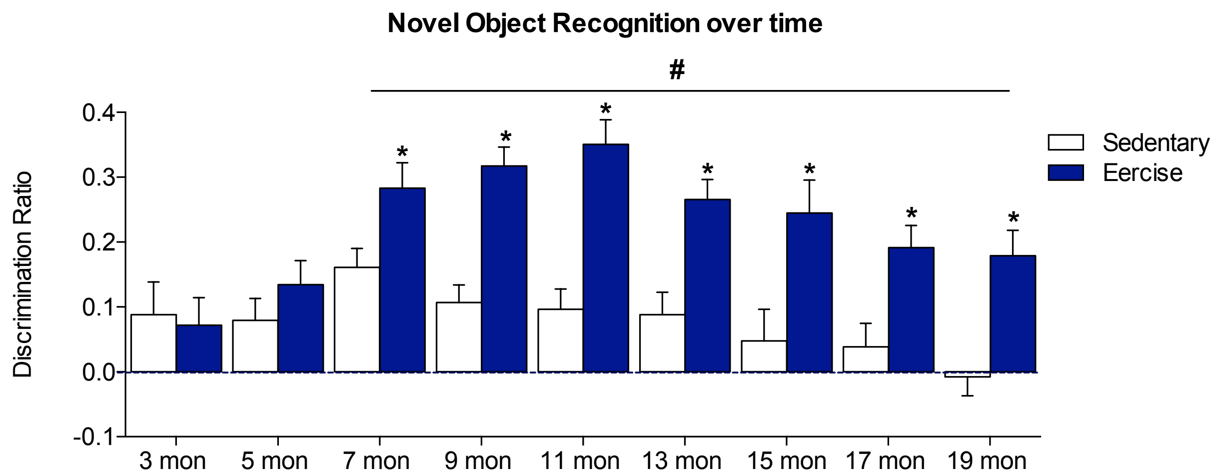


Figure 3.12 - Performance in NOR task 8 months after exercise cessation
In the last NOR assessment, mice had stopped exercising 8 months previously, and were 19 months old. In the acquisition trial of NOR, mice explored the 2 objects equally **(A)**. During testing, still only exercise mice displayed a significant preference for the novel object (Object C) and *post hoc* analysis revealed that exercise mice still explored the novel object more when compared to sedentary group **(B)**. All data are expressed as mean ± SEM (n=18). ***p<0.001, Object A vs. Object C, $^{+++}$*p*<0.001, Object C exercise group vs Object C sedentary group, two-way ANOVA with Bonferroni *post hoc* analysis.



Object Displacement over time

#

Discrimination Ratio

0.5
0.4
0.3
0.2
0.1
0.0

*

3 mon
5 mon
7 mon
9 mon
11 mon
13 mon
15 mon
17 mon
19 mon

Sedentary
Eercise

DR. All data are expressed as mean ± SEM (n=18). **p*<0.05, sedentary vs exercise from the same age, #*p*<0.05, DR exercise group compared to DR exercise baseline and 5 months old, two-way RM ANOVA with Bonferroni *post hoc* analysis.

3.3.2 Persistent effects of long-term exercise on spatial memory throughout the lifespan: The Object Displacement (OD) Task

Spatial-memory also was assessed every 2 months from the commencement of the experiment. The Object displacement (OD) task was used to assess spatial-memory and at the first OD assessment animals were 5 months (Figure 3.14 A). In the acquisition trial of the first OD task, 2 months after exercise commencement, when mice were 5 months old, there was no difference in the exploration of the three objects (objects A, B and C) in both sedentary and exercise groups ($p=0.4889$, two-way ANOVA, SED: Obj. A: 32.79±0.62%, Obj. B: 32.68±0.60%, Obj. C: 34.47%; EX: Obj. A: 33.31±0.73%, Obj. B: 33.53±0.59%, Obj. C: 33.10±0.84%, Bonferroni *post-hoc*, Figure 3.14 B). However, in the testing phase, twenty-four hours after the acquisition, statistical analysis showed a difference between exploration of the displaced object (object C) compared to the stationary objects (objects A and B) in both groups ($p<0.001$ two-way ANOVA, Object C vs. Object A and Object B, SED: 31.73±1.81%, Obj. B: 31.63±0.99%, Obj. C: 36.74±1.37%; EX: Obj. A: 29.89±0.96%, Obj. B: 28.37±0.90%, Obj. C: 41.63±1.03%, Bonferroni *post-hoc*, Figure 3.14 C). Further, in the first OD testing there was an interaction between the group and the exploration of objects. Bonferroni *post-hoc* analysis revealed that the exercise group explored the displaced object significantly more than the sedentary group ($p<0.001$, two-way ANOVA Object C exercise group vs. Object C sedentary group, Figure 3.14 C).

Four months after the commencement of exercise protocol animals were 7 months old. At this point, OD task was performed for the second time to assess spatial-memory after 4 months of exercise (Figure 3.15 A and B). In the acquisition, mice from both groups explored the 3 objects similarly ($p=0.8793$, two-way ANOVA, SED: Obj. A: 33.94±0.66%, Obj. B: 32.47±0.68%, Obj. C: 33.68±0.78%; EX: 33.26±1.46%, Obj. B: 33.74±0.96%, Obj. C: 32.95±1.14%, Bonferroni *post-hoc*, Figure 3.15 A). In contrast, during the second OD testing, performed twenty-four hours after acquisition, statistical analysis showed a difference between the exploration of the displaced (object C) and the stationary objects (objects A and B) in sedentary and exercise groups ($p<0.001$, two-way ANOVA, Object C vs. Object

A and Object B, SED: Obj. A: 29.73±0.73%, Obj. B: 27.63±0.63%, Obj. C: 47.74±0.89%; EX: Obj. A: 22.84±0.72%, Obj. B: 24.58±1.61%, Obj. C: 52.47±1.47%, Bonferroni *post-hoc*, Figure 3.15 B). Moreover, exercise group explored the displaced object significantly more compared to sedentary group ($p<0.001$, two-way ANOVA, Object C exercise group vs. Object C sedentary group, Figure 3.15 B).

Third and fourth OD were performed 6 and 8 months after exercise commencement, when mice were 9 (Figure 3.16 A and B) and 11 months old (Figure 3.17 A and B), respectively. In both, there was no difference in the exploration of any objects during the acquisition phase (3rd OD training - $p=0.4595$, SED: Obj. A: 32.89±0.68%, Obj. B: 33.47±.47%, Obj. C: 33.45±0.525; EX: Obj. A: 33.05±0.73%, Obj. B: 33.06±0.60%, Obj. C: 33.95±0.52, Figure 3.16 A; and 4th OD training - $p=0.6780$, SED: Obj. A: 32.89±1.07%, Obj. B: 32.28±0.74%, Obj. C: 34.83±1.05%; EX: Obj. A: 33.59±0.99%, Obj. B: 33.65±.26%, Obj. C: 32.84±0.83%, two-way ANOVA, Bonferroni *post-hoc*, Figure 3.17 A). Furthermore, during testing, in both assessments, sedentary and exercise mice explored more the displaced object (Object C) than the stationary objects (3rd OD testing - $p<0.001$, SED: Obj. A: 27.95±0.69%, Obj. B: 29.84±0.77%, Obj. C: 41.16±1.06; EX: Obj. A: 25.16±0.58%, Obj. B: 25.42±.65%, Obj. C: 49.58±0.79%, Figure 3.16 B; and 4th OD testing - $p<0.001$, SED: Obj. A: 27.39±1.39%, Obj. B: 29.78±1.70, Obj. C: 44.78±1.29%; EX: Obj. A: 21.53±1.33%, Obj. B: 22.94±1.45%, Obj. C: 55.59±1.96%, two-way ANOVA Object C vs. Object A and Object B, Figure 3.17 B). Furthermore, similar to the previous OD testing, statistical analysis showed that mice from exercise group explored more the displaced object compared to mice from sedentary group ($p<0.001$, two-way ANOVA, Object C exercise group vs. Object C sedentary group, Figure 3.16 B and Figure 3.17 B).

At this point, mice stopped exercising and were kept for 10 more months in the absence of exercise, as described above. The next OD assessment was performed 2 months after exercise cessation, when mice were 13 months old (5th OD assessment, Figure 3.18 A and B). Similar to previous results, in the acquisition trial, mice from both groups explored the three objects equally ($p=0.5236$, two-way

ANOVA, SED: Obj. A: 32.61±.96%, Obj. B: 33.89±0.64, Obj. C: 33.67±1.03%; EX: Obj. A: 32.170±1.30%, Obj. B: 33.23±1.49%, Obj. C: 34.12±1.72%, Bonferroni *post-hoc*, Figure 3.18 A). Again, during the testing, mice from both groups explored significantly more the object displaced (p<0.001, SED: Obj. A: 29.78±1.20%, Obj. B: 2.33±1.33%, Obj. C: 37.72±1.50%; EX: Obj. A: 24.35±1.22%, Obj. B: 26.29±1.28%, Obj. C: 49.35±1.50%, two-way ANOVA Object C vs. Object A and Object B, Figure 3.18 B) and exercised mice explored even more the displaced object when compared to sedentary mice (p<0.001, two-way ANOVA, Object C exercise group vs. Object C sedentary group, Figure 3.18 B).

Following, the next OD task was performed when mice were 15 months old, 4 months after exercise cessation (6th OD assessment, Figure 3.19 A and B). During acquisition, mice from both groups explored similarly the 3 objects (p=0.9036, two-way ANOVA, SED: Obj. A: 33.44±1.27%, Obj. B: 2.83±1.15%, Obj. C: 33.83±1.25%; EX: Obj. A: 33.76±0.84%, Obj. B: 3.35±1.28%, Obj. C: 32.76±0.96%, Bonferroni *post-hoc*, Figure 3.19 A). In the testing phase, mice from both groups still explored more the displaced object compared to objects A and B (p<0.001, SED: Obj. A: 33.50±1.42%, Obj. B: 29.11±1.26%, Obj. C: 38.00±.47%; EX: Obj. A: 26.41±1.24%, Obj. B: 26.82±1.46%, Obj. C: 46.76±2.16%, two-way ANOVA Object C vs. Object A and Object B, Figure 3.19 B). Statistical analysis also demonstrated that exercise mice explored the displaced object more compared to sedentary mice (p<0.001, two-way ANOVA, Object C exercise group vs. Object C sedentary group, Figure 3.19 B).

After that, OD was assessed 6 and 8 months after exercise cessation, when mice were 17 (7th OD assessment, Figure 3.20 A and B) and 19 months old (8th OD assessment, Figure 3.21 A and B), respectively. On both occasions, there was no difference in the exploration of the 3 objects during acquisition (7th OD training - p=0.1417, SED: Obj. A: 34.11±1.05%, Obj. B: 31.72±.06%, Obj. C: 34.11±.09%; EX: Obj. A: 31.88±1.26%, Obj. B: 33.23±0.94%, Obj. C: 34.88±0.83%, Figure 3.20 A; and 8th OD training - p=0.5851, SED: Obj. A: 32.50±1.34%, Obj. B: 34.83±1.26%, Obj. C: 32.67±1.18%; EX: Obj. A: 33.50±1.16%, Obj. B: 35.37±1.06%. Obj. C: 31.25±1.33%, two-way ANOVA, Bonferroni *post-hoc*, Figure

3.21 A). During testing in the 2 last OD assessments, only mice from the exercise group explored the displaced object significantly more than the stationary objects A and B (7th OD testing - $p<0.001$, SED: Obj. A: 31.89±1.73%, Obj. B: 30.72±1.80%, Obj. C: 37.5±1.28%; EX: Obj. A: 27.82±2.33%, Obj. B: 27.64±2.09%, Obj. C: 44.58±2.65%, two-way ANOVA, Object C vs. Object A and Object B, Figure 3.20 B; and 8th OD testing - $p<0.001$, SED: Obj. A: 29.11±1.53%, Obj. B: 35.72±1.37%, Obj. C: 36.22±1.55%; EX: Obj. A: 25.12±1.41%, Obj. B: 30.94±1.49%, Obj. C: 44.18±2.11%, two-way ANOVA Object C vs. Object A and Object B, Bonferroni *post-hoc*, Figure 3.21 B). On both occasions, statistical analysis demonstrated an increased exploration of the displaced object by exercise group when compared to sedentary group ($p<0.05$, two-way ANOVA, Object C exercise group vs. Object C sedentary group, Figure 3.20 B and Figure 3.21 B).

Finally, after all the OD assessments, the DR (how much mice prefer the displaced object rather the stationary objects) was calculated for the OD and results over time were analysed. From 5 months old until 19 months old, DR was higher in exercise mice compared to sedentary mice for the same age ($p<0.05$, DR 3 months old SED: 0.07±0.03, EX: 0.08±0.02; 5 months old SED: 0.08±0.03, EX: 0.16±0.02; 7 months old SED: 0.19±0.01, EX: 0.31±0.03; 9 months old SED: 0.17±.02, EX: 0.31±0.02; 11 months old SED: 0.23±0.02, EX: 0.40±0.04; 13 months old SED: 0.09±0.03, EX: 0.30±0.03; 15 months old SED: 0.09±.03, EX: 0.25±.04; 17 months old SED: 0.08±0.02, EX: 0.20±.05; 19 months old SED: 0.05±0.03, EX: 0.19±0.04, two-way RM ANOVA, sedentary vs. exercise from the same age, Figure 3.22). Also, statistical analysis showed a difference between the DR in exercise mice in all ages compared with their own baseline DR ($p<0.05$, DR exercise group compared to DR exercise baseline, Figure 3.22).

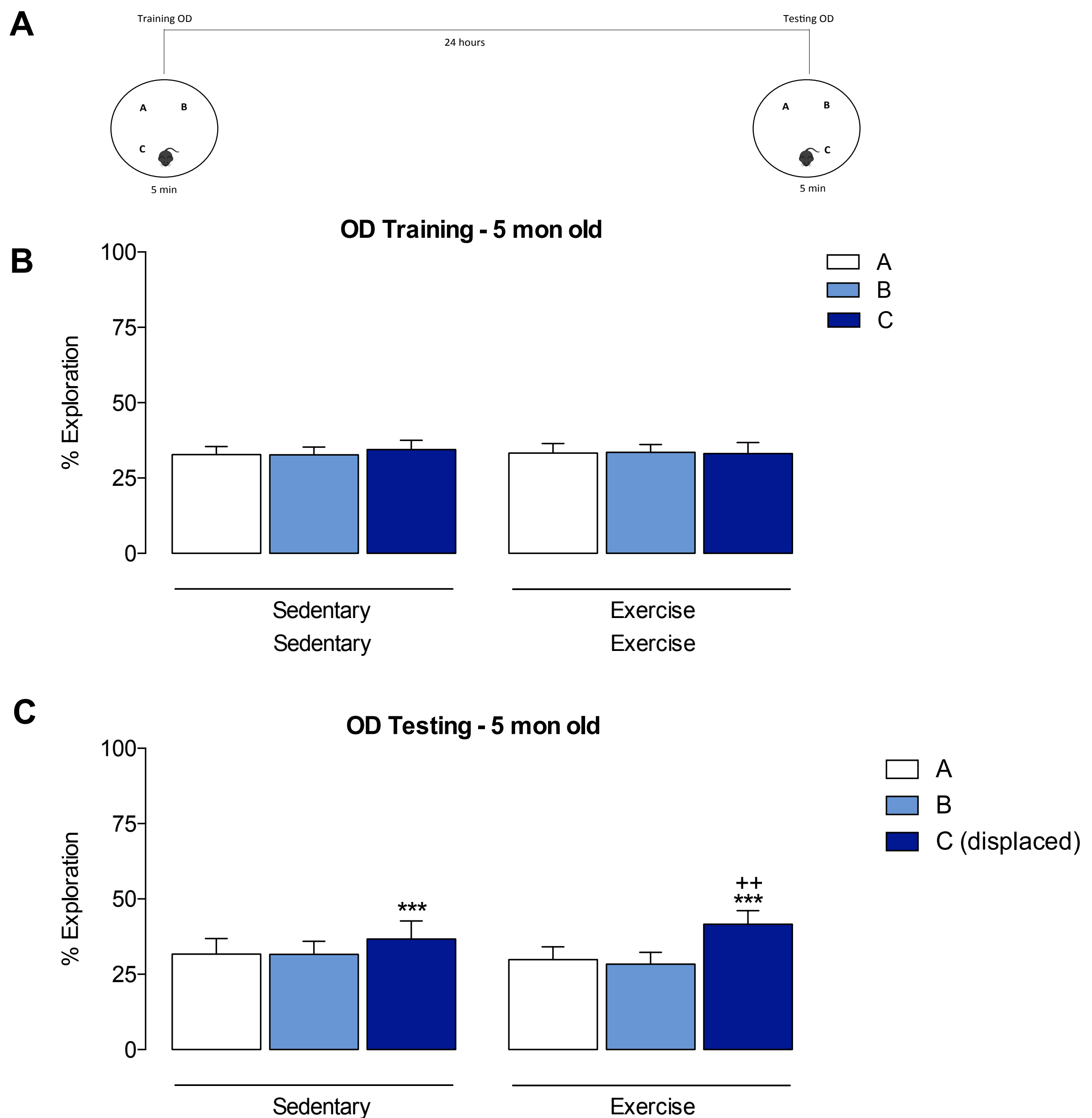


Figure 3.14 - Performance in OD task after 2 months of exercise

A schematic of the timeline utilized every 2 months to test mice in the OD task. This involved one 5 min acquisition trial followed by a 5 min testing trial, 24 hrs later **(A)**. In the acquisition trial, 2 months after of exercise commencement, when mice were 5 months old, there was no difference in the exploration of the 3 objects **(B)**. In the test, both groups displayed a significant preference for the displaced object (Object C) and exercise mice explored more the displaced object when compared to sedentary mice **(C)**. All data are expressed as mean ± SEM (n=19). ***p<0.001, Object A vs Object C, $^{+++}$*p*<0.001, Object C exercise group vs Object C sedentary group, two-way ANOVA with Bonferroni *post hoc* analysis.

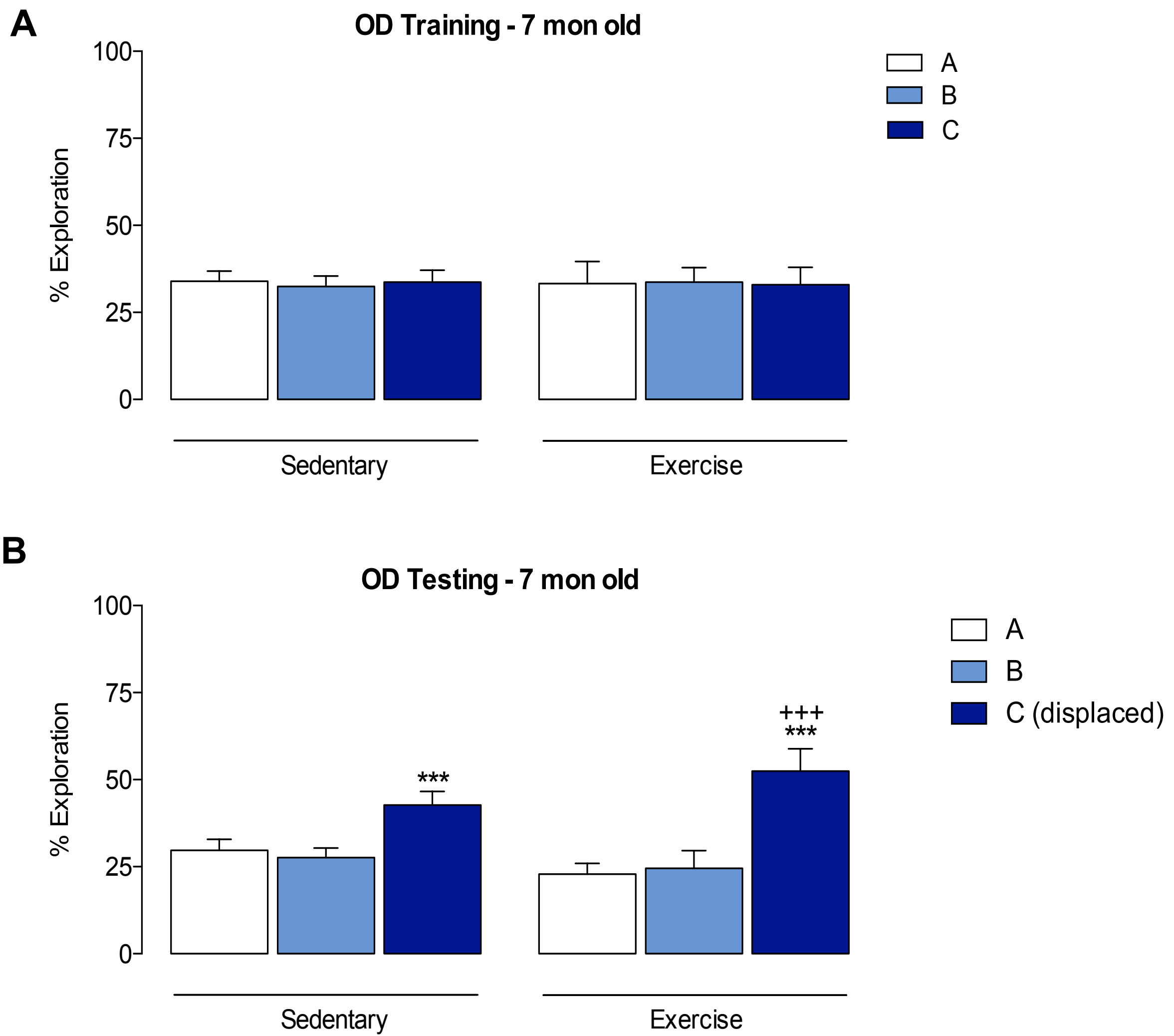


Figure 3.15 - Performance in OD task after 4 months of exercise

In the acquisition trial, 4 months after of exercise commencement, when mice were 7 months old, there was no difference in the exploration of the 3 objects **(A)**. In the test, both groups displayed a significant preference for the displaced object (Object C) and exercise mice explored more the displaced object when compared to sedentary mice **(B)**. All data are expressed as mean ± SEM (n=19). ***p<0.001, Object A vs Object C, +++*p*<0.001, Object C exercise group vs Object C sedentary group, two-way ANOVA with Bonferroni *post hoc* analysis.

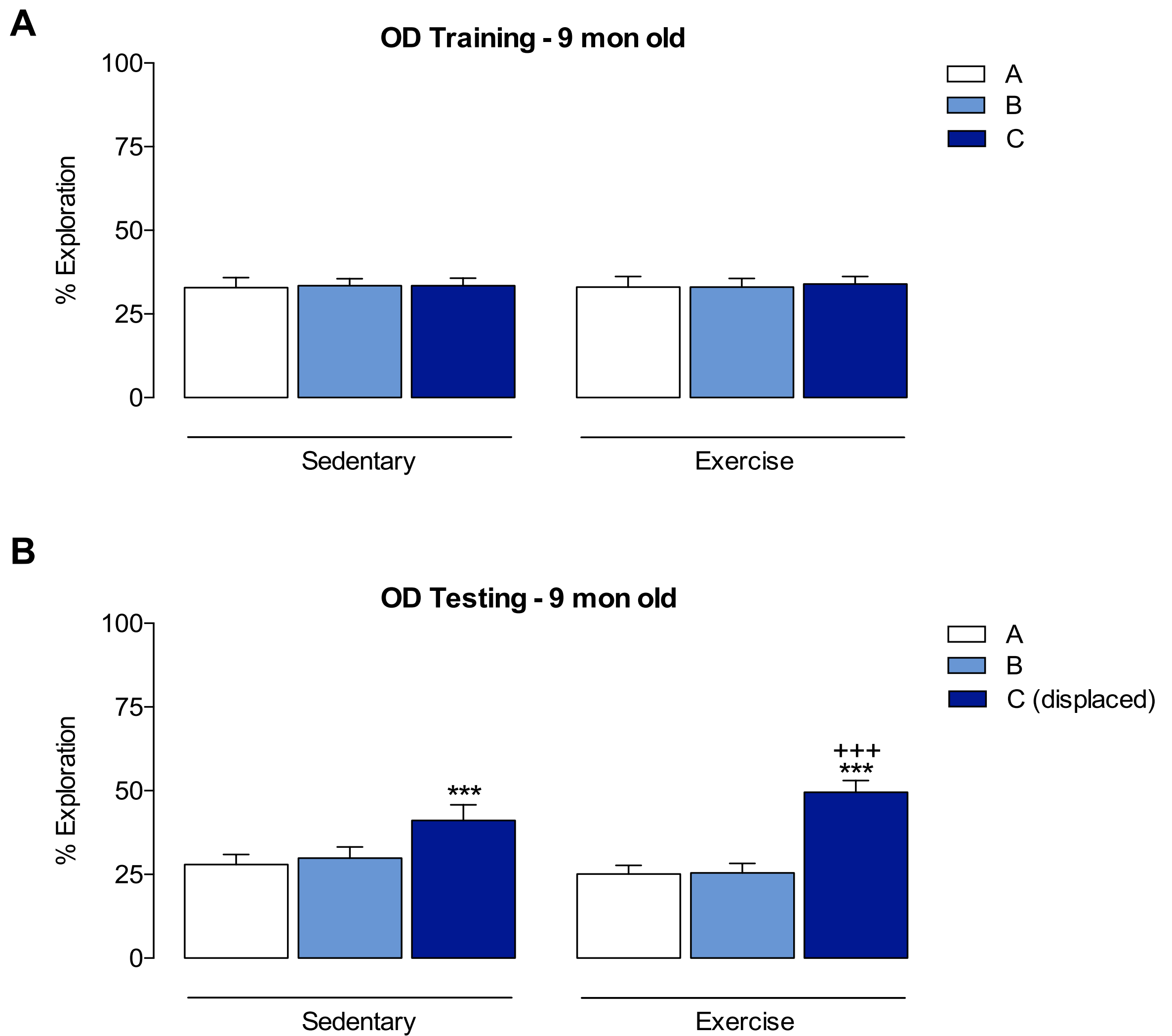


Figure 3.16 - Performance in OD task after 6 months of exercise

In the acquisition trial, 6 months after of exercise commencement, when mice were 9 months old, there was no difference in the exploration of the 3 objects **(A)**. In the test, both groups displayed a significant preference for the displaced object (Object C) and exercise mice explored more the displaced object when compared to sedentary mice **(B)**. All data are expressed as mean ± SEM (n=19). ***p<0.001, Object A vs Object C, $^{+++}$*p*<0.001, Object C exercise group vs Object C sedentary group, two-way ANOVA with Bonferroni *post hoc* analysis.

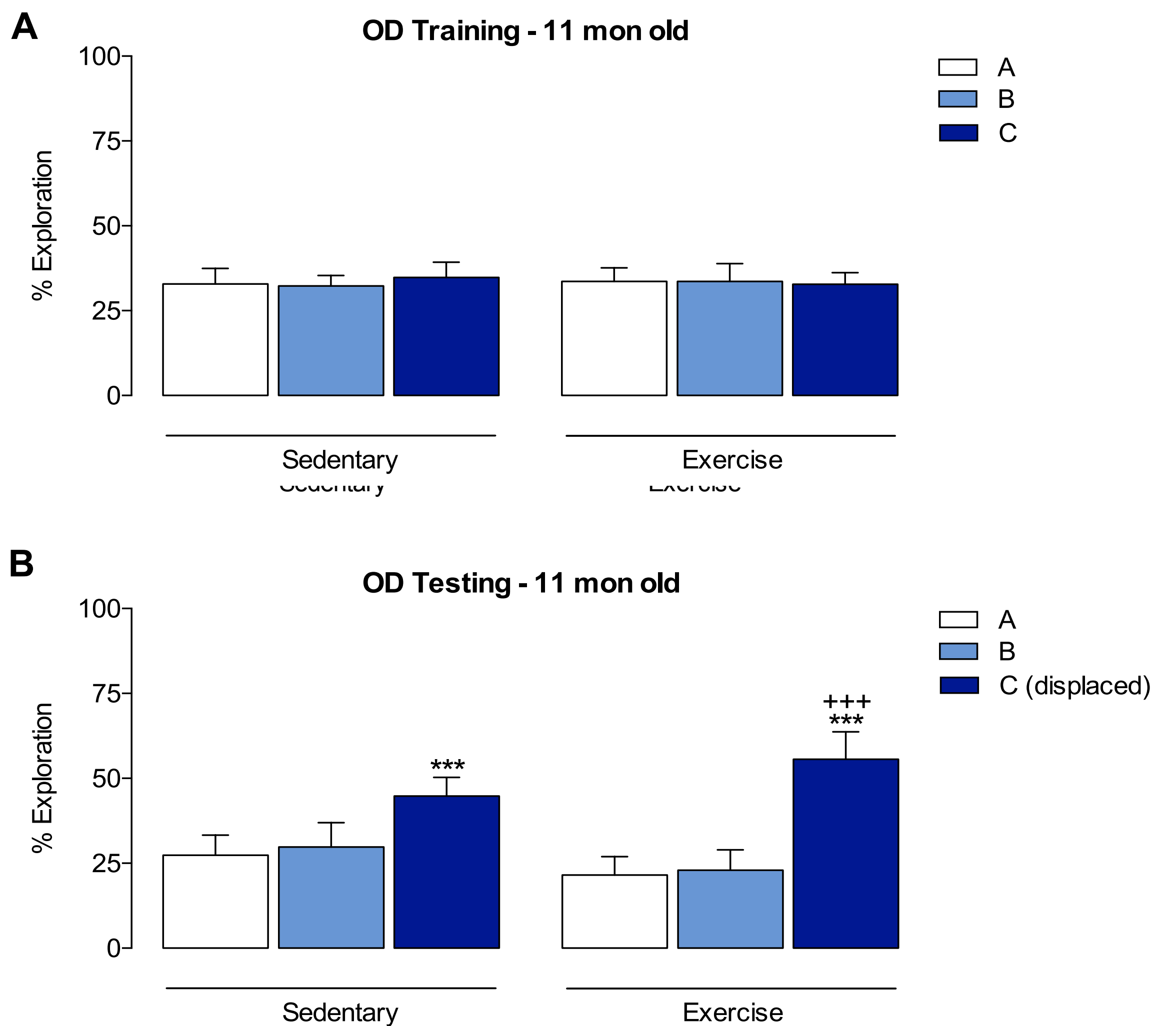


Figure 3.17 - Performance in OD task after 8 months of exercise

In the acquisition trial, 8 months after of exercise commencement, when mice were 11 months old, there was no difference in the exploration of the 3 objects **(A)**. In the test, both groups displayed a significant preference for the displaced object (Object C) and exercise mice explored more the displaced object when compared to sedentary mice **(B)**. All data are expressed as mean ± SEM (n=19). ***p<0.001, Object A vs Object C, $^{+++}$*p*<0.001, Object C exercise group vs Object C sedentary group, two-way ANOVA with Bonferroni *post hoc* analysis.

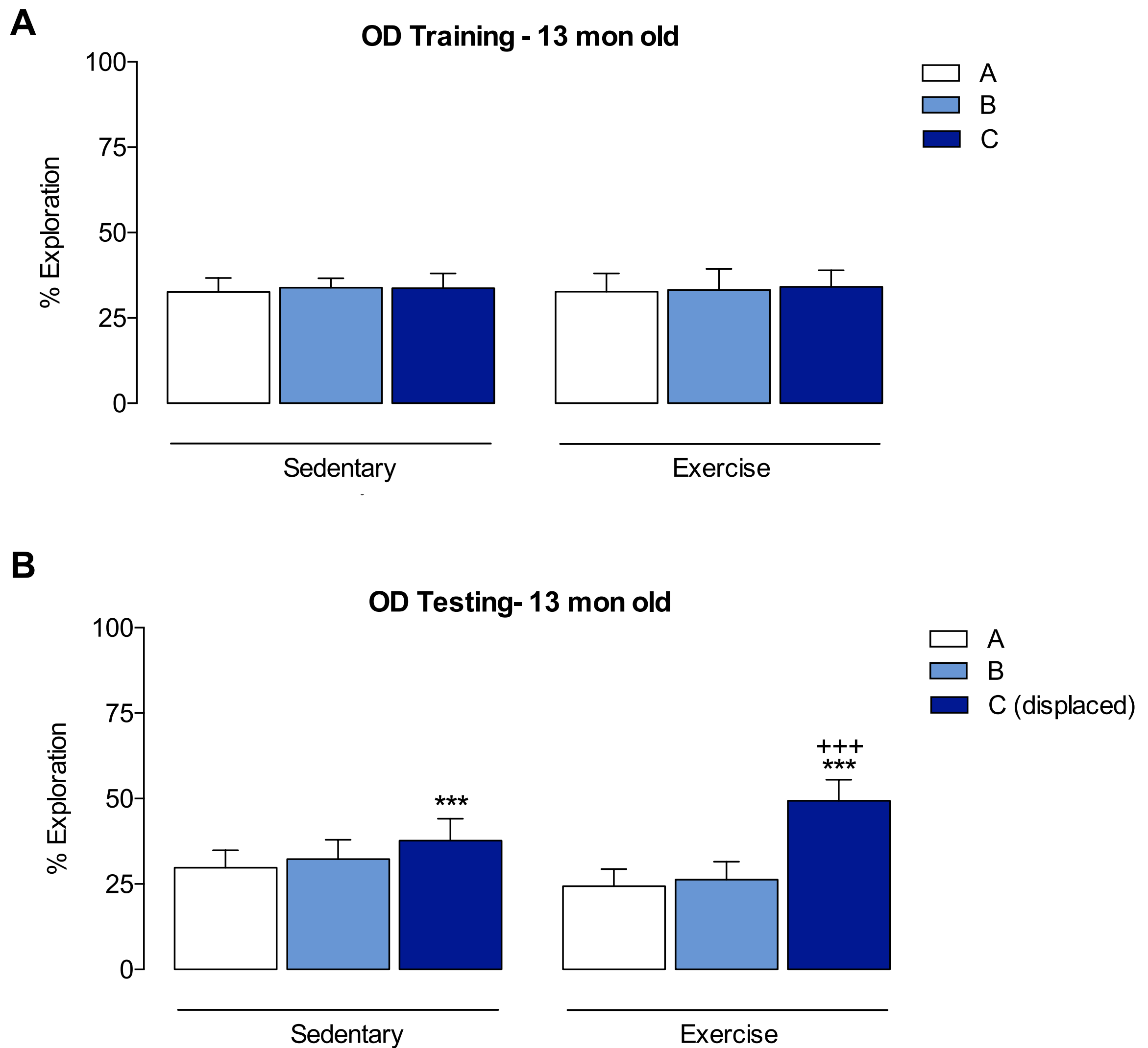


Figure 3.18 - Performance in OD task 2 months after exercise cessation

At this point, mice had stopped exercising 2 months previously, and were 13 months old. In the acquisition trial of OD, mice explored the 3 objects equally **(A)**. During testing, both groups still displayed a significant preference for the displaced object (Object C) and exercise group explored more the displaced object when compared to sedentary group **(B)**. All data are expressed as mean ± SEM (n=18). ***p<0.001, Objects A and B vs Object C, $^{+++}$*p*<0.001, Object C exercise group vs. Object C sedentary group, two-way ANOVA with Bonferroni *post hoc* analysis.

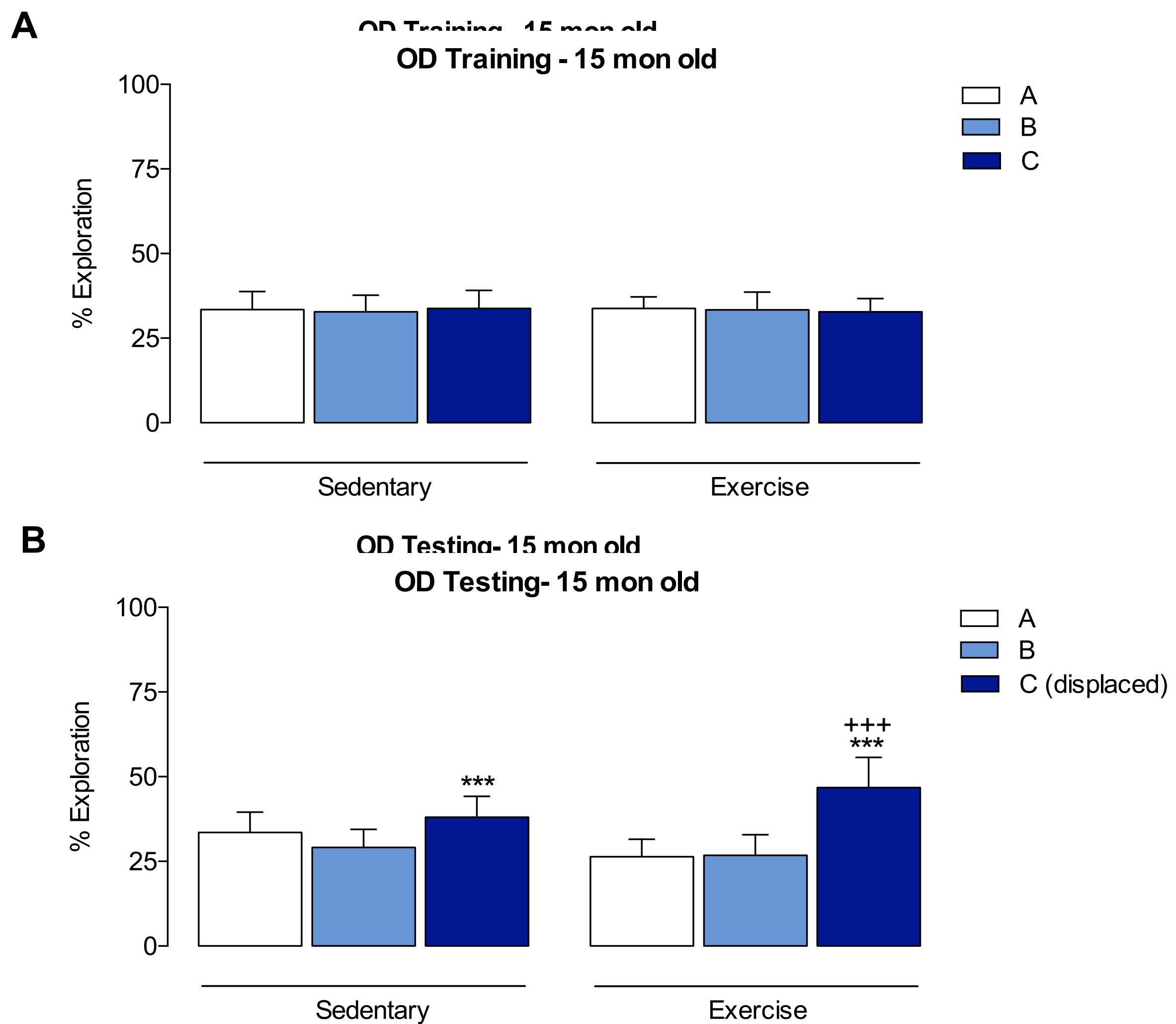


Figure 3.19 - Performance in OD task 4 months after exercise cessation

At this point, mice had stopped exercising 4 months previously, and were 15 months old. In the acquisition trial of OD, mice explored the 3 objects equally **(A)**. During testing, both groups still displayed a significant preference for the displaced object (Object C) and exercise group explored more the displaced object when compared to sedentary group **(B)**. All data are expressed as mean ± SEM (n=18). $^{***}p<0.001$, Objects A and B vs Object C, $^{+++}p<0.001$, Object C exercise group vs. Object C sedentary group, two-way ANOVA with Bonferroni *post hoc* analysis

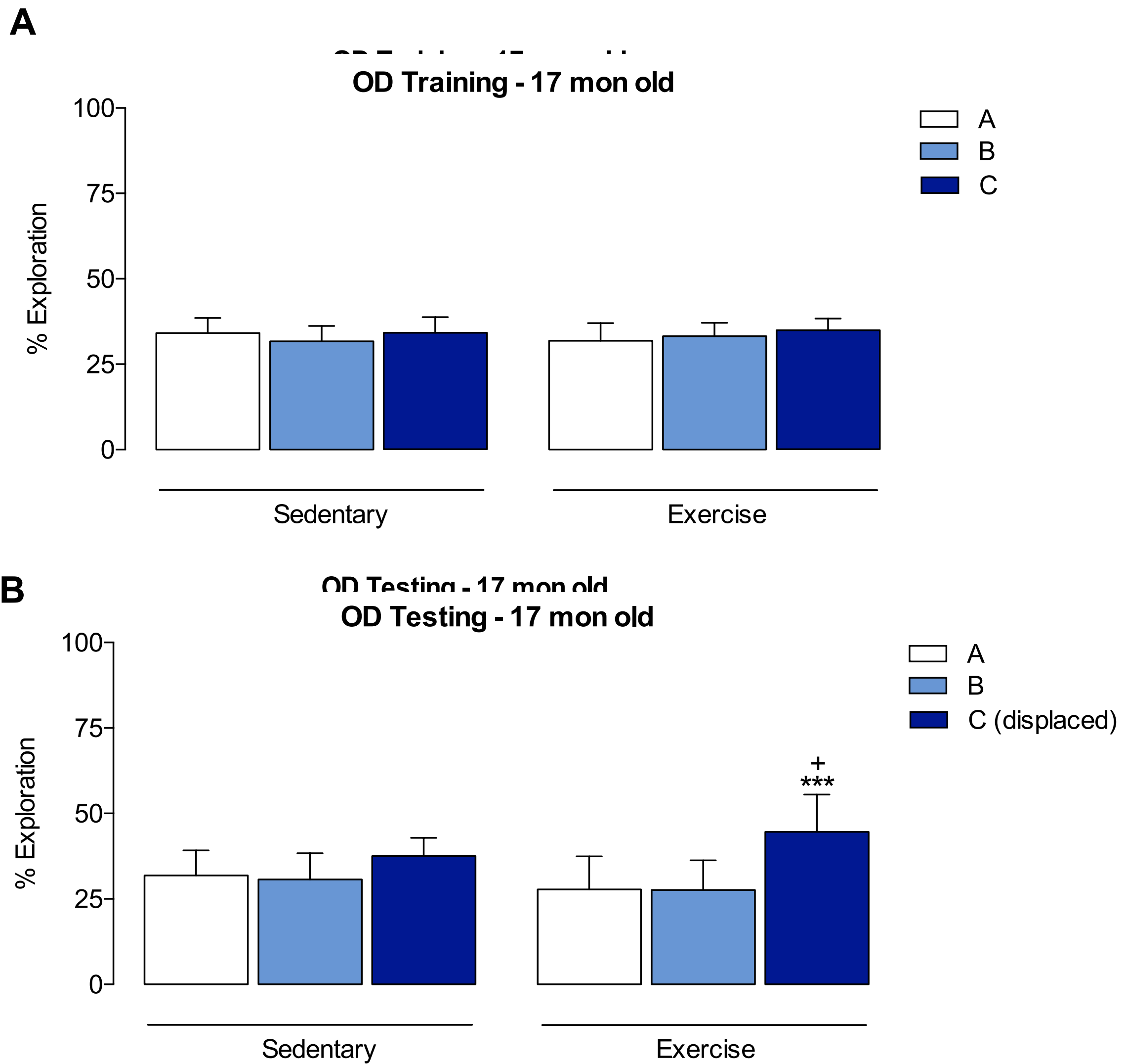


Figure 3.20 - Performance in OD task 6 months after exercise cessation

At this point, mice had stoped exercising 6 months previously, and were 17 months old. In the acquisition trial of OD, mice explored the 3 objects equally **(A)**. During testing, only exercised mice still demonstrated a preference for the displaced object (Object C) and exercise group still explored more the displaced object when compared to sedentary group **(B)**. All data are expressed as mean ± SEM (n=18). ***p<0.001, Objects A and B vs Object C, $^{+++}$*p*<0.001, Object C exercise group vs. Object C sedentary group, two-way ANOVA with Bonferroni *post hoc* analysis.

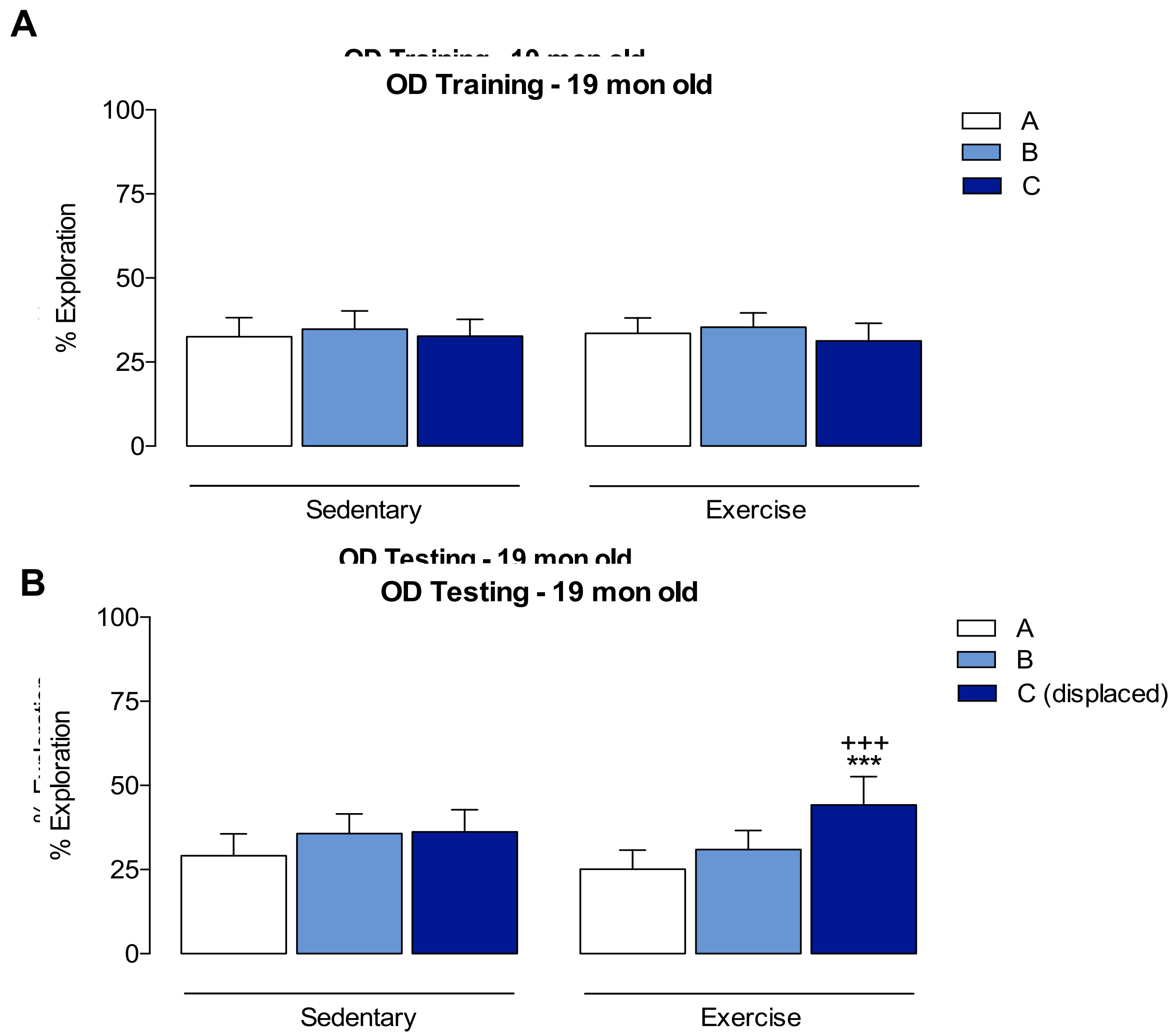


Figure 3.21 - Performance in OD task 8 months after exercise cessation

The last OD assessment was performed when mice had stopped exercising 8 months previously and were 19 months old. In the acquisition trial of OD, mice explored the 3 objects equally **(A)**. During testing, only exercised mice still demonstrated a preference for the displaced object (Object C) and exercise group still explored more the displaced object when compared to sedentary group **(B)**. All data are expressed as mean ± SEM (n=18). ***p<0.001, Objects A and B vs Object C, $^{+++}$*p*<0.001, Object C exercise group vs. Object C sedentary group, two-way ANOVA with Bonferroni *post hoc* analysis.

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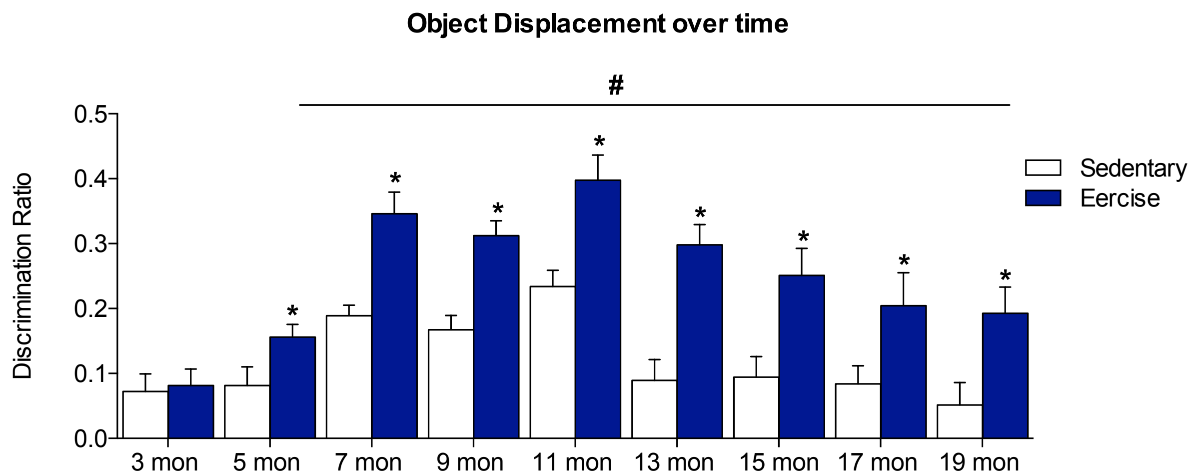


Figure 3.22 - Analysis of OD task over time

DR (how much mice preferred the displaced object compared to the stationary objects) was calculated and analysed over time for the OD task. A baseline assessment was performed before mice were grouped assigned. After that, OD was performed each 2 months. At 11 months old mice stopped exercising and OD learning was tested every 2 months, until 19 months old. From 5 months old, 2 months after exercise commencement, until 19 months old, 8 months after exercise cessation, statistical analysis demonstrated a significant difference in the DR of exercise mice compared to sedentary mice at same age in the OD task. Also, from 5 months old until 19 months the DR of exercise mice was significantly higher compared to their baseline. All data are expressed as mean ± SEM (n=18). *$p<0.05$, sedentary vs exercise from the same age, $^{\#}p<0.05$, DR exercise group compared to DR exercise baseline and 5 months old, two-way RM ANOVA with Bonferroni *post hoc* analysis.

3.3.3 Persistent effects of long-term exercise on spatial learning and memory in ageing: The Morris Water Maze (MWM) Task

The last memory task mice performed was the Morris Water Maze (MWM) task. At this point mice had been tested in NOR and OD tasks, the OF task and the EPM task and were aged between 20 and 21 months old. The test consisted of 5 days of training with a hidden platform, as described above in section 3.2.4.5. During the first 4 days of training in the MWM, mice from exercise and sedentary groups showed similar latency to find the platform (p=0.2618, two-way RM ANOVA, Day 1 SED: 41.94±2.92s and EX: 47.40±2.75s; Day 2 SED: 44.26±4.20s and EX: 44.32±2.75s; Day 3 SED: 31.55±3.11s and EX: 29.84±3.95s; and Day 4 SED: 29.55±4.60s and EX: 20.87±3.97s, Bonferroni *post-hoc*, Figure 3.23 A). However, on day 5, the last day of training in the MWM, mice from exercise group showed a significant decrease in the latency to find the platform compared to sedentary mice (p=0.0377, two-way RM ANOVA Sedentary vs Exercise, SED: 32.60±1.96s, EX: 19.50±3.22s , Bonferroni *post-hoc*, Figure 3.23 A).

Statistical analysis showed no difference in the swimming speed between the groups over the 5 days of training (p=0.5474, two-way RM ANOVA, Day 1 SED: 15.77±0.40 cm/s and EX: 15.34±0.42 cm/s; Day 2 SED: 15.53±0.40 cm/s and EX: 15.29±0.53 cm/s; Day 3 SED: 16.75±0.59 cm/s and EX: 16.91±0.61 cm/s; Day 4 SED: 15.45±1.04 cm/s and EX: 16.54±0.90 cm /s; and Day 5 SED: 14.69±0.83 and EX: 16.18±0.67 cm/s, Bonferroni *post-hoc*, Figure 3.23 B).

Twenty-four hours following the last training session, the probe trial was performed. The platform was removed from the maze, and the time spent in the target quadrant, crossing the annulus area and swimming speed were analysed. In this trial, exercise mice spent more time in the target quadrant compared to the other quadrants, while the sedentary group did not (p=0.0127, two-way RM ANOVA, time spent in the target quadrant vs other quadrants exercise, Target quadrant SED: 28.33±2.32s and EX: 36.93±3.26s; Opposite quadrant SED: 22.13±2.32s and EX: 24.93±2.60s; Left quadrant SED: 32.06±2.56s and

EX:22.13±1.51s; and Right quadrant SED: 17.26±2.64s and EX: 15.96±1.90s, Bonferroni *post-hoc*, Figure 3.24 A and B).

Also, during the probe trial, exercise mice made significantly more crossings of the annulus area compared to sedentary mice (p=0.0239, Student's *t*-Test, SED: 1.67±0.44 times and EX: 3.10±0.38 times, Figure 3.25 A). Furthermore, no difference in the swimming speed was observed in the probe trial (p=0.4017, Student's *t*-Test, SED: 16.80±0.93 cm/s and EX: 17.97±0.99 cm/s, Figure 3.25 B).

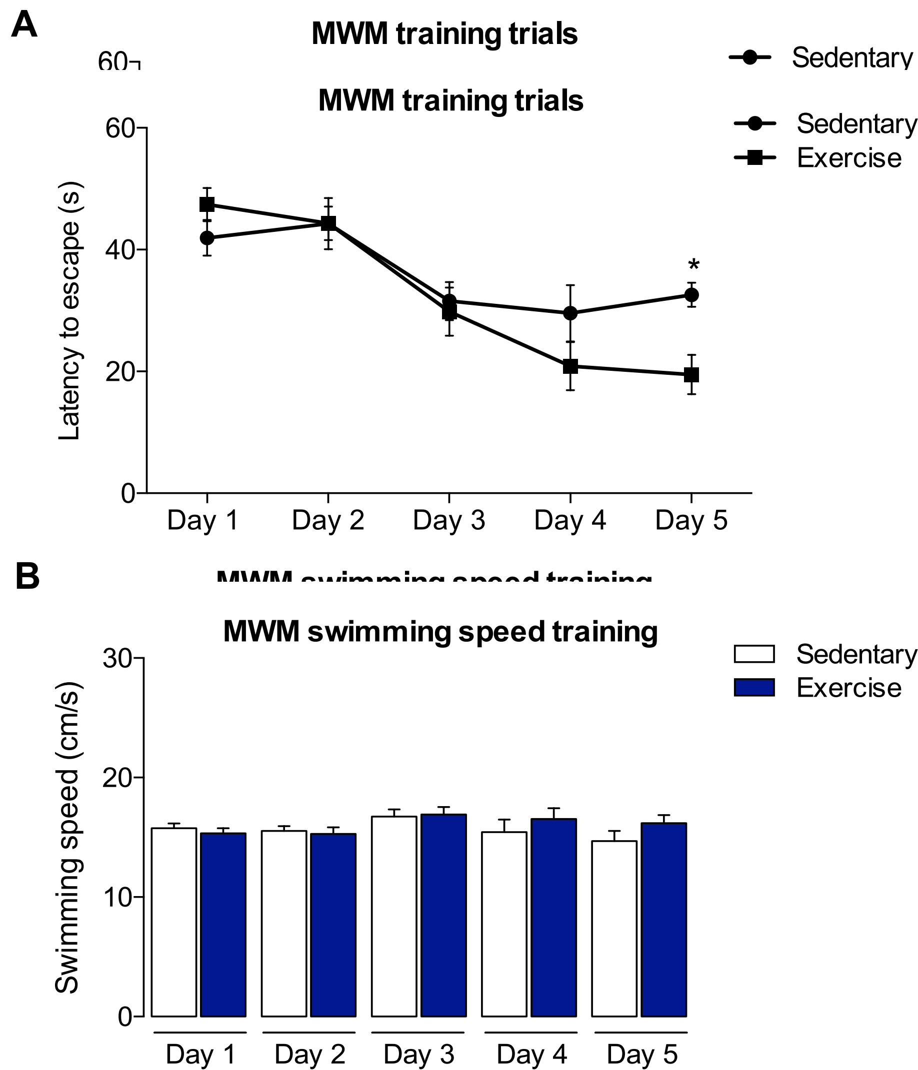


Figure 3.23 - Performance in the training trial of MWM between 20 and 21 months old
MWM training was performed for 5 consecutive days, consisting of 4 trials per day. On the last day of MWM training (Day 5) exercise mice showed a decrease in the latency to find the hidden platform, compared to sedentary mice **(A)**. There was no difference in the swimming speed during the 5 days of MWM training **(B)**. All data are expressed as mean ± SEM (n=10). *p<0.05, Sedentary vs Exercise, two-way RM ANOVA with Bonferroni *post hoc* analysis.

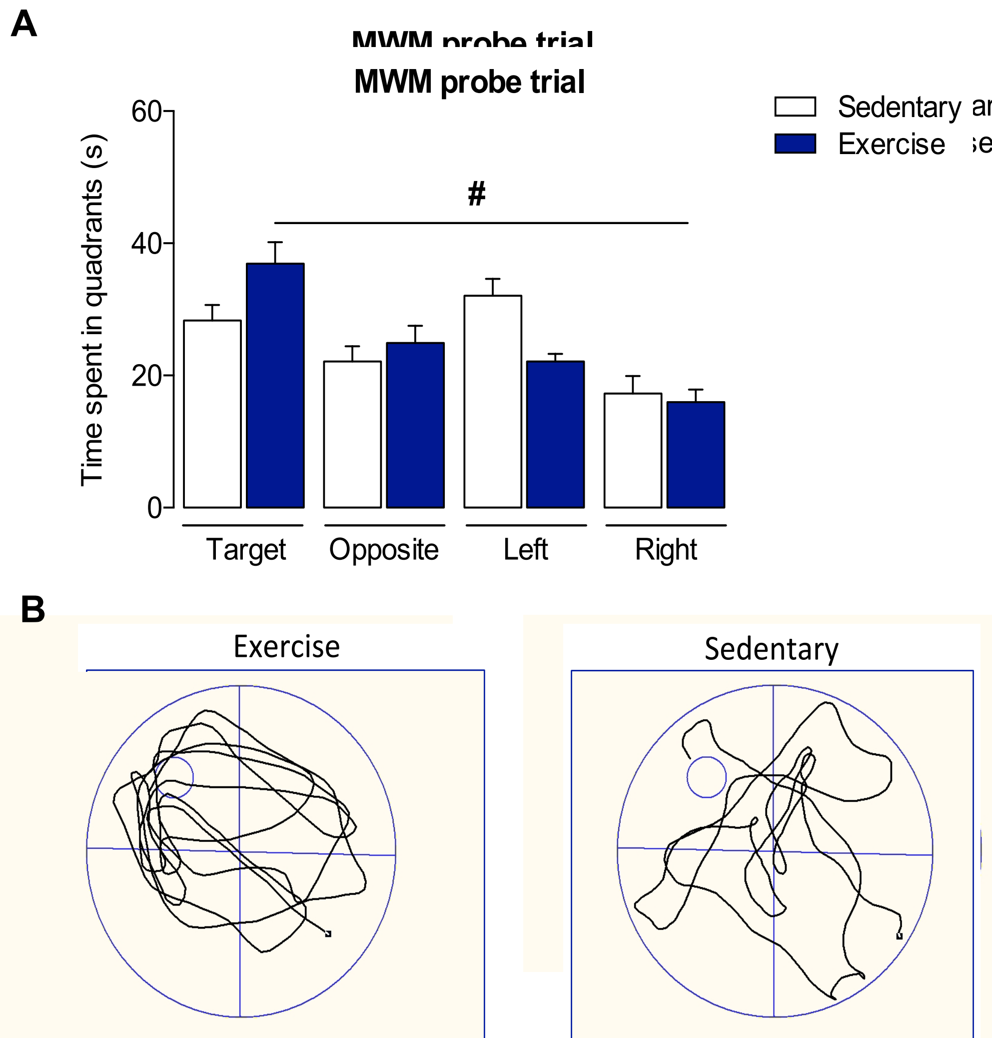


Figure 3.24 - Performance in the probe trial of MWM between 20 and 21 months old (time in the quadrants)

MWM probe trial was performed 24 hours after the last training session, consisting of one-single trial. Exercised mice spent more time in the target quadrant when compared to other quadrants **(A)**. Representative image showing the pathway in the MWM probe trial of exercise and sedentary mice **(B)**. All data are expressed as mean ± SEM (n=10). *p<0.05, Sedentary vs Exercise, $^{\#}$*p*<0.05, time spent in the target quadrant compared to all quadrants in exercise group, two-way RM ANOVA with Bonferroni *post hoc* analysis.

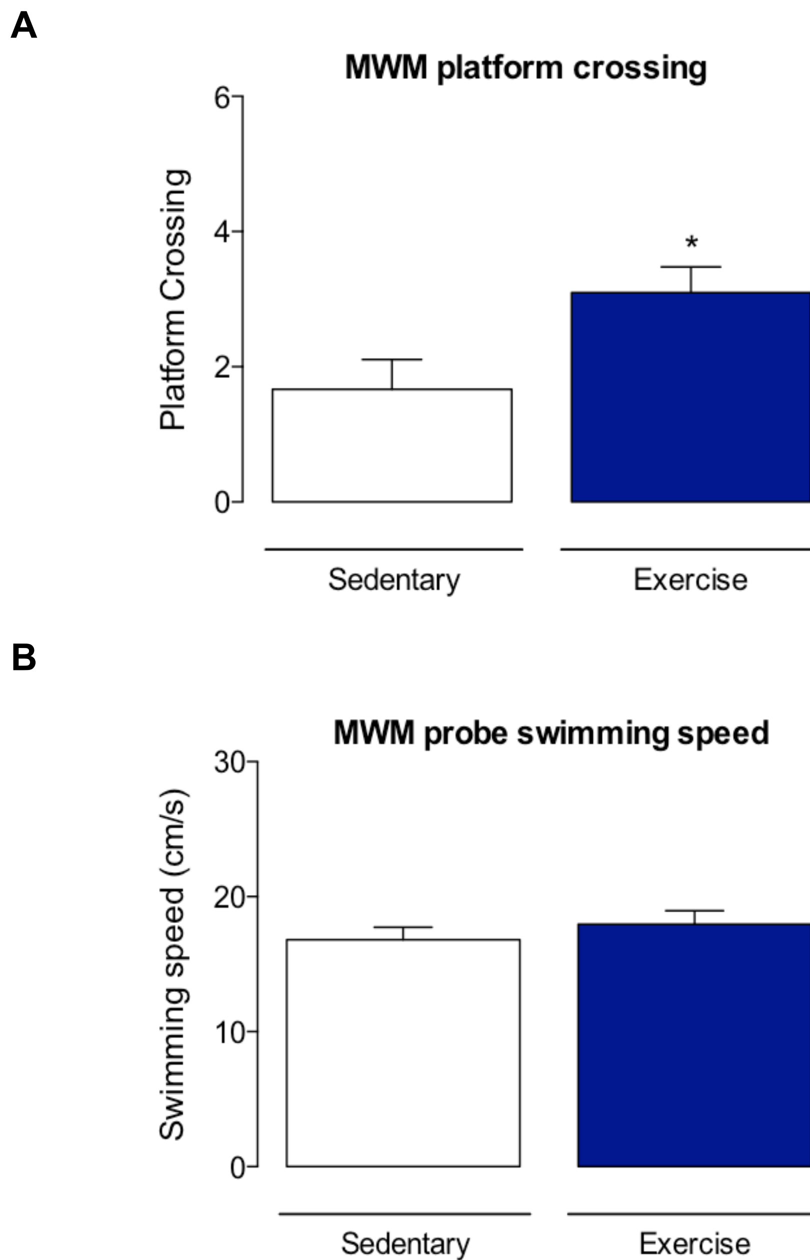


Figure 3.25 - Performance in the probe trial of MWM between 20 and 21 months old (crossing annulus area)

MWM probe trial was performed 24 hours after the last training session, consisting of one-single trial. Mice from exercise group crossed the annulus area more times during the probe trial when compared to sedentary group **(A)**. There was no difference in the swimming speed between the groups, in the MWM probe trial **(B)**. All data are expressed as mean ± SEM (n=10). *p<0.05, Sedentary vs Exercise, Student's *t*-Test.

3.3.4 Persistent effects of long-term exercise on anxiety and depression-like behaviour in ageing: The Open Field, the Elevated Plus maze and the Tail Suspension Tasks

Exploratory and locomotor activities were assessed 4 days after the last NOR and OD tasks, when mice were 19 months old. The Open Field (OF) task was used, to assess whether ageing and/or physical exercise would affect locomotor and exploratory activity of mice, that could interfere with the behaviour activity in the behaviour tasks employed. Both groups performed similarly in the open field, with no significant changes observed in the parameters analysed, including crossing (p=0.7294, Student's *t*-Test, SED: 90.89±6.80 and EX: 87.94±4.66, Figure 3.26 A) and rearing (p=0.8369, Student's *t*-Test, SED: 24.94± 2.71 and EX: 24.25±1.80, Figure 3.26 B).

The day after the OF task, mice were submitted to the EPM task. This task was employed to evaluate anxiety-like behaviour, when mice were between 19 and 20 months old. Statistical analyses demonstrated that there was no difference in the number of entries in the open arm between the groups (p=0.1282, Student's *t*-Test, SED: 0.72±0.28 entries and EX: 1.37±0.31 entries, Figure 3.27 A). However, the number of entries in the closed arm and the total number of entries of exercise group was higher that sedentary group (closed arm entries - p=0.0214, Student's *t*-Test, SED: 7.05±1.00 and EX: 10.5±1.00, Figure 3.27 B; and total entries - p=0.0215, Student's *t*-Test, SED: 7.78±1.18 and EX: 11.88±1.21, Figure 3.27 C). Moreover, statistical analysis demonstrated that there was no difference in the time spent in the open arm between the groups (p=0.3197, Student's *t*-Test, SED: 1.68±0.84s and EX: 2.92±0.88s, Figure 2.28 A). On the other hand, exercise mice spent less time in the closed arm compared to sedentary mice (p=0.0089, Student's *t*-Test, SED: 91.68±1.65s and EX: 85.31±1.56s, Figure 3.28 B). Analysis of the anxiety score (time spent in the open and closed arms divided by the total time of arms exploration) showed a lower score for the exercise group compared to sedentary group (p=0.0315, Student's *t*-Test, SED:0.90±0.02 and EX:0.82±0.02, Figure 3.28 C).

Finally, mice underwent the last behaviour assessment, when they were approximately 21 months old. The TST was used to measure depression-like behaviour. The immobility time of the exercise group during the 5 minutes of testing was significant lower than the immobility time of the sedentary group ($p<0.001$, Student's *t*-Test, SED: 167.2±6.39s and EX: 129.2±5.92s, Figure 3.29), suggesting that exercise mice showed a less depressive-like behaviour.

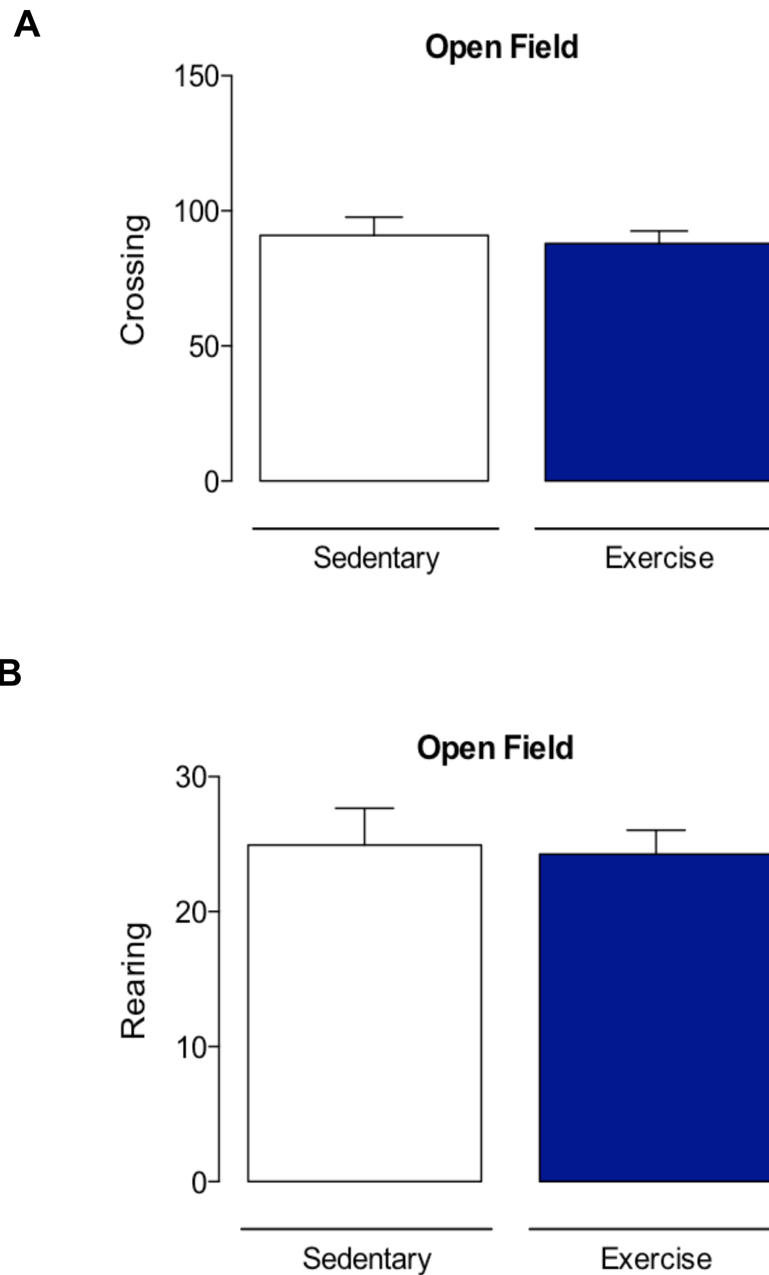


Figure 3.26 - Locomotor and exploratory activity in the OF at 19 months old
Locomotor and exploratory activity were assessed using the OF task. No difference was observed in the number of crossings and rearings between the groups **(A and B)**. All data are expressed as mean ± SEM (n=18), Student's *t*-Test.

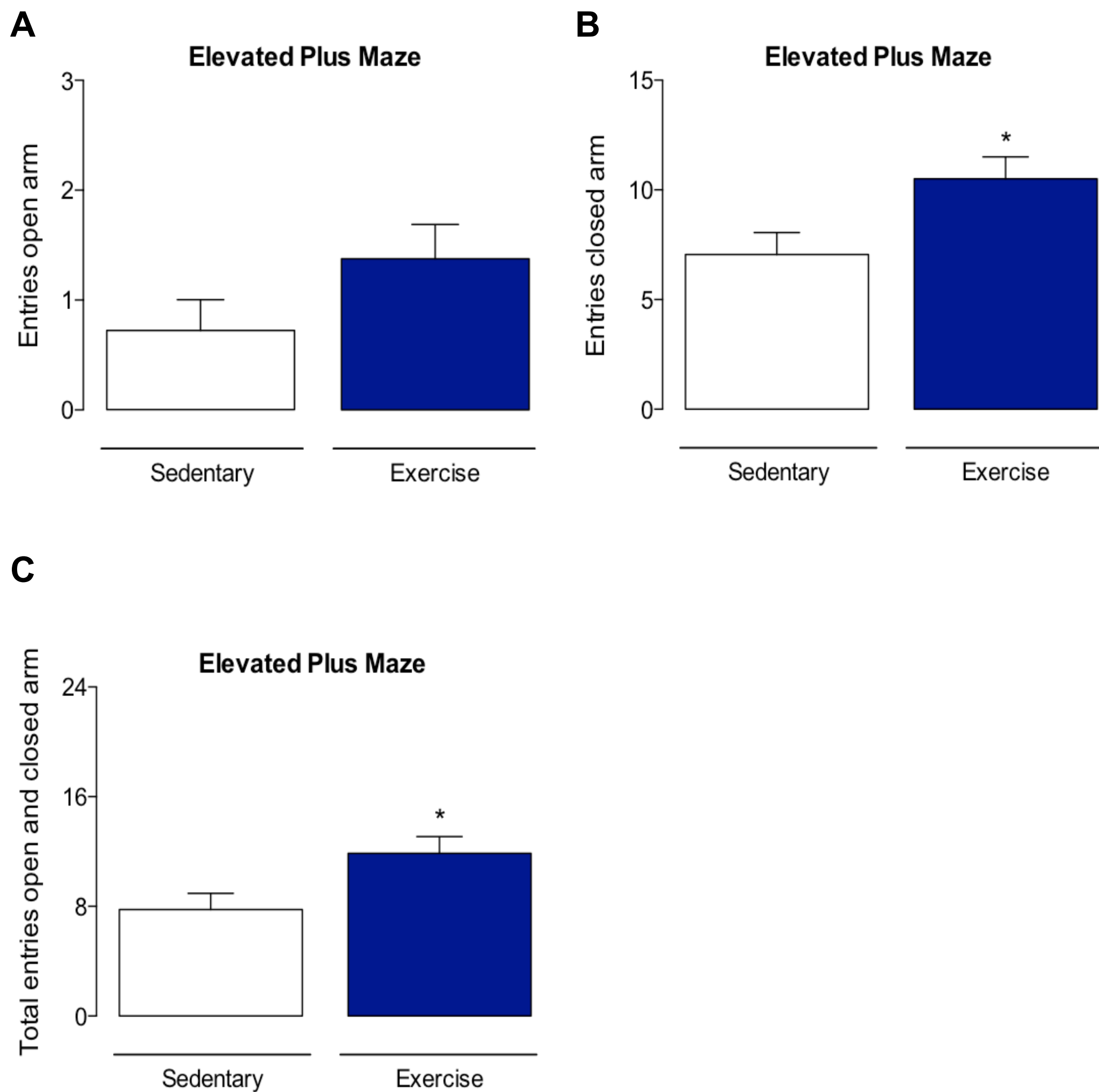


Figure 3.27 - Anxiety-like behaviour in the EPM at between 19 and 20 months old (entries in the arms)

Anxiety-like behavior was assessed using the EPM task. There was no difference between groups in the number of entries in the open arms **(A).** Statistical analysis demonstrated a significant increase in the number of entries in the closed arm and in the total number of entries in exercise mice compared to sedentary mice **(B and C)**. All data are expressed as mean ± SEM (n=18), *p<0.05, Sedentary vs Exercise, Student's *t*-Test.

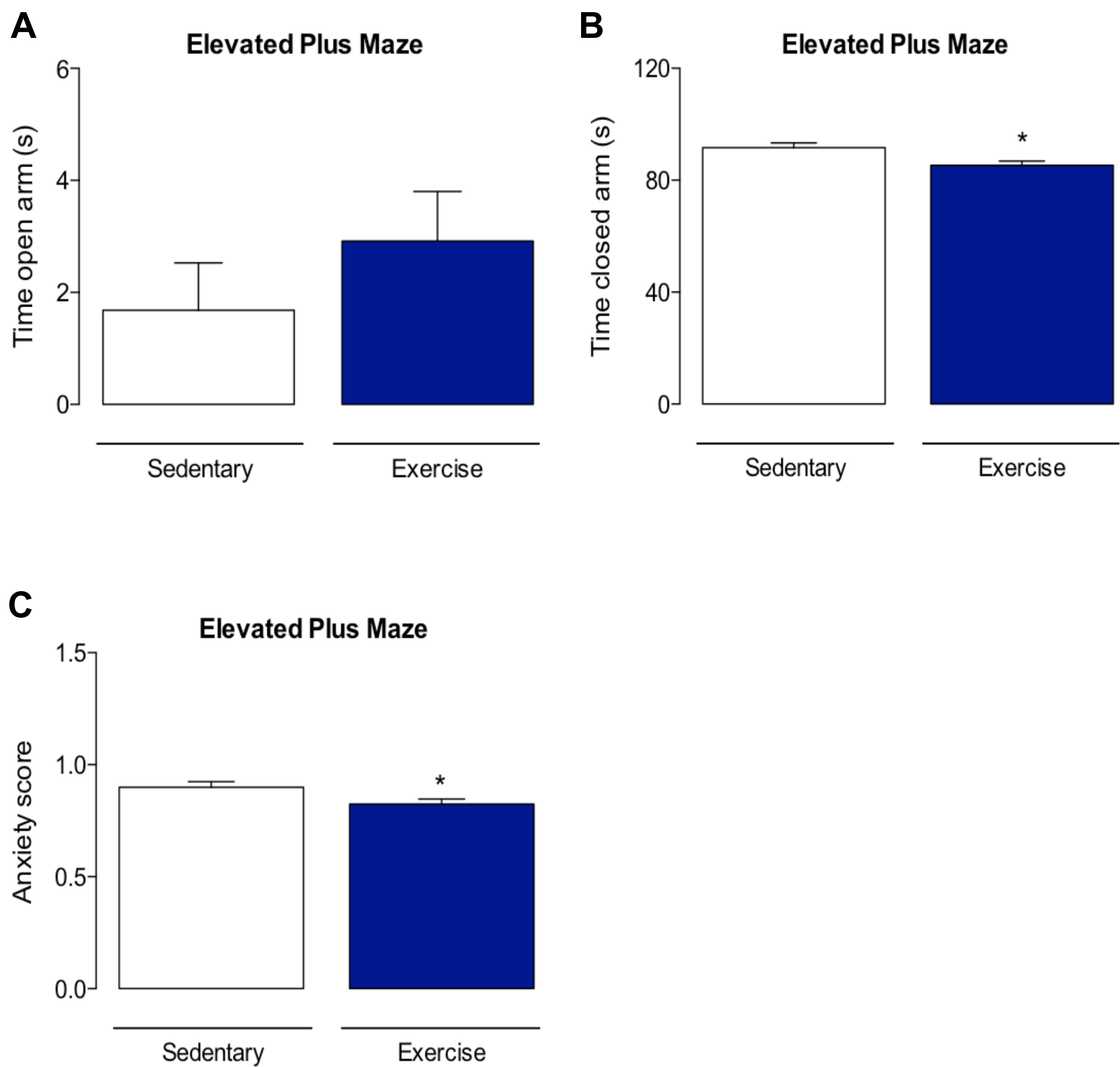


Figure 3.28 - Anxiety-like behaviour in the EPM between 19 and 20 months old (time in the arms)

Anxiety-like behavior was assessed using the EPM task. There was no difference between groups in the time spent in the open arms **(A).** Statistical analysis showed that exercised mice spent significantly less time in the closed arm **(B)**. Also, analysis of the anxiety score (time spent in open and closed arm by the total time of exploration) demonstrated a lower anxiety score for exercise group compared to sedentary group **(C)**. All data are expressed as mean ± SEM (n=18), *p<0.05, Sedentary vs Exercise, Student's *t*-Test.

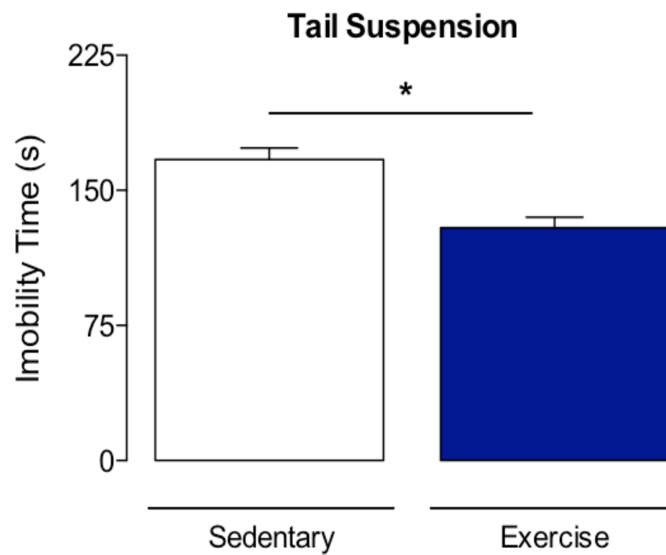


Figure 3.29 - Depressive-like behaviour in the TS task at 21 months old

TS task was used to assess depressive-like behavior at 21 months old. Exercise mice showed a decreased immobility time in the tail suspension task when compared to sedentary mice, suggesting exercise is attenuating age-induced depression-like behaviour. All data are expressed as mean ± SEM (n=18). *p<0.05, Sedentary vs Exercise, Student's *t*-Test.

3.4 Discussion

The main objective of this chapter was to investigate whether exercise during youth and middle-age would protect against age-related cognitive decline in the mouse. Firstly, we established the effect of 8 months of treadmill running, between young-adult and middle-age, on recognition and spatial memory throughout the lifespan, assessing mice performance on NOR and OD tasks, respectively, every 2 months until old age. Secondly, we assessed space referential learning and memory in old age, using the MWM task, to confirm the persistent effects of exercise on age-related impairment in spatial memory. Finally, we determined the effects of this early-life exercise on age-related anxiety and depression-like behaviour, using the EPM and TST, respectively.

The data presented here demonstrate that long-term forced exercise facilitates recognition memory (NOR) and spatial memory (OD) throughout the lifespan and protects against age-induced impairments in space referential learning and memory (MWM), as well as reducing anxiety and depression-like behaviour in old age, even 10 months after exercise cessation. Although there are many studies in the literature showing the beneficial effects of forced and voluntary exercise on cognition and brain plasticity in both humans and animals (Anderson et al., 2000, Ang et al., 2006, Marlatt et al., 2012), only few have assessed how long these effects persist throughout the lifespan after exercise cessation (Morgan et al., 2018).

3.4.1 Recognition memory throughout the mouse lifespan

Recognition memory was assessed every 2 months, with the first assessment at 5 months old (2 months after exercise commencement) and the last assessment at 19 months old (8 months after the end of exercise protocol), The task used was the NOR task, which has been widely employed to examine declarative memory in animals (Leger et al., 2013) and whose utility relies on the premise that animals have a preference for novelty (Rutten et al., 2008a). The effects of physical exercise in facilitating recognition memory on the NOR task in young healthy animals have been reported previously (Bechara et al., 2014, Hopkins et al., 2011)

as well as its effects in preventing and/or restoring age-related cognitive impairments (Leger et al., 2013). Our findings in the NOR task complement these published studies, since we have shown exercise to enhance recognition memory in the NOR task from 7 months old, after 4 months of exercise commencement, until 19 months old, even when mice went 8 months without exercise.

Our findings demonstrate that 2 months after exercise commencement, when mice were 5 months old, both sedentary and exercised mice explored the novel object significantly more than the familiar object in the testing phase, confirming the premise of a natural preference for the new object instead of the familiar object in this task (Rutten et al., 2008a, Leger et al., 2013). However, we found that after 2 months of exercise, exercise did not enhance recognition memory in comparison with sedentary mice. Previously in our laboratory, it has been demonstrated that only 7 consecutive days of treadmill running enhances young-adult rats' performance in two different variations of the NOR (2- and 3-objects variant of NOR task) (Griffin et al., 2009, O'Callaghan et al., 2007). However, in the 3-objects variation of the NOR task, sedentary rats were unable to discriminate between one novel and the two familiar objects, suggesting that 3 objects adds more difficult to the NOR task (Griffin et al., 2009). In our study, for the NOR assessment, we employed the easier 2-objects variant of the NOR task. Also, in the present study, animals exercised only 30 minutes per day, 3 times per week during the 2 months and also, we used mice instead of rats. Therefore, these differences could explain the lack of exercise enhancement in recognition memory in young animal compared to sedentary observed 2 months after exercise commencement.

We could not observe exercise enhancing recognition memory after 2 months of exercise, but our results demonstrate that 4, 6 and 8 months after the beginning of exercise, when mice were 7, 9 and 11 months old, respectively, the sedentary and exercised mice still explored the novel object significantly more than the familiar object in the testing phase. In addition, from 4 months until 8 months of undergoing exercise, mice from the exercise group explored the new object more when compared to sedentary mice, suggesting exercise enhanced recognition memory over time in comparison with sedentary mice. Most interestingly, our results demonstrated, for the first time, that the effects of exercise in enhancing recognition

memory persist even 8 months after exercise cessation. Even though mice stopped exercise from 11 months old until 19 months old, the exercise effect was still observed, while sedentary mice started to present cognitive impairment from 17 months old. Only a few studies have demonstrated the persistent effects of exercise in enhancing memory after exercise cessation (Morgan et al., 2018)), but not in the NOR task and not for a prolonged time in the lifespan. A recent study has shown early-life exposure to 6 weeks of voluntary exercise enhanced fear condition response in rats, 4 months after exercise cessation, suggesting that early experience can build cognitive reserve (Shevtsova et al., 2017). Our findings are in agreement with this, and for the first time we demonstrate that exercise-induced enhancement and protection from age-induced impairments in recognition memory persists even 8 months after exercise had stopped.

3.4.2 Spatial learning and memory throughout the lifespan

Spatial memory was also assessed every 2 months, with the first assessment at 5 months old (2 months after exercise commencement) and the last assessment at 19 months old (8 months after the end of exercise protocol). The OD task has been widely used to assess spatial memory in rodents (Murai et al., 2007) and, similar to the NOR, this task also takes advantage of the natural tendency of rodents to investigate novelty (Ricceri et al., 2000). Also, at 20 and 21 months old, mice were tested in the MWM task; this spatial learning task was included at the end of the protocol to confirm that the effects observed throughout the lifespan on spatial memory as tested by the OD task were not a practice effect resulting from repetition of the OD task.

Our findings demonstrate that exercise enhanced spatial memory in the OD task throughout the lifespan, from the first assessment at 5 months old, after 2 months of exercise commencement. Most interestingly, we demonstrated for the first time that this effect persists until 19 months old, even when mice did not exercise for 8 months. This exercise-induced persistent enhancement of spatial learning was confirmed by results from the MWM task, assessed almost 10 months after exercise cessation. These results support the published studies that have used a variety of tasks, such as the MWM, the Y-maze, the radial arm maze and the OD

task to demonstrate that spatial learning and memory is highly modulated and enhanced by physical exercise, (Creer et al., 2010, Bechara et al., 2014, Fordyce and Farrar, 1991, Marlatt et al., 2012).

Spatial learning and memory can be enhanced after short or long periods of exposure to exercise training. For example, previous work from our laboratory has investigated the cognitive effects of 7 consecutive days of treadmill running and observed enhancement of spatial memory in rats in the OD task (Bechara et al., 2014). Also, 8 months of exercise initiated at middle-age demonstrated a protective effect of exercise against age-related impairment in spatial learning and memory in the MWM (O'Callaghan et al 2009). Several other groups have investigated the effect of long term forced and voluntary exercise on spatial learning and memory. For example, 14 months of treadmill exercise has been shown to enhance performance of mice in the MWM (Wang et al., 2015), while 11 months of voluntary wheel running from 3 until 14 months old, enhances spatial memory in the Barnes Maze in mice (Morgan et al., 2018). However, to the best of our knowledge, no other studies have shown the persistent effect of exercise on spatial memory after exercise cessation and that exercise can continue to enhance spatial memory, even while sedentary mice present age-related cognitive impairment. Taken together, our results suggest that exercise early in life builds cognitive reserve and protects against age-induced impairment in spatial learning and memory.

3.4.3 Anxiety and depression-like behavior in age and exercise

Anxiety and depression-like behaviours were assessed at old age, after exercise cessation. We employed the EPM and the TST respectively, tasks commonly used to measure anxiety and depression-like behaviour in animals (Steru et al., 1985). We also evaluated exploratory and locomotor activities in aged mice in the OF task, to assess any impairment in these parameters that may explain alterations in performance of cognitive tasks. While changes in locomotor activity have been associated with ageing and with exercise in the C57BL/6 mice in the OF task (Morgan et al., 2018), our results demonstrated no effect of either ageing or exercise on these parameters. The maintenance of locomotor and exploratory activity observed in both groups of aged mice, could be explained by the constant

exposure of these mice to not only to the exercise in the case of exercise group, but the contact with a novel environment, as both groups had been removed from their home cage, and similarly to the exercise mice, sedentary mice were exposed to the stationary treadmill. Indeed, positive effects of enrichment in the absence of exercise have been reported in aged animals (Birch and Kelly, 2018).

Anxiety-like behaviour was attenuated in exercised mice at 19 months, as was depressive-like behaviour, evaluated at 21 months old, demonstrating that the persistent positive effects of exercise, long after its cessation, are not limited to cognitive behaviour, but also to emotional behaviour. In the EPM test, we demonstrated an increase in the total number of arm entries and a decrease in the anxiety score in the exercise compared to sedentary group, suggesting anxiety-like behaviour was decreased in exercise group, even 8 months after the end of exercise. Our findings support previous studies in the literature, which have reported exercise to decrease anxiety in young mice (Binder et al., 2004) and to prevent an age-induced increase in anxiety-like behaviour in middle-aged and aged mice (Morgan et al., 2018). The reduction observed in anxiety-like behaviour in aged mice reported here, suggests that the persistent anxiolytic effect of exercise could be associated with the resilience that is also reflected in the maintenance of cognitive functions.

Finally, our results demonstrated that exercise decreased immobility time in the TST, suggesting exercised mice show reduced depressive-like behaviour compared to sedentary mice, even 10 months after the end of exercise. This supports a previous study which demonstrated that adult mice that had ad libitum access to running wheels for three or four weeks showed reduced immobility time in the forced swim test (FST) (Duman et al., 2008; Cunha et al., 2013). However, a recent study demonstrated no effects of 11 months of voluntary exercise in the immobility time in middle-age to aged mice tested in the TS test (Morgan, et al. 2018), although these mice were housed in pairs. Since the mice in the present study were group-housed and exercised on treadmills, our contrasting results suggest that the type of exercise and the housing conditions could contribute to this sustained effect of exercise on depression-like behaviour at age.

3.4.4 Summary

In summary, our results demonstrate that exercise enhances recognition memory (NOR task) and spatial learning and memory (OD task), over time; whereas a persistent effect of exercise on spatial memory was confirmed in the MWM task, observed between 20 and 21 months old (Table 3.1). Moreover, we observe no changes in exploratory and locomotor activities associated either with age or exercise at 19 months old, measured in the OF task. However, our data suggested an anxiolytic effect of exercise between 19 and 20 months old in the EPM test, as well as an anti-depressive effect at 21 months old in the TS test (Table 3.1).

The mechanisms underlining exercise-induced learning and memory improvements across the lifespan, as well as its role in reducing anxiety and depression-like behavior, have been widely investigated following a variety of exercise protocols and in animals of different ages. The recognition memory and spatial learning tasks assessed here are dependent on a well-functioning hippocampus and associated cortical regions including the perirhinal and entorhinal cortices. Many studies have suggested that the beneficial effects of exercise in enhancing cognition, and also mood and affect, could be mediated by changes in the expression of key proteins in the hippocampus, changes in inflammatory profile, increase of hippocampal neurogenesis, and enhancement of signaling pathways involved in neurotrophin expression and synaptic plasticity (O'Callaghan et al., 2007, Berchtold et al., 2010, Voss et al., 2013, van Praag et al., 2005). To explore these possibilities, in the next chapter, we investigate the possible mechanisms underlying exercise-induced behavior enhancement and protection against age-induced cognitive impairment that we have observed in our study over time, and the potential contribution of cognitive reserve on this persistent effect, even after exercise cessation.

	5 mon old		7 mon old		9 mon old		11 mon old		13 mon old		15 mon old		17 mon old		19 mon old		20 mon old		21 mon old	
	Sed	Ex	Sed	Ex	Sed	Ex	Sed	Ex	Sed	Ex	Sed	Ex	Sed	Ex	Sed	Ex	Sed	Ex	Sed	Ex
NOR	↑	↑	↑	↑↑	↑	↑↑	↑	↑↑	↑	↑↑	↓	↑↑	↓	↑↑	↓	↑↑	—	—	—	—
OD	↑	↑↑	↑	↑↑	↑	↑↑	↑	↑↑	↑	↑↑	↓	↑↑	↓	↑↑	↓	↑↑	—	—	—	—
EPM	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	↓	↑	—	—
MWM	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	↓	↑	—	—
TS	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	↓	↑

Table 3.1 - Effect of exercise on learning and memory, anxiety and depression-like behaviour throughout the lifespan

Illustrative table with the outcome effect of exercise on Novel Object Recognition (NOR) task, Object Displacement (OD) task, Elevated Plus Maze (EPM) test, Morris Water Maze (MWM) task and Tail Suspension (TS) test in the different time points of assessment. (↑) Normal performance of mice in the task; (↑↑) Enhanced performance of mice in the task; (↓) Poor performance of mice in the task; (—) no task assessment at the time point.

Chapter 4

Potential mechanisms underlying the persistent effects of exercise on learning and memory throughout the lifespan: the role of inflammation and neurogenesis and the cognitive reserve hypothesis

Chapter 4: Potential mechanisms underlying the persistent effects of exercise on learning and memory throughout the lifespan: the role of inflammation and neurogenesis and the cognitive reserve hypothesis

4.1 Introduction

Ageing results in dysfunction and changes in several brain functions and possibly, the most remarkable and debilitating of these changes is the decline in the cognitive function. However, people can age differently and sometimes, even though morphological and molecular changes in the brain are observed in aged individuals, these people will not experience the manifestation of cognitive decline associated with ageing. In order to explain this frequent discrepancy between an individual's measured level of age-related brain changes and the functional and cognitive deficits that are expected to result from the process of ageing, the reserve hypothesis was proposed (Stern, 2002, Stern, 2009, Barulli and Stern, 2013).

Over the years, evidence has supported the existence of this reserve capacity and suggested that synaptic plasticity and hippocampal neurogenesis might contribute to this reserve (Kempermann, 2008). Also, increased capability reserve has been linked to maintaining cognitive function during ageing and studies suggested that this reserve could be modulated by external factors and life exposure, such as engagement in physical and social activities (Stern, 2002, Cheng, 2016). Indeed, physical exercise has been widely reported as playing an important role in cognition, hippocampal neurogenesis and in counteracting age-related cognitive dysfunctions and age-related diseases.

Ageing is well-documented to negatively affect hippocampal neurogenesis and synaptic plasticity (Lazarov et al., 2010) and exercise has been shown to rescue age-related impairment in neurogenesis (van Praag et al., 2005). In many of these studies, exercise-induced protection against age-related decrease in hippocampal neurogenesis was associated with increased synaptic plasticity and improvement in cognitive function (van Praag, 2009, Vaynman and Gomez-Pinilla, 2005, Voss et al., 2013). In addition, a recent study reported that six weeks of early-age exposure to a running wheel enhanced hippocampal neurogenesis and cognition

in adulthood, even four months after exercise cessation, supporting the hypothesis that neurogenesis and plasticity contribute to increased cognitive reserve (Shevtsova et al., 2017)

A positive NSCs microenvironment is crucial to the promotion of neurogenesis and several age-related changes, such as decreased expression of neurotrophins (BDNF) and growth factors (Igf-1, VEGF) and low-grade inflammation could contribute to the negative effect of ageing in neurogenesis. Exacerbated inflammation is detrimental to neurogenesis; one study has shown that LPS, which stimulates microglia activation, impaired hippocampal neurogenesis and that systemic administration of minocycline, which inhibits microglia activation, restored hippocampal neurogenesis in rats (Ekdahl et al., 2003), suggesting the involvement of microglia and inflammation in the process of hippocampal neurogenesis. Studies have shown that the negative effects of activated microglia and astrocytes on newborn neurons are most likely to be mediated by the action of cytokines released from cells in the M1 and A1 state, such as IL-1β or IL-6, TNF-α and iNOS (Vallieres et al., 2002, Monje et al., 2003). In contrast, evidence suggests that anti-inflammatory cytokines, such as IL-10 and growth factors support neurogenesis (Battista et al., 2006), suggesting that an “alternative” and anti-inflammatory microglia activation (M2 state) contribute positively to hippocampal neurogenesis.

Normal ageing is associated with increased microglial’ activity and low-grade chronic inflammation. Also, it is known that microglia in the aged brain are primed towards a inflammatory phenotype, characterised by increased expression of pro-inflammatory cytokines, such as IL-1β and IL-6 (Sierra et al., 2007, Dilger and Johnson, 2008). A study has demonstrated that incubation medium from cultured microglial cells prepared from aged mice was less effective in inducing neurogenesis in vitro compared to medium from adult mice (Walton et al., 2006), suggesting that microglia from aged mice modified the inflammatory profile contributing to age-induced decline in neurogenesis.

In this context, as exercise has been widely described to be anti-inflammatory, this is an important tool to potentially counteract age-related inflammation. The beneficial effects of exercise in restoring age-related impairment in neurogenesis

have been shown to be at least partially linked to its anti-inflammatory effects. Indeed, it has been shown that five weeks of voluntary exercise attenuated the inflammation-induced impairments in neurogenesis in aged mice treated with LPS (Littlefield, et al. 2015). Also, exercise has been reported to prime "alternative" microglia activation in aged mice. Six weeks of exercise reduced the inflammatory response of aged hippocampal microglia cells treated with LPS, demonstrated by reduction in the levels of IL-1β, TNF-α and IL-6, and protected against age-induced cognitive impairment (Barrientos et al., 2011). Taken together, this evidence strongly supports the link between inflammation, neurogenesis and age-related cognitive decline and the role of exercise to prevent or reverse these age-related changes.

Thus, exercise in early life could contribute to maintaining a positive microenvironment for NSCs throughout the lifespan, by regulating expression of neurotrophins and growth factors and controlling inflammation, thereby promoting neurogenesis and an increased cognitive reserve, which makes the brain resilient to age-induced cognitive decline. In the previous chapter, we demonstrated that long-term forced exercise enhanced spatial and recognition memory throughout the mouse lifespan and reduced anxiety and depression-like behaviour, even 10 months after exercise cessation. Therefore, the aim of this chapter was to investigate the mechanisms mediating the persistent effect of exercise in cognitive function during the mouse lifespan, focusing on the contribution of inflammation and neurogenesis to modulation of cognitive reserve

4.2 Methods

4.2.1 Animals

Three-month-old male C57BL/6 mice obtained from Charles River Laboratories U.K. Ltd. were used in all experiments. Mice were given one week to acclimatize to the Bioresources Unit and were handled daily for five minutes during this period. All experiments were performed in accordance with National and European directives on the protection of animals (European Union (Protection of Animals used for Scientific Purposes) Regulations 2012 (SI 543/2012), and European Union (Protection of Animals used for Scientific Purposes) (Amendment) Regulations 2013 (SI 434/2013), under a license issued by the Health Products Regulatory Authority and were approved by the Animal Research Ethics Committee, Trinity College Dublin. Animals were group-housed, four/five per cage, in Tecniplast 1285LN individually ventilated cages (W x D x H - 396 x 215 x 172 mm) with food and water available *ad libitum*. All animals were maintained in a facility with controlled ambient temperature (22°C ± 2°C) and humidity (55% ± 5%) under a 12:12-hour light-dark cycle.

4.2.2 Experimental design

The tissue samples analysed in this chapter are form the mice used for the behavioural assessment, reported in the previous chapter. Three months old male C57BL/6 mice (n=38) were randomly assigned to either sedentary control group (SED) or exercise group (EX). Exercise group animals (n=19) underwent one hour of forced moderate exercise in a treadmill, three times per week, for a period of eight months. Sedentary control animals (n=19) for this study were placed in a stationary treadmill for an equivalent time period. At the end of this period of exercise, the adult-young mice were middle-aged (11 months). At this point, animals ceased exercise and were maintained in the facility for a period of approximately 10 months (Figure 4.1), until they achieved old age (21 months old). At old age (21 months), just after all the behaviour tests, mice were scanned in a Structural Magnetic Resonance Image Scanner (MRI). During the 18 months of experiment, mice were injected with the thymidine analogue 5'bromo-2-

deoxyuridine (BrdU - 50mg/kg) at different time points to allow later assessment of neurogenesis. At 21 months of age, mice were sacrificed; the brain was removed for assessment of BrdU labelling and cell phenotype by immunohistochemistry, assay of mRNA levels and protein expression of different targets of interest. Also, peripheral tissues, such as liver, muscle, blood, caecum and faeces samples were collected for analyses of peripheral changes induced by exercise. To analyse the effect of ageing, brain and peripheral tissue samples were harvested from a group of 3 month old male C57BL/6 mice (n=16) to act as a control group in the biochemistry analysis.

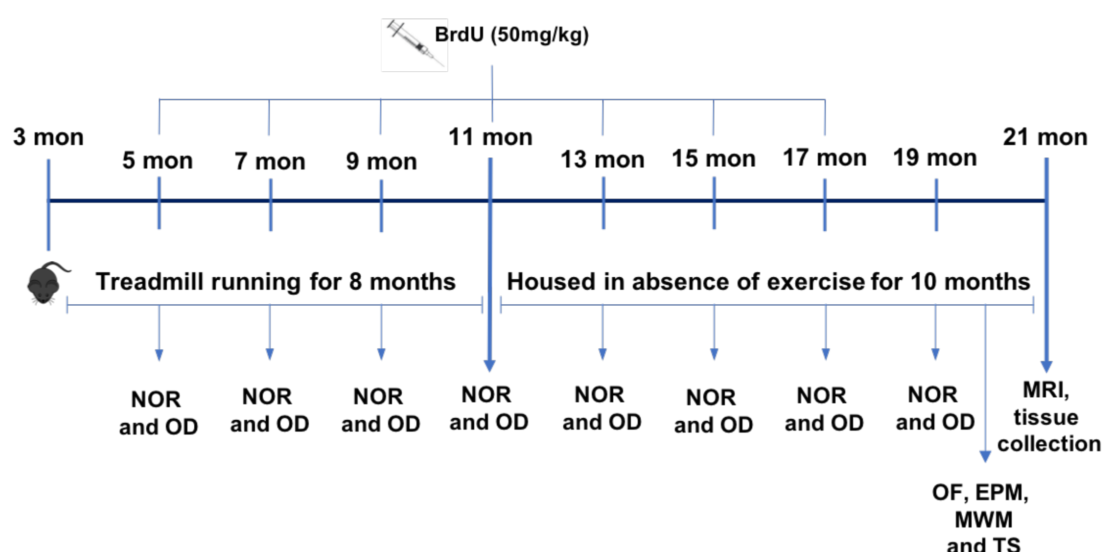


Figure 4.1 - Experiment timeline

Timeline showing physical exercise protocol, MRI and tissue collection

4.2.3 Treadmill exercise protocol

Animals were randomly assigned to either sedentary control group (n=19) or exercise group (n=19). All animals were familiarized to motorized rodent treadmills (Exercise 3/6 treadmill, Columbus Instruments) for one week prior to the commencement of the exercise protocol. Following familiarization, animals were exercised three times per week for a period of eight months. The exercise protocol consisted of one hour of running (belt speed 6-12 m/min, at zero inclination). Sedentary control animals were placed in stationary treadmills for the same period as the exercise group, to control for possible effects of handling, stress or novelty.

The treadmills are equipped with wire loops at one end of the belt through which a mild electric shock can be delivered to encourage animals to run. In our experiment, animals ran in the absence of the electric shock, just a gentle hand prodding was used to motivate animals to run.

4.2.4 BrdU administration

Mice were injected intraperitoneally with the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU; 50mg/kg in 0.89% w/v saline solution) for five consecutive days every 2 months, following the behavioural tasks, as described in detail in section 2.7. The last BrdU injection was administrated three months before the end of the experiment.

4.2.5 Magnetic resonance image (MRI)

Mice were anaesthetized and maintained with 1-2 % isofluorane and placed on an MRI-compatible support cradle. The cradle was then positioned within the 7 Tesla MRI Scanner (Bruker BioSpec 7T system with a 1H MRI Cryoprobe, AVANCE III HD architecture and Paravision 6 Software). Animals were imaged for structural MRI, using a variety of magnetic resonance imaging techniques ("MRI pulse sequences"), as described in detail in section 2.10. At the end of the experiment, animals were allowed to recover from anaesthesia and returned to the home cage. Manual volume measurements were performed blinded using MIPAV software (NHI, USA) to manually trace regions of interest slice by slice along the coronal plane. Regions of interest analysed were both hemispheres of hippocampus and total brain volume and data were expressed in mm^3, as described in section 2.10.3.

4.2.6 Tissue collection and preparation

After all the behavioural assessment and the MRI assay, one set of mice (n=24, 3 months, sedentary and exercise) was anaesthetised by intraperitoneal injection of pentobarbital sodium (1g/kg) and euthanized via transcardial perfusion with PBS. Hippocampus and a sample of prefrontal cortex were dissected free from the brain,

while samples of liver, gastrocnemius muscle, blood, bone marrow, caecum and faeces were removed and stored for later analysis. Microglia and astrocytes were purified and isolated from the remainder of the brain tissue, except for the cerebellum, as described in detail in section 2.11.3.

Tissue samples (hippocampus, cortex, liver and muscle) were homogenized for western blot and ELISA and samples were stored at -20° C until required. For RNA analysis, samples (hippocampus, cortex and liver) were placed in RNase-free tubes and stored at -80° C until RNA extraction. Another set of mice (n=16, 3 months old, sedentary and exercise) was sacrificed by transbbbcardial perfusion with PBS, followed by paraformaldehyde (PFA, 4% (w/v)) fixation; brains were removed, processed as mentioned in detail in section 2.16; and then were analysed by immunohistochemistry.

4.2.7 Analysis of protein expression by ELISA

This technique was used to examine the concentration of cytokines (see table 2.1 in section 2.13) in samples of hippocampus (IL-1β, TNF-α and IL-10 and BDNF), liver (IL-1β and TNF-α) and muscle (IL-1β and TNF-α). Tissue homogenates were prepared as described in section 2.11.1 and protein content of the samples was analysed by the BCA method, as described in section 2.12. Samples and standards were analysed using commercially available IL-1β, TNF-α, IL-10 and BDNF Duoset® ELISA development systems kit (R&D Systems Europe, Oxon, United Kingdom) and the ELISAs were performed as described in detail in section 2.13.

4.2.8 Analysis of mRNA expression by polymerase chain reaction (RT-PCR)

RNA was isolated from hippocampus, prefrontal cortex, astrocytes and microglial cells using a Nucleospin RNAII kit (Macherey-Nagel, Germany) and samples were reverse transcribed into complementary DNA (cDNA) using the ABI High Capacity cDNA archive kit (Applied Biosystems, Ireland) as described in section 2.13. Gene expression of targets was assessed using “Taqman gene expression assays” (Applied Biosystems, Ireland; see table 2.3 in section 2.15.4). RT-PCR was

performed using Step One Plus TM Software (Applied Biosystems) with β-actin used as endogenous control for each of the targets. Data were quantified using the ΔΔCT method and expressed as relative quotient (RQ) values. The RT-PCR procedure is described in detail in section 2.15.

4.2.9 Analysis of protein expression by western immunoblotting

To assess the persistent effect of exercise in ageing on synaptogenesis, expression of synaptic density markers, including synapsin-1 and PSD-95, was measured in the hippocampus using western immunoblotting technique. We also analyzed the expression of TrkB receptor and NF-κB (for dilutions, see Table 2.2 in section 2.14.3) in hippocampal tissue using western immunoblotting. Tissue homogenates were prepared as described in section 2.11.1 and protein content of the samples was analysed by the BCA method, as described in section 2.12. The procedure for western immunoblotting is described in detail in section 2.14.

4.2.10 Quantification of protein expression by immunohistochemistry

This technique, in conjunction with fluorescent microscopy, was used to assess neurogenesis by immunolabeling of BrdU in the dentate gyrus of hippocampus of animals. Cells were double-stained with BrdU and neuronal nuclear antigen (NeuN), glial fibrillary acidic protein (GFAP) or ionized calcium-binding adapter molecule 1 (Iba-1) to establish whether BrdU-labelled cells were differentiated into mature neuronal or glial cell phenotypes, respectively (for antibodies and dilutions, see table 2.4 in section 2.16.2). This technique was also used to establish the number of positive GFAP and Iba-1 cells in the CA1 and CA3 regions of hippocampus. Quantification of staining was conducted blind using stereological sampling. Immunohistochemistry and data analysis is described in detail in section 2.16.

4.2.11 Statistical analysis

All data were analysed and grouped using Microsoft Excel, and then imported into Graphpad Prism 5. Data are expressed and presented as mean ± standard error of the mean (SEM). Analysis was carried out using one-way ANOVA (Analysis of Variance) when one factor was compared among three or more groups. When data did not follow a normal distribution, non-parametric test was used (Kruskal-Wallis), in conjunction with Dunn’s multiple comparisons test. In addition to this, data were further analysed using Bonferroni *post hoc* tests. Statistical significance was accepted at $p<0.05$.

4.3 Results

4.3.1 Effects of age and exercise on mRNA expression of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) in the hippocampus and cortex

10 months after exercise cessation, mRNA expression of pro-inflammatory cytokines in hippocampal and prefrontal cortical tissue was analysed by RT-PCR. A significant difference in mRNA expression of the pro-inflammatory cytokine IL-1β was observed in the hippocampus (p=0.0002, one-way ANOVA, Figure 4.2 A) and cortex (p=0.0242, one-way ANOVA, Figure 4.2 B). In hippocampus, *post-hoc* analysis showed an increase in IL-1β in sedentary mice compared to 3 month old mice while exercise attenuated the age-induced increase (RQ 3 months old: 0.87±0.12, SED: 2.28±0.23, EX: 1.43±0.18, Bonferroni *post-hoc*, Figure 4.2 A). Similarly, in the cortex, post-hoc analysis also revealed an increase in IL-1β mRNA in the sedentary group compared to 3 months, but exercise had no significant effect (RQ 3 months old: 0.92±0.09, SED: 2.07±0.44, EX: 1.43±0.08, Bonferroni *post-hoc*, Figure 4.2 B).

There was significant difference between groups in the expression of TNF- α in the hippocampus (p=0.0215, one-way ANOVA, Figure 4.3 A) and cortex (p=0.0461, one-way ANOVA, Figure 4.3 B). *Post-hoc* analysis showed that age induced an increase in mRNA expression of TNF-α in the hippocampus of sedentary mice, but not exercised mice (RQ 3 months old: 1.02±0.07, SED: 3.21±0.85, EX: 2.24±0.22, Bonferroni *post-hoc*, Figure 4.3 A). In the cortex, post-hoc analysis also revealed a significant effect of age (RQ 3 months old: 0.97±0.14, SED: 3.97±1.07, EX: 2.15±0.15, Bonferroni *post-hoc*, Figure 4.3 B).

Also, in the hippocampus, statistical analysis demonstrated a difference in mRNA expression of IL-6 (p=0.0178 one-way ANOVA, Figure 4.4 A) and *post-hoc* analysis revealed an age-related increase in IL-6 mRNA only in sedentary mice, with no change in the exercise group (RQ 3 months old: 0.25±0.01, SED: 0.53±0.0.10, Ex: 0.37±0.03, Bonferroni *post-hoc*, Figure 4.4 A). However, in the cortex, no changes in mRNA expression of IL-6 were observed between groups

(p=0.1637, one-way ANOVA, RQ 3 months old: 1.02±0.09, SED: 1.31±0.15, Ex: 1.32±.0.09, Bonferroni *post-hoc,* Figure 4.4 B).

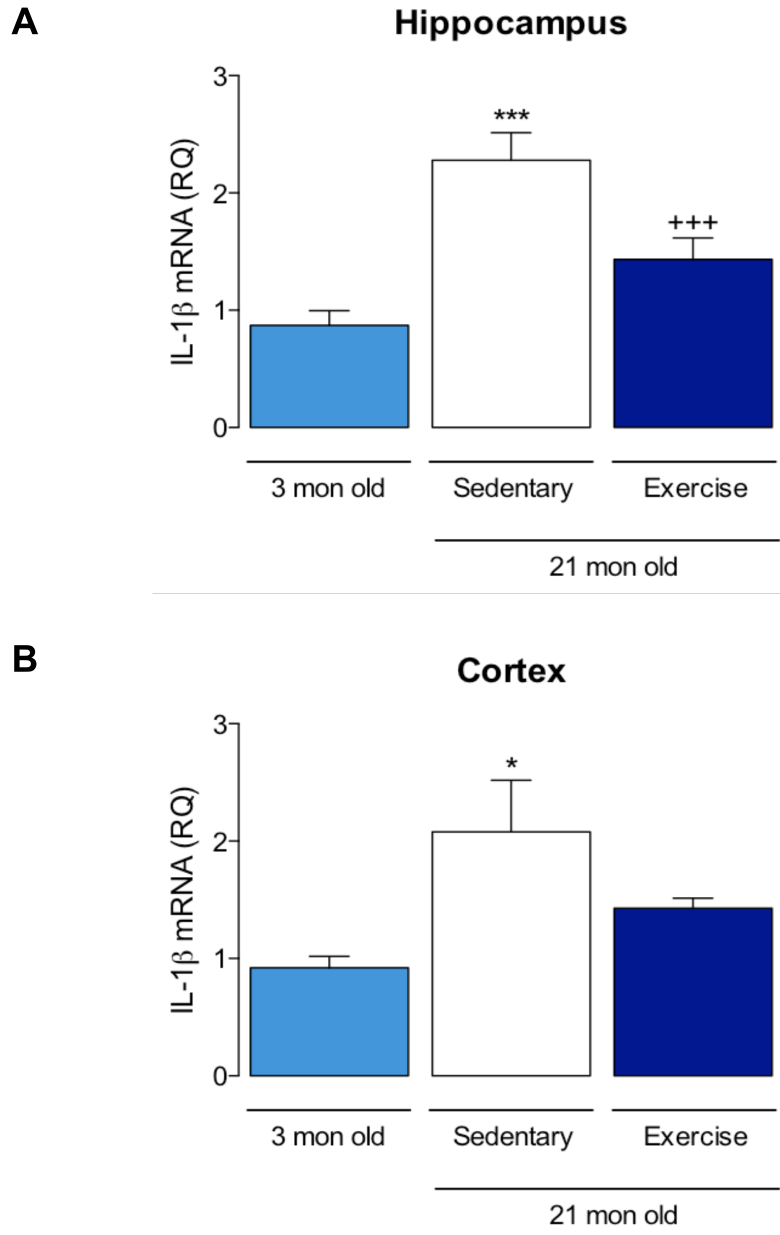


Figure 4.2 - Effects of age and exercise on mRNA expression of IL-1β in the hippocampus and in the cortex

Ten months after exercise cessation, hippocampal and cortical mRNA expression of IL-1β was assessed by RT-PCR. Expression of IL-1β was increased in hippocampus and cortex of age-SED mice, but exercise attenuated this increase in both regions **(A, B)**. Data are presented as mean ± SEM (n=7 to 8). ***$p<0.001$, 3 months old vs. SED; *$p<0.05$, 3 months old vs. SED, $^{+++}p<0.001$, SED vs. EX; one-way ANOVA with Bonferroni *post hoc* analysis

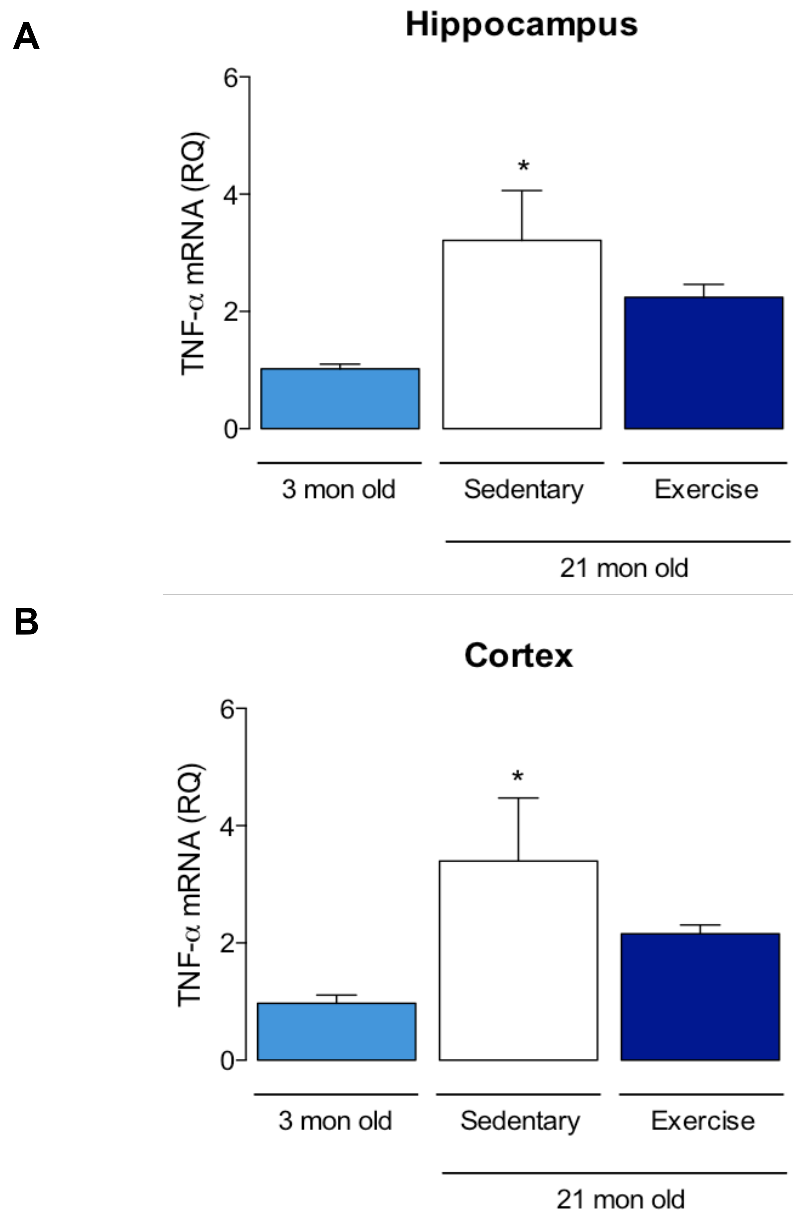


Figure 4.3 - Effects of age and exercise on mRNA expression of TNF-α in the hippocampus and in the cortex

Ten months after exercise cessation, hippocampal and cortical mRNA expression of TNF-α was assessed by RT-PCR. Expression of TNF-α was increased in hippocampus and cortex of age-SED mice, but not in age-EX mice **(A, B)**. Data are presented as mean ± SEM (n=7 to 8). *p<0.05, 3 months old vs. SED; one-way ANOVA with Bonferroni *post hoc* analysis.

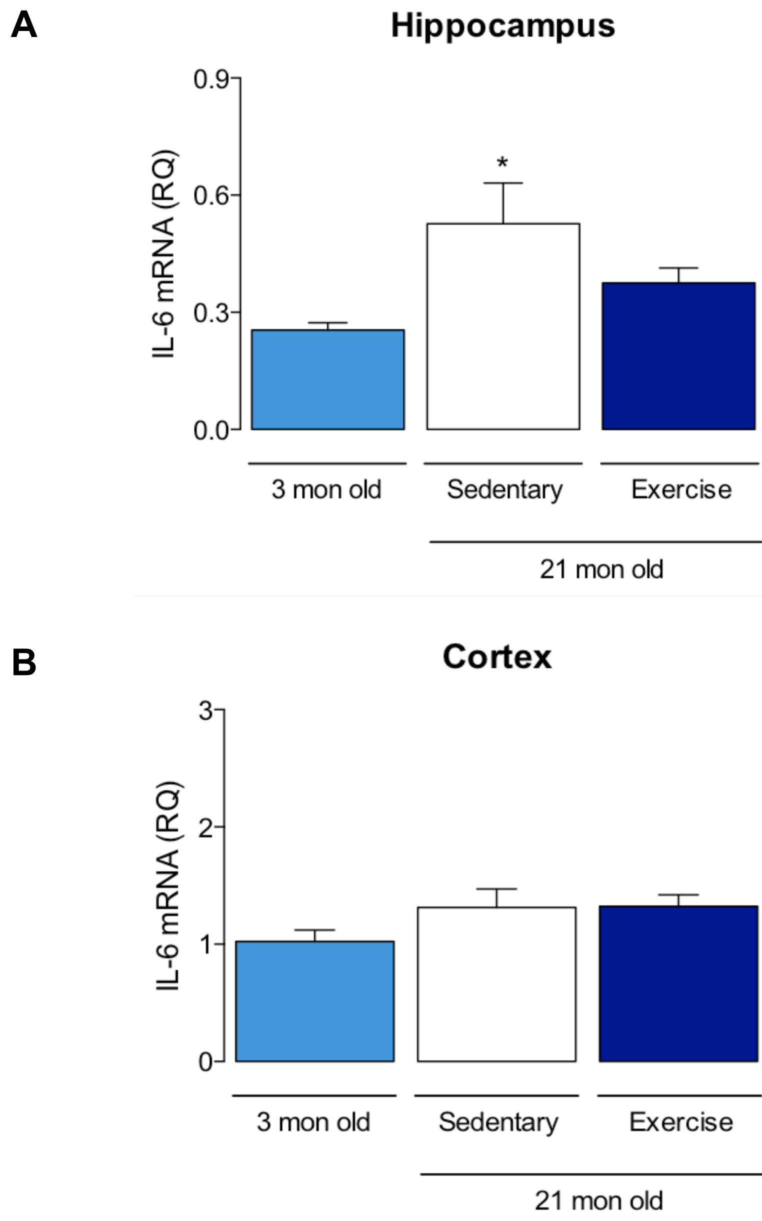


Figure 4.4 - Effects of age and exercise on mRNA expression of IL-6 in the hippocampus and in the cortex

Ten months after exercise cessation, hippocampal and cortical mRNA expression of IL-6 was assessed by RT-PCR. Expression of IL-6 was increased in hippocampus of age-SED mice, but not in age-EX mice **(A)**. In the cortex, IL-6 mRNA expression was unaffected either by age or exercise **(B)**. Data are presented as mean ± SEM (n=7 to 8). *p<0.05, 3 months old vs. SED; one-way ANOVA with Bonferroni *post hoc* analysis.

4.3.2 Effects of age and exercise on mRNA expression of the anti-inflammatory cytokine IL-10 in the hippocampus and cortex

10 months after exercise cessation, mRNA expression of the anti-inflammatory cytokine IL-10 in hippocampal and prefrontal cortical tissue was analysed by RT-PCR. Statistical analysis showed a difference in the expression of IL-10 mRNA in the hippocampus ($p=0.027$, one-way ANOVA, Figure 4.5 A) and cortex ($p=0.0237$, one-way ANOVA, Figure 4.5 B).

In the hippocampus, *post-hoc* analysis revealed that, even 10 months after exercise cessation, mRNA expression of IL-10 was increased in the exercise group compared to the 3 month old and sedentary groups (RQ 3 months old: 0.78±0.27, SED: 0.55±0.34, EX: 4.02±0.32, Bonferroni *post-hoc,* Figure 4.5 A). In the cortex, *post-hoc* analysis showed a significant increase in mRNA expression of IL-10 in the exercise group compared to the 3 months old group only (RQ 3 months old: 1.03±0.24, SED: 1.28±0.16, EX: 2.96±0.76, Bonferroni *post-hoc,* Figure 4.5 B).

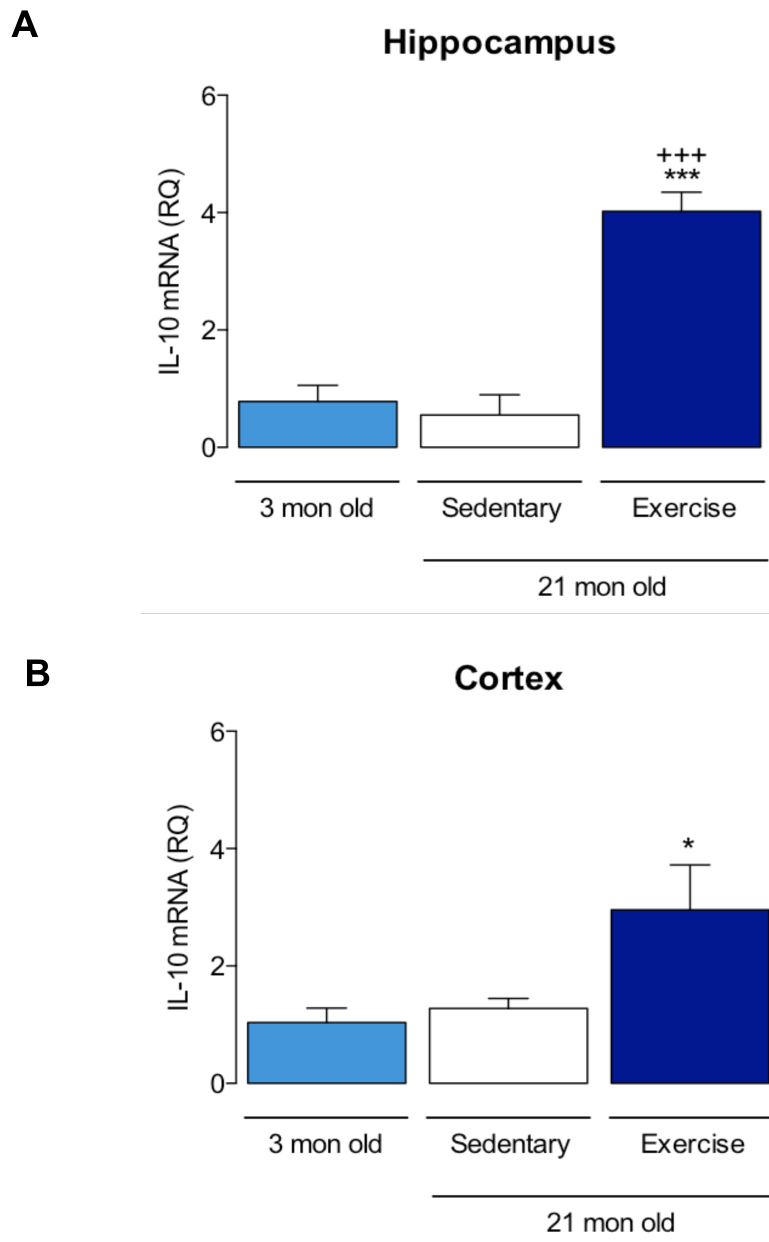


Figure 4.5 - Effects of age and exercise on mRNA expression of IL-10 in the hippocampus and in the cortex

Ten months after exercise cessation, hippocampal and cortical mRNA expression of IL-10 was assessed by RT-PCR. Expression of IL-10 was increased in hippocampus and cortex only of age-EX mice, but not in age-SED **(A, B)**. Data are presented as mean ± SEM (n=7 to 8). ***p<0.001, 3 months old vs. EX; *p<0.05, 3 months old vs. EX, $^{+++}$p<0.001, SED vs. EX; one-way ANOVA with Bonferroni *post hoc* analysis.

4.3.3 Effects of age and exercise on protein expression of pro-inflammatory cytokines (IL-1β, TNF-α) and anti-inflammatory cytokine (IL-10) in the hippocampus

After analysis of mRNA expression of pro- and anti-inflammatory cytokines in the hippocampus and cortex, we decided to analyse the protein expression of these cytokines in the hippocampus by ELISA assay. Analysis of IL-1β concentration demonstrated that there was a trend toward a higher expression of IL-1β in the hippocampus of sedentary mice, however, no significant difference was observed between groups ($p=0.2644$, one-way ANOVA, 3 months old: 2.25±4.78 pg/mg, SED: 13.92±6.14 pg/mg, EX: 10.43±3.8 pg/mg, Bonferroni *post-hoc,* Figure 4.6 A). Similarly, no significant difference was observed in the concentration of TNF-α in the hippocampus ($p=0.1418$, one-way ANOVA, 3 months old: 202.9±27.36 pg/mg, SED: 263.1±18.51 pg/mg, EX: 264.3±24.93 pg/mg, Bonferroni *post-hoc,* Figure 4.6 B).

Mimicking the pattern seen in IL-10 mRNA expression (Figure 4.5 A), there was a significant increase in the concentration of IL-10 in the hippocampus ($p=0.0117$, one-way ANOVA, Figure 4.6 C). Post-hoc analysis revealed an increase in the concentration of IL-10 in the hippocampus of exercise mice, compared to 3 months old and sedentary mice (3 months old: 0.202±4.81 pg/mg, SED: 0.736±2.43 pg/mg, EX: 19.28±5.25 pg/mg, Bonferroni *post-hoc*, Figure 4.6 C).

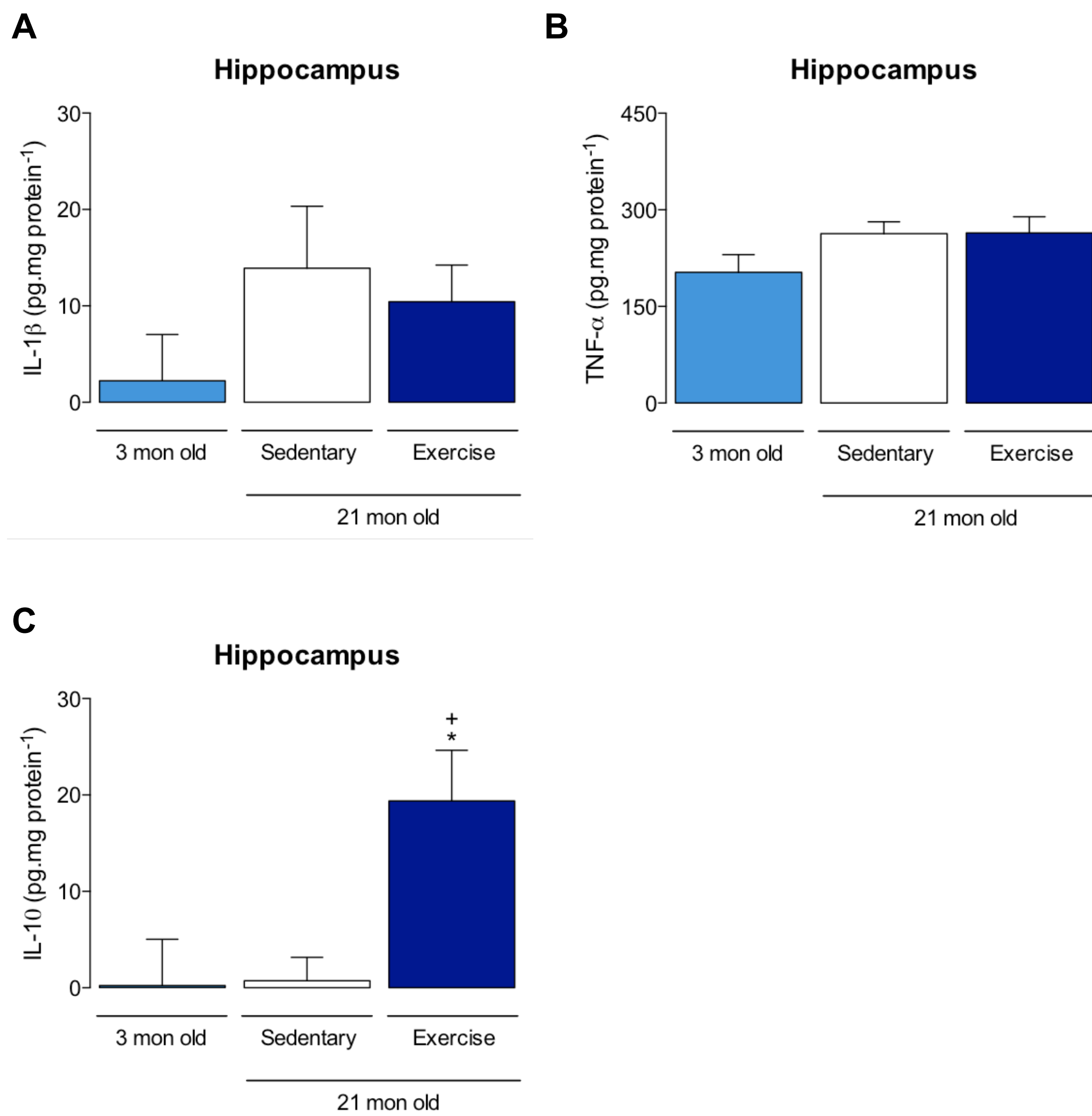


Figure 4.6 - Effects of age and exercise on protein content of IL-1β, TNFα and IL-10 in the hippocampus

Ten months after exercise cessation, hippocampal concentrations of IL-1β, TNF-α and IL-10 were assessed by ELISA. Concentrations of IL-1β and TNF-α, in the hippocampus, were not affected either by age or exercise **(A, B)**. However, concentration of IL-10 was increased in age-EX group **(C)**. Data are presented as mean ± SEM (n=7 to 8). *p<0.05, 3 months old vs. EX, ⁺p<0.05, SED vs. EX; one-way ANOVA with Bonferroni *post hoc* analysis.

4.3.4 Effects of age and exercise on mRNA expression of the glial cell activation markers GFAP, Iba-1 and CD11b in the hippocampus and cortex

10 months after exercise cessation, mRNA expression of the glial cell markers GFAP, Iba-1 and CD11b, in hippocampal and prefrontal cortical tissue, were analysed by RT-PCR. Statistical analysis demonstrated a significant difference in the mRNA expression of GFAP in the hippocampus (p=0.0009, one-way ANOVA, Figure 4.7 A) and the cortex (p=0.001, one-way ANOVA, Figure 4.7 B). In the hippocampus, *post-hoc* analysis revealed an age-induced increase in GFAP mRNA in both sedentary and exercise groups (RQ 3 months old: 1.01±0.05, SED: 1.49±0.11, EX: 1.37±0.07, Bonferroni *post-hoc*, Figure 4.7 A). Similarly, in the cortex, age also increased the expression of GFAP, in both sedentary and exercise mice (RQ 3 months old: 0.68±0.05, SED: 1.15±0.09, EX: 1.10±0.09, Bonferroni *post-hoc*, Figure 4.7 B).

Statistical analysis showed a significant difference in the expression of Iba-1 in the hippocampus (p=0.003, one-way ANOVA, Figure 4.8 A), but not in the cortex (p=0.3674, one way ANOVA, fold change 3 months old: 1.01±0.07, SED: 0.95±0.07, EX: 1.08±0.05, Bonferroni *post-hoc*, Figure 4.8 B). In the hippocampus, *post-hoc* analysis demonstrated that Iba-1 expression was increased only in sedentary mice, but not in exercise mice, compared to the 3 month old group (RQ 3 months old: 1.01±0.05, SED: 1.32±0.05, EX: 1.16±0.06, Bonferroni *post-hoc*, Figure 4.8 B).

Moreover, CD11b mRNA expression was significantly different in the hippocampus (p=0.0004, one-way ANOVA, Figure 4.9 A), but not in the cortex (p=0.3508, one way ANOVA, RQ 3 months old: 1.00±0.04, SED: 1.11±0.07, EX: 1.12±0.06, Bonferroni *post-hoc*, Figure 4.9 B). *Post-hoc* analysis showed an age-induced increase in hippocampal mRNA expression of CD11b in both sedentary and exercise groups, compared to 3 months old group (RQ 3 months old: 1.00±0.05, SED: 1.40±0.07, EX: 1.28±0.05, Bonferroni *post-hoc*, Figure 4.9 A).

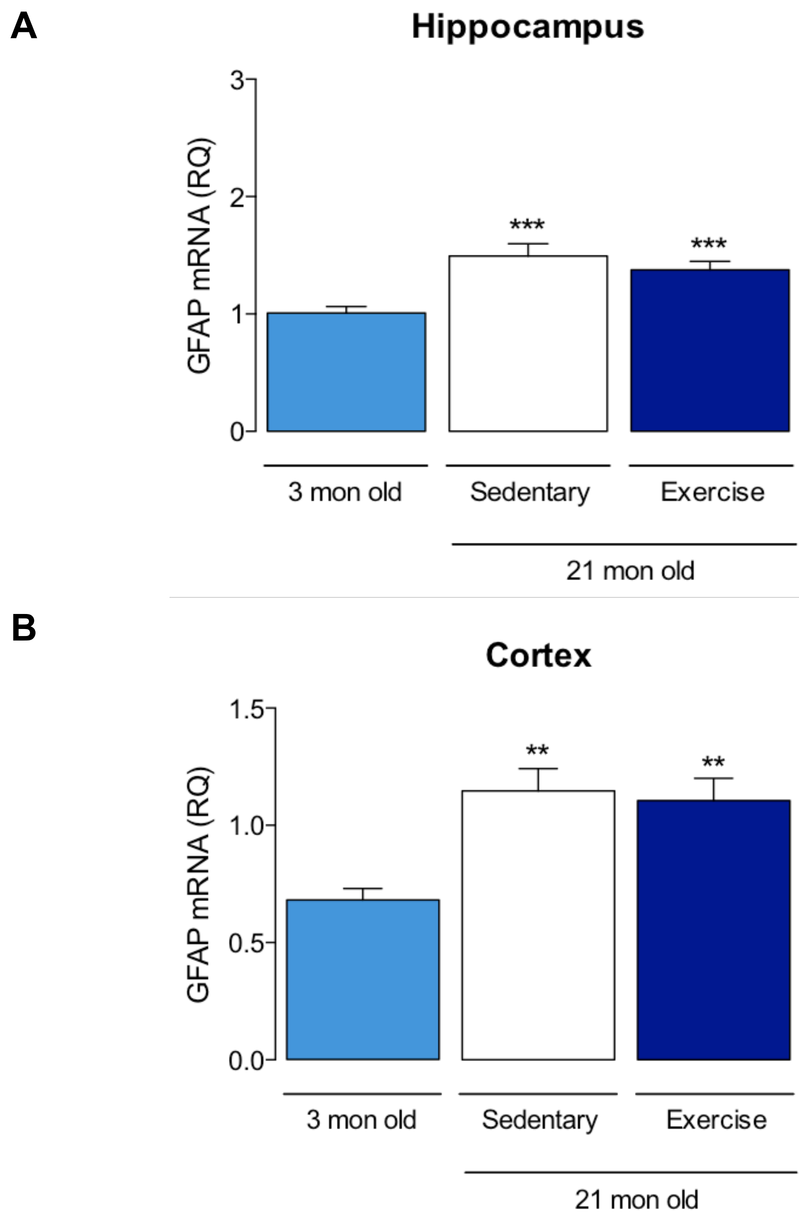


Figure 4.7 - Effects of age and exercise on mRNA expression of GFAP in the hippocampus and in the cortex

Ten months after exercise cessation, hippocampal and cortical mRNA expression of GFAP was assessed by RT-PCR. Expression of GFAP was increased in hippocampus and cortex of age-SED and age EX mice **(A, B)**. Data are presented as mean ± SEM (n=7 to 8). ***p<0.001, 3 months old vs. SED and EX; **p<0.01, 3 months old vs. SED and EX; one-way ANOVA with Bonferroni *post hoc* analysis.

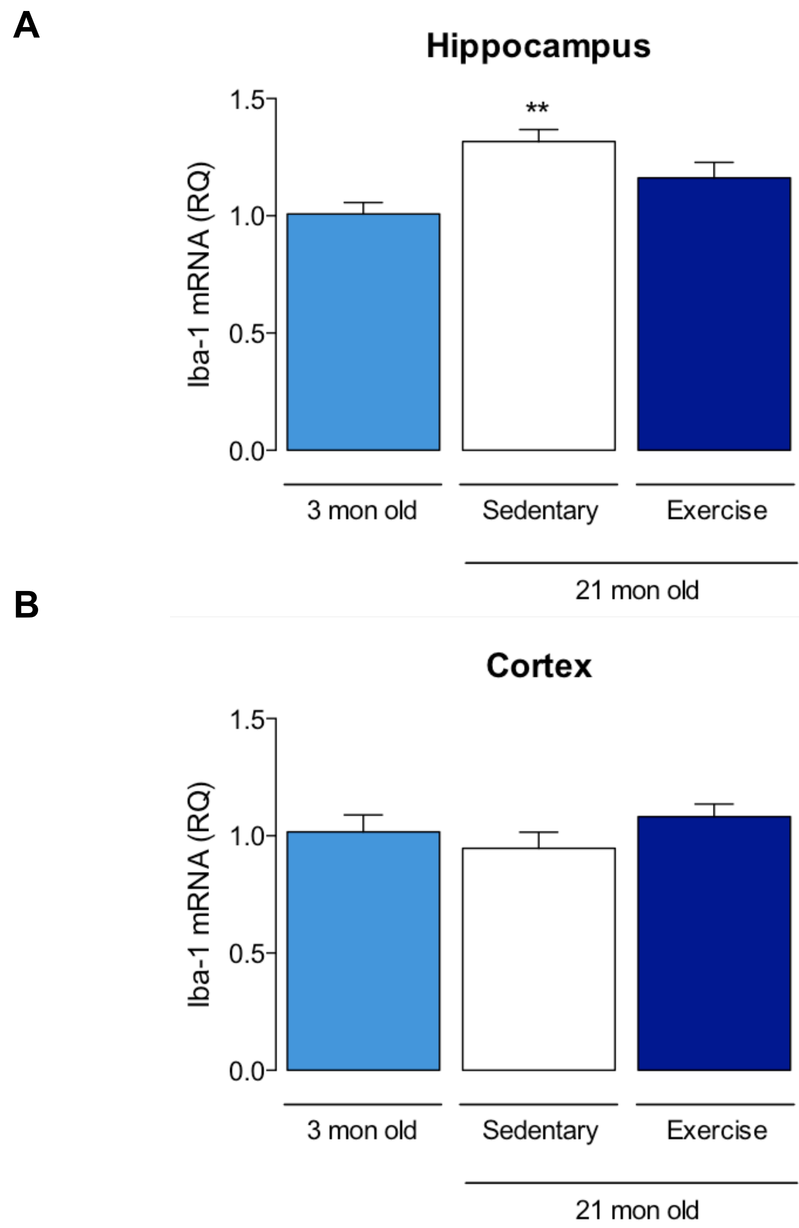


Figure 4.8 - Effects of age and exercise on mRNA expression of Iba-1 in the hippocampus and in the cortex

Ten months after exercise cessation, hippocampal and cortical mRNA expression of Iba-1 was assessed by RT-PCR. Expression of Iba-1 was increased in hippocampus of age-SED and age EX mice, but not in age-EX mice **(A)**. In the cortex, Iba-1 mRNA expression was not affected either by age or exercise **(B)**. Data are presented as mean ± SEM (n=7 to 8). **p<0.01, 3 months old vs. SED; one-way ANOVA with Bonferroni *post hoc* analysis.

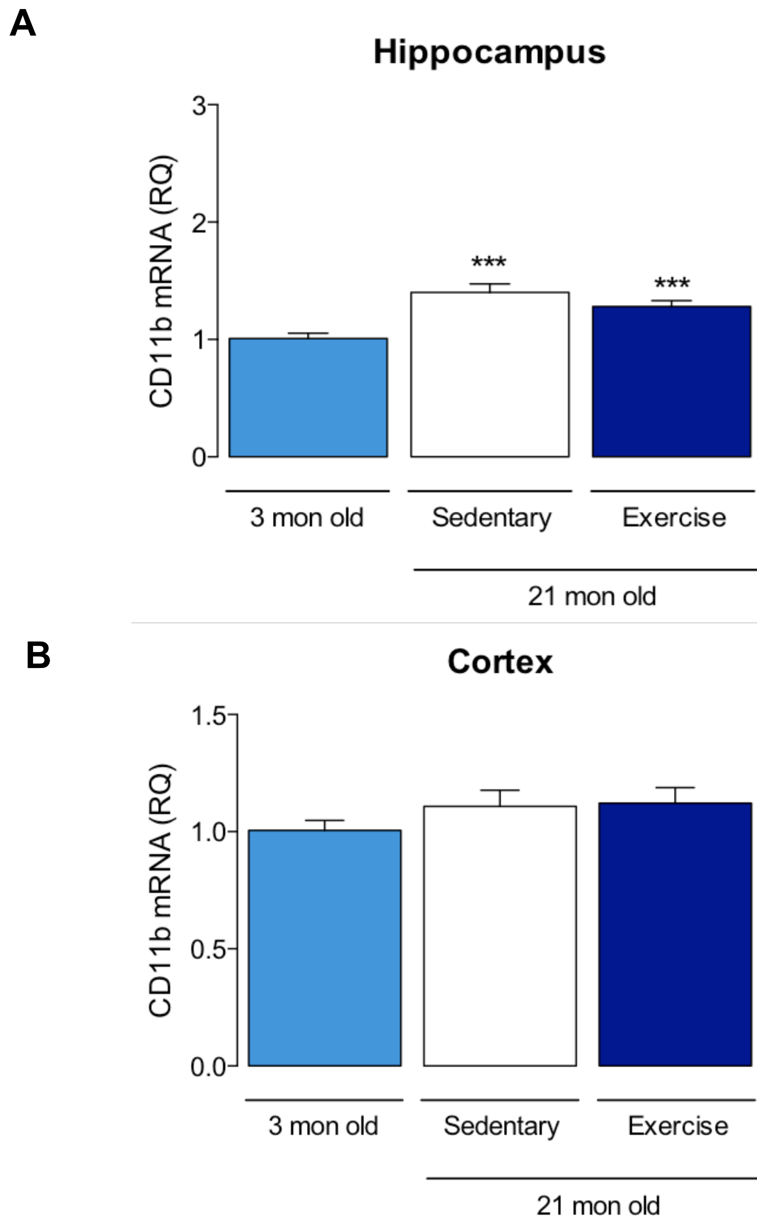


Figure 4.9 - Effects of age and exercise on mRNA expression of CD11b in the hippocampus and in the cortex

Ten months after exercise cessation, hippocampal and cortical mRNA expression of CD11b was assessed by RT-PCR. Expression of CD11b was increased in hippocampus of age-SED and age EX mice **(A)**. In the cortex, CD11b mRNA expression was not affected either by age or exercise **(B)**. Data are presented as mean ± SEM (n=7 to 8). ***p<0.001, 3 months old vs. SED and EX; one-way ANOVA with Bonferroni *post hoc* analysis.

4.3.5 Effects of age and exercise on mRNA expression of iNOS, Arginase-1 and Mrc1 in the hippocampus and cortex

The mRNA expression of inflammatory markers iNOS, Arg-1 and Mrc1 was assessed in the hippocampus and cortex by RT-PCR. Statistical analysis showed a significant difference in expression of iNOS in the hippocampus (p=0.0301, one-way ANOVA, Figure 4.10 A), but not in the cortex (p=0.4695, one-way ANOVA, RQ 3 months old: 1.02±0.08, SED: 1.03±0.19, EX: 0.79±0.15, Bonferroni *post-hoc*, Figure 4.10 B). Post-hoc analysis revealed a decrease in iNOS mRNA in the hippocampus of exercise compared to sedentary mice (RQ 3 months old: 0.95±0.03, SED: 1.12±0.17, EX: 0.70±0.04, Bonferroni *post-hoc*, Figure 4.10 A).

Statistical analysis also demonstrated a significant increase in the expression of Arg-1 in hippocampus (p=0.0312, one-way ANOVA, Figure 4.11 A), but not in cortex (p=0.2505, one-way ANOVA, RQ 3 months old: 0.98±0.10, SED: 0.95±0.10, EX: 1.57±0.47, Bonferroni *post-hoc*, Figure 4.11 A). In the hippocampus, *post-hoc* analysis revealed an increase in Arg-1 expression in sedentary group, but not the exercise group (RQ 3 months old: 1.06±0.13, SED: 1.88±1.35±0.14, Bonferroni *post-hoc*, Figure 4.11 B). Mrc1 mRNA levels were not affected, by age nor exercise, in the hippocampus (p=0.2343, one-way ANOVA, RQ 3 moths old: 1.08±0.07, SED: 1.32±0.2, EX: 1.55±0.23, Figure 4.12 A) or the cortex (p=0.9926, one-way ANOVA, RQ 3 months old: 1.01±0.06, SED: 1.01±0.11, EX: 0.99±0.11, Bonferroni *post-hoc*, Figure 4.12 B).

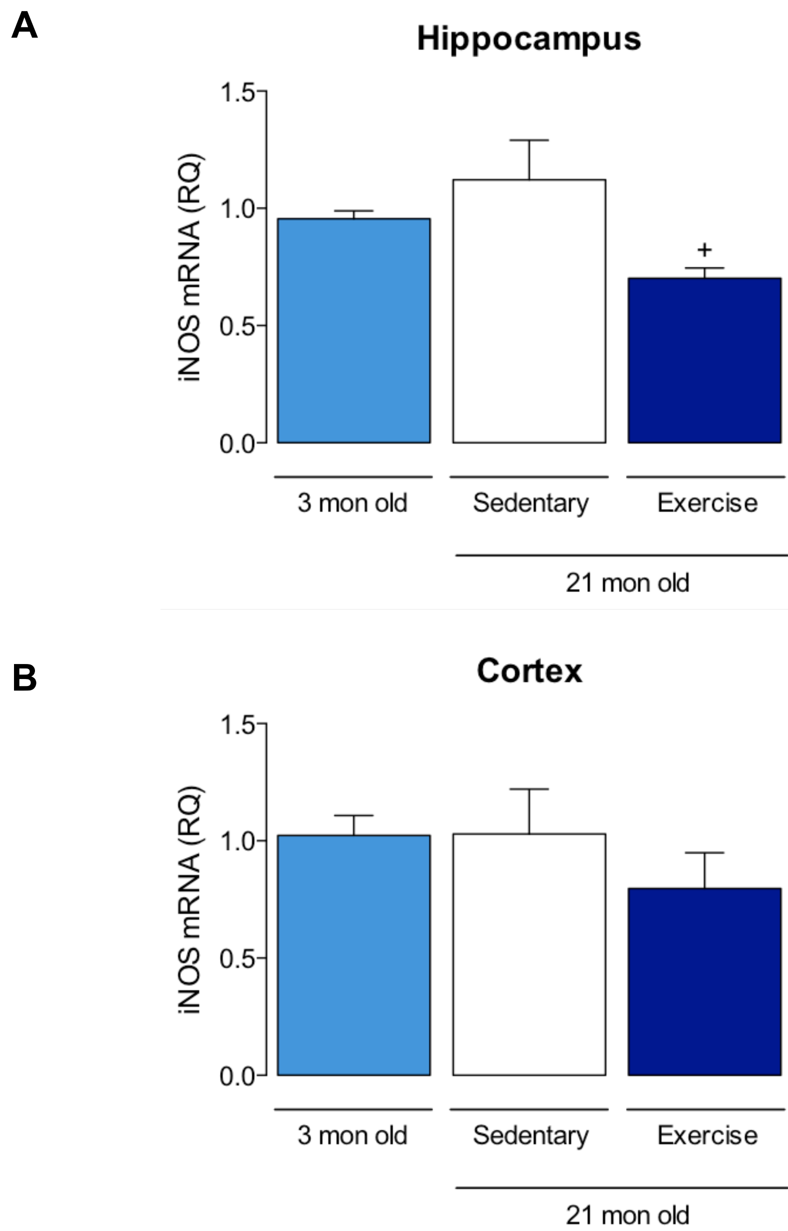


Figure 4.10 - Effects of age and exercise on mRNA expression of INOS in the hippocampus and in the cortex

Ten months after exercise cessation, hippocampal and cortical mRNA expression of INOS was assessed by RT-PCR. Expression of INOS mRNA was decreased in age-EX group **(A)** In the cortex, iNOS mRNA expression was not affected either by age or exercise **(B)**. Data are presented as mean ± SEM (n=7 to 8). $^{+}p<0.05$, SED vs. EX; one-way ANOVA with Bonferroni *post hoc* analysis.

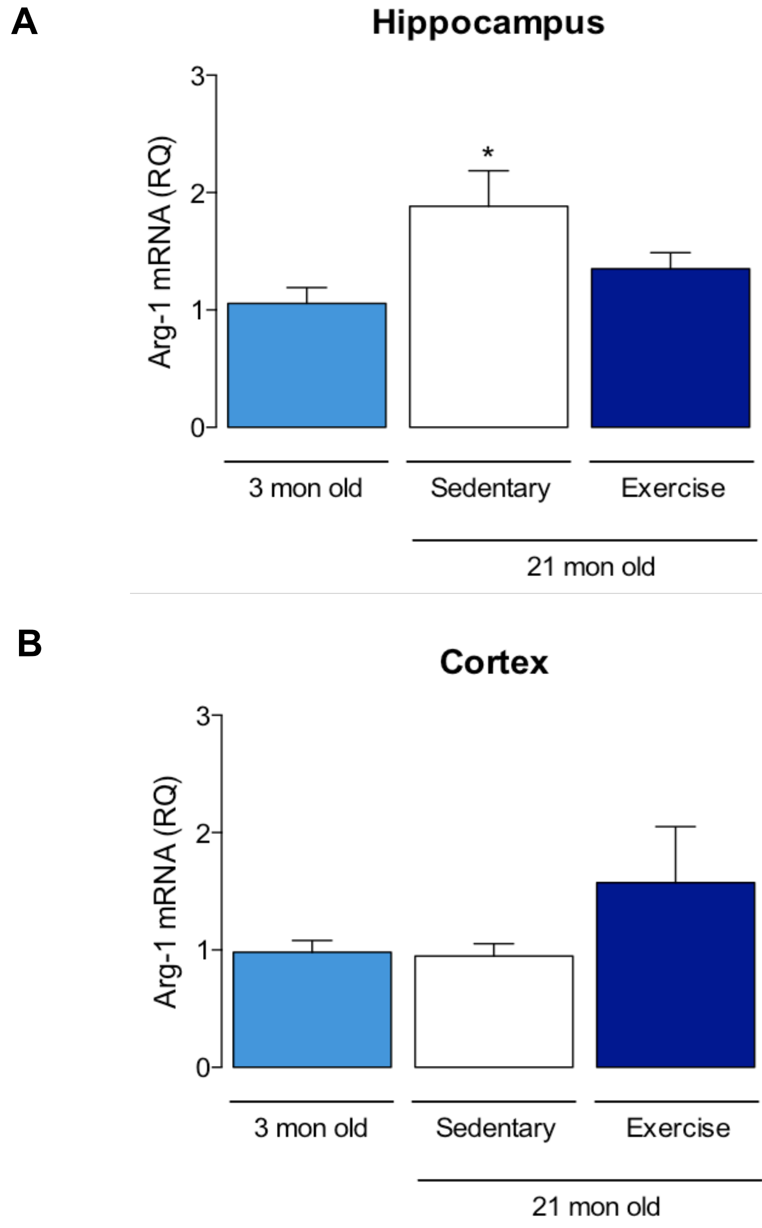


Figure 4.11 - Effects of age and exercise on mRNA expression of Arg-1 in the hippocampus and in the cortex

Ten months after exercise cessation, hippocampal and cortical mRNA expression of Arg-1 was assessed by RT-PCR. Expression of Arg-1 was increased in the hippocampus of age-SED, but not in age-EX group **(A)**. In the cortex, Arg-1 mRNA expression was not affected either by age or exercise **(B)**. Data are presented as mean ± SEM (n=7 to 8). *p<0.05, 3 months old vs. SED; one-way ANOVA with Bonferroni *post hoc* analysis.

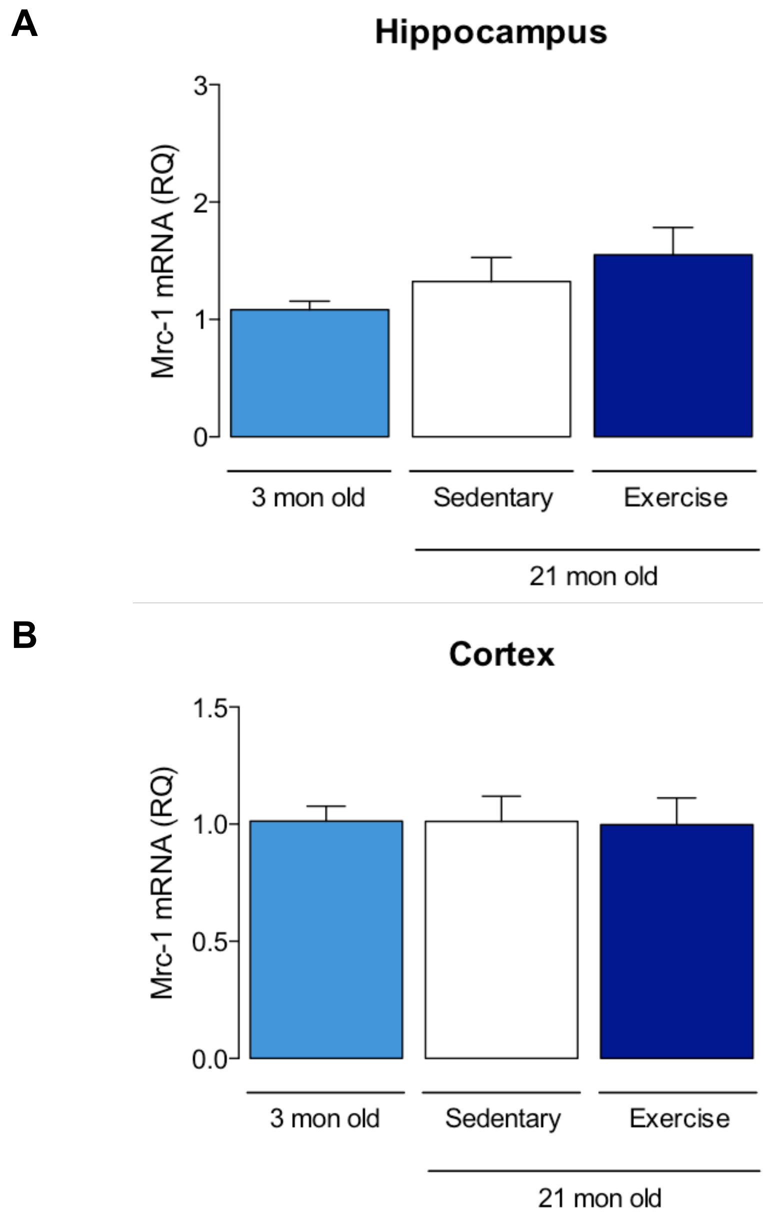


Figure 4.12 - Effects of age and exercise on mRNA expression of Mrc1 in the hippocampus and in the cortex

Ten months after exercise cessation, hippocampal and cortical mRNA expression of Mrc1 was assessed by RT-PCR. Expression of Mrc1, in the hippocampus and cortex, was not affected either by age or exercise **(A, B)**. Data are presented as mean ± SEM (n=7 to 8); one-way ANOVA with Bonferroni *post hoc* analysis.

4.3.6 Effects of age and exercise on mRNA expression of Cx3cl1, Cx3cr1 and CD44, in the hippocampus and cortex

The mRNA expression of inflammatory mediators, Cx3cl1, Cx3cr1 and CD44 was assessed in the hippocampus and cortex by RT-PCR. Statistical analysis showed no significant difference in mRNA expression of Cx3cl1 in the hippocampus ($p=0.1170$, one way ANOVA, RQ 3 months old: 1.00 ± 0.03, SED: 0.95 ± 0.04, EX: 0.89 ± 0.02, Bonferroni *post-hoc*, Figure 4.13 A), however there was a significant difference in the cortex ($p=0.0022$, one-way ANOVA, Figure 4.13 B). Post hoc analysis revealed, in the cortex, an age-induced reduction in Cx3cl1 mRNA in both sedentary and exercise groups (RQ 3 month old: 1.00 ± 0.05, SED: 0.81 ± 0.02, EX: 0.85 ± 0.03, Bonferroni *post-hoc*, Figure 4.13 B).

Expression of Cx3cr1 mRNA was altered in the hippocampus ($p=0.0422$, one-way ANOVA, Figure 4.13 C), but not the cortex ($p=0.2164$, one-way ANOVA, RQ 3 months old: 1.00 ± 0.03, SED: 1.11 ± 0.05, EX: 1.00 ± 0.05, Bonferroni *post-hoc*, Figure 4.13 D). In the hippocampus, Bonferroni *post-hoc* analysis showed an age-induced decrease in Cx3cr1 mRNA in sedentary and exercise mice (RQ 3 months: 1.00 ± 0.03, SED: 1.17 ± 0.06, EX, 0.05, Figure 4.13 C). Expression of CD44 was not altered, by ageing nor exercise, in either hippocampus ($p=0.3962$, one way ANOVA, RQ 3 months old: 1.07 ± 0.15, SED: 1.27 ± 0.12, EX: 1.06 ± 0.08, Bonferroni *post-hoc*, Figure 4.14 A) or cortex ($p=0.1887$, one-way ANOVA, RQ 3 months old: 1.03 ± 0.09, SED: 0.98 ± 0.08, EX: 1.18 ± 0.06, Bonferroni *post-hoc*, Figure 4.14 B).

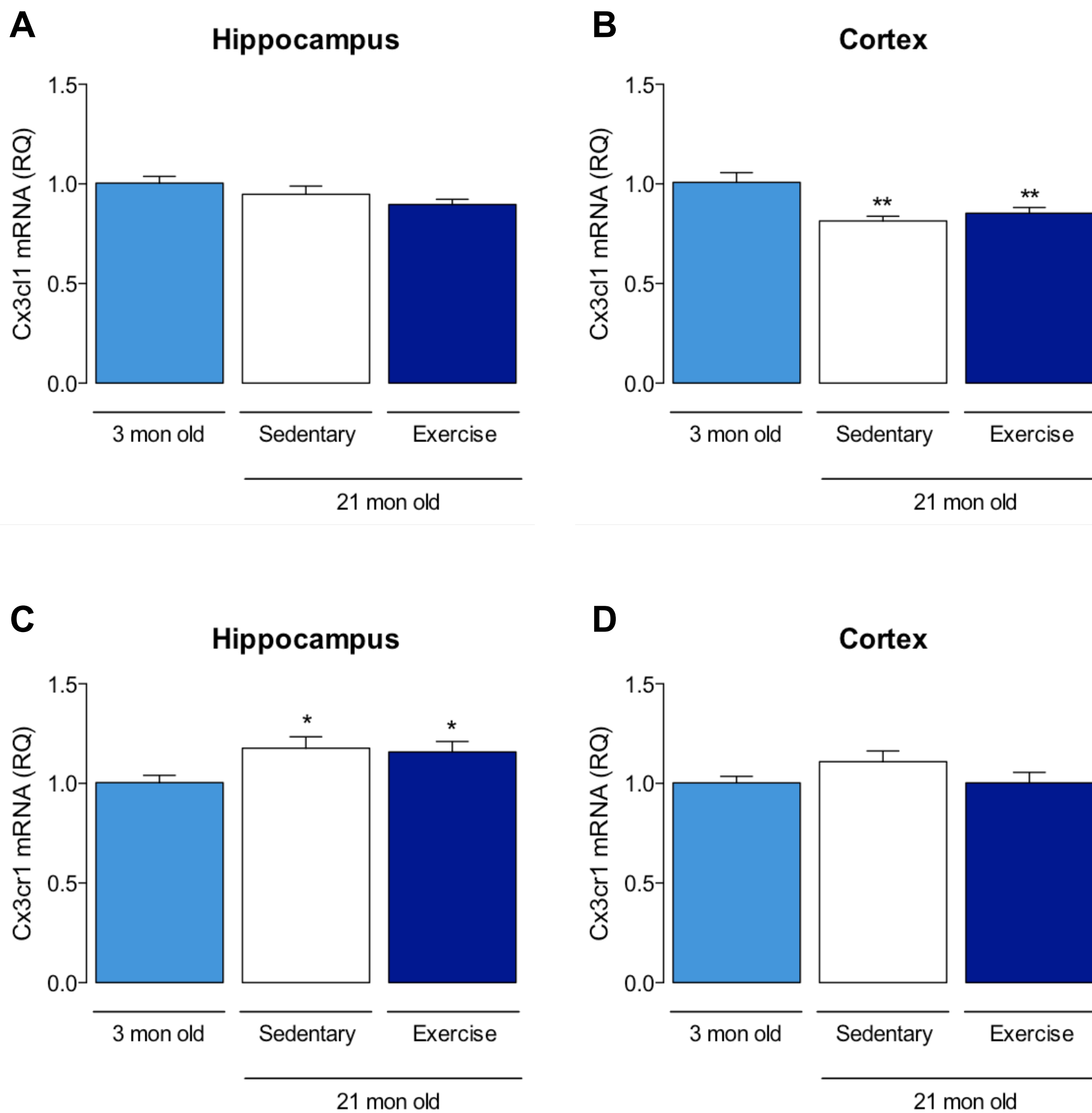


Figure 4.13 - Effects of age and exercise on mRNA expression of Cx3cl-1 and Cx3cr1 in the hippocampus and in the cortex

Ten months after exercise cessation, hippocampal and cortical mRNA expression of Cx3cl1 and Cx3cr1 were assessed by RT-PCR. Expression of Cx3cl1 was not affected either by age or exercise **(A)**. In the cortex, Cx3cl1 was increased in the hippocampus of age-SED and age-EX groups **(B)**. Expression of Cx3cr1 was increased in the hippocampus of age-SED and age-EX groups **(C)**. In the cortex, Cx3cr1 was not affected either by age or exercise **(D)**. Data are presented as mean ± SEM (n=7 to 8). **p<0.01, 3 months old vs. SED and EX, *p<0.05, 3 months old vs. SED and EX; one-way ANOVA with Bonferroni *post hoc* analysis.

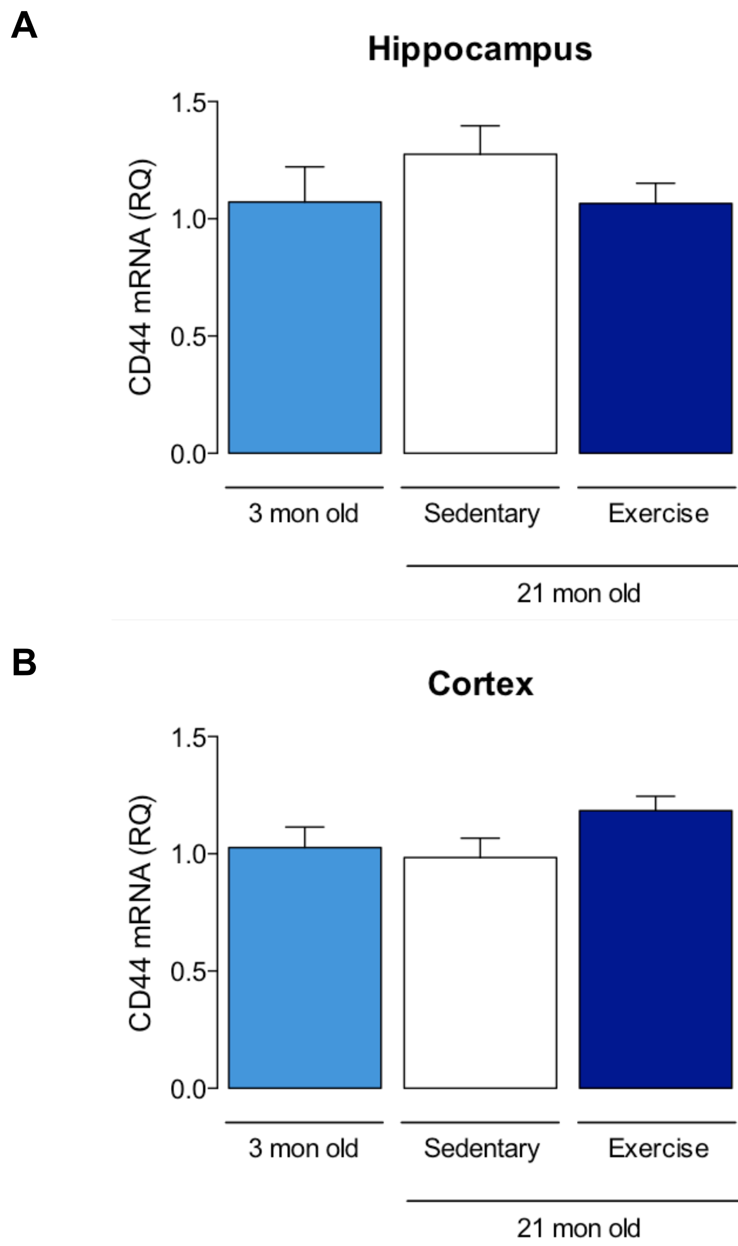


Figure 4.14 - Effects of age and exercise on mRNA expression of CD44 in the hippocampus and in the cortex

Ten months after exercise cessation, hippocampal and cortical mRNA expression of CD44 was assessed by RT-PCR. Expression of CD44, in the hippocampus and cortex, was not affected either by age or exercise **(A, B)**. Data are presented as mean ± SEM (n=7 to 8); one-way ANOVA with Bonferroni *post hoc* analysis.

4.3.7 Effects of age and exercise on mRNA expression of apoptosis-related proteins, Bax and Bcl-2 and cell proliferation markers Ki67 in the hippocampus and cortex

We also investigated mRNA expression of Bax and Bcl-2, proteins responsible for regulating apoptosis, and mRNA expression of the cell proliferation marker, Ki67 by RT-PCR. Statistical analysis showed no significant difference in the mRNA expression of Bax in hippocampus ($p=0.3725$, one way ANOVA, RQ 3 months old: 1.00 ± 0.02, SED: 1.04 ± 0.03, EX: 0.98 ± 0.03, Bonferroni *post-hoc*, Figure 4.15 A) or cortex ($p=0.0717$, one-way ANOVA, RQ 3 months old: 1.01 ± 0.06, SED: 0.84 ± 0.03, EX: 0.86 ± 0.06, Bonferroni *post-hoc*, Figure 4.15 B). Similarly, no difference was observed between the groups in Bcl-2 mRNA in the hippocampus ($p=0.6635$, one-way ANOVA, RQ 3 months old: 1.01 ± 0.06, SED: 0.99 ± 0.08. EX: 0.94 ± 0.03, Bonferroni *post-hoc*, Figure 4.15 C). However, there was a significant difference in Bcl-2 mRNA in the cortex ($p=0.0097$, one-way ANOVA, Figure 4.15 D). Bonferroni *post-hoc* analysis revealed an increase in Bcl-2 mRNA in the cortex of exercised mice compared to sedentary mice (RQ 3 months old: 0.96 ± 0.03, SED: 0.85 ± 0.04, EX: 1.06 ± 0.04, Figure 4.15 D).

Also, statistical analysis demonstrated an age-induced decrease in Ki67 mRNA, in sedentary and exercise groups, in both hippocampus ($p<0.0001$, one-way ANOVA, RQ 3 months old: 1.03 ± 0.09, SED: 0.47 ± 0.07, EX: 0.47 ± 0.06, Figure 4.16 C) and cortex ($p<0.0001$, one way ANOVA, RQ 3 months old: 1.04 ± 0.10, SED: 0.36 ± 0.02, EX: 0.38 ± 0.03, Bonferroni *post-hoc*, Figure 4.16 D).

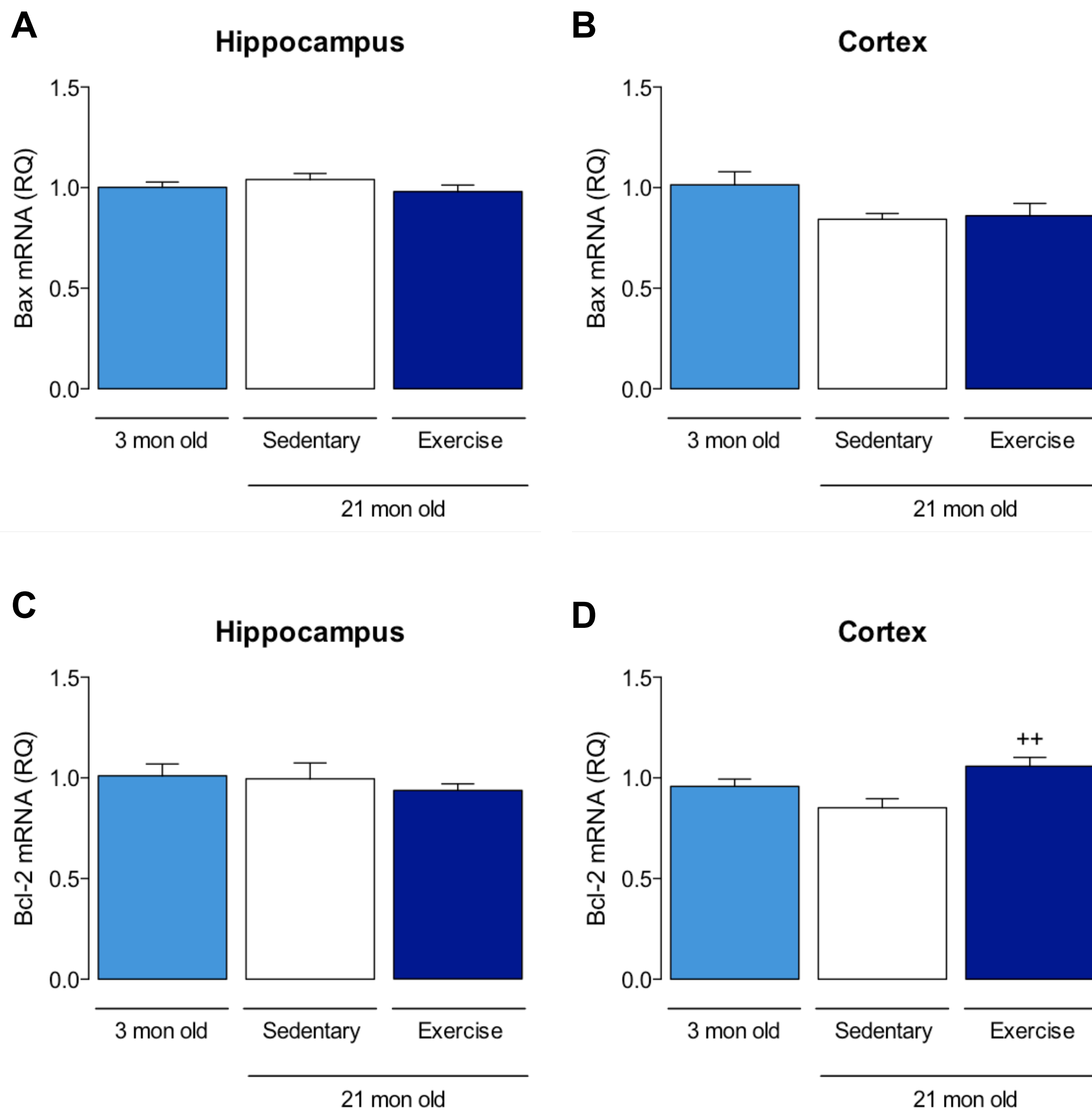


Figure 4.15 - Effects of age and exercise on mRNA expression of Bax and Bcl-2 in the hippocampus and in the cortex

Ten months after exercise cessation, hippocampal and cortical mRNA expression of Bax and Bcl-2 and were assessed by RT-PCR. Expression of Bax in the hippocampus and cortex was not affected either by age or exercise **(A, B)**. Similarly, expression of Bcl-2, in the hippocampus, was not affected either by age or exercise **(C)**. In the cortex, Bcl-2 mRNA levels were increased in the age-EX, but not in age-SED group **(D)**. Data are presented as mean ± SEM (n=7 to 8). $^{+}$p<0.05, SED vs. EX; one-way ANOVA with Bonferroni *post hoc* analysis.

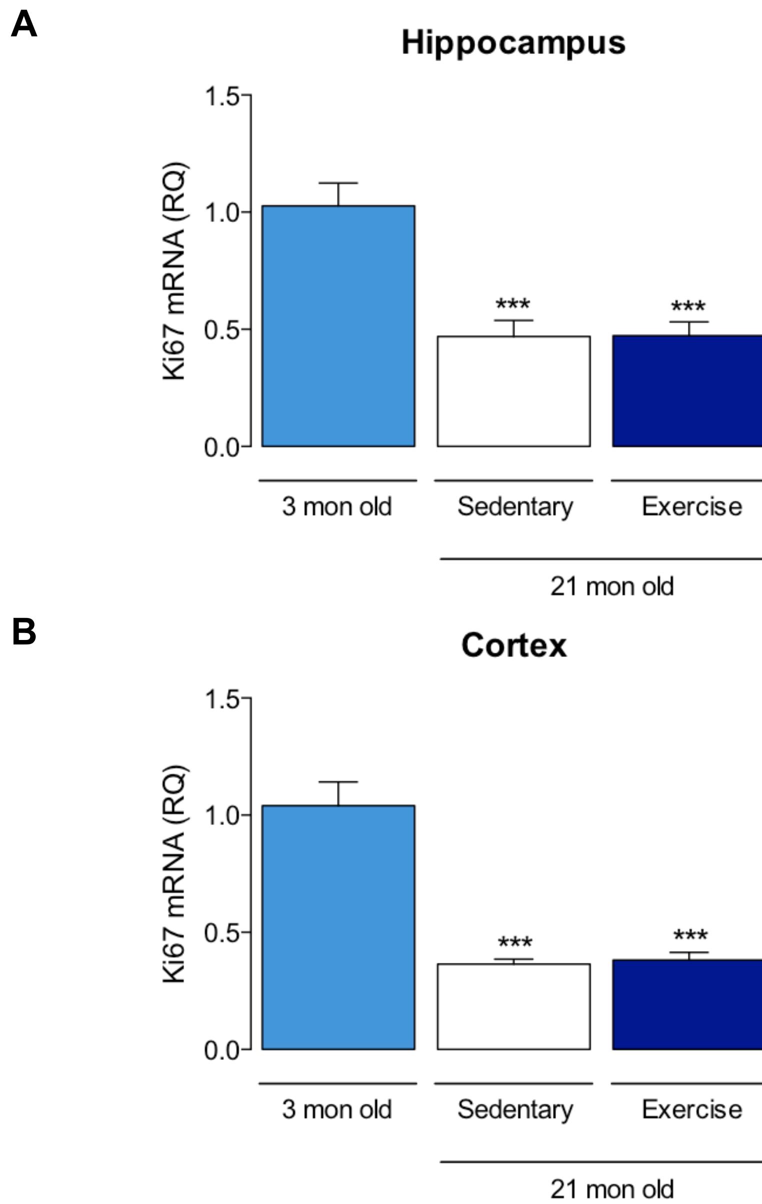


Figure 4.16 - Effects of age and exercise on mRNA expression of Ki67 in the hippocampus and in the cortex

Ten months after exercise cessation, hippocampal and cortical mRNA expression of Ki67 was assessed by RT-PCR. Expression of ki67 was decreased in the hippocampus and cortex of age-SED and age-EX groups **(A, B)**. ***p<0.001, 3 months old vs. SED and EX, *p<0.05, 3 months old vs. SED and EX; one-way ANOVA with Bonferroni *post hoc* analysis.

4.3.8 Effects of age and exercise on mRNA and protein expression of BDNF, TrkB and p75 receptors in the hippocampus and cortex

Hippocampal and cortical expression and concentration of BDNF were measured using RT-PCR and ELISA assay, respectively. Statistical analysis demonstrated no significant difference in mRNA expression of BDNF in the hippocampus ($p=0.3599$, one-way ANOVA, RQ 3 months old: 1.00±0.04, SED: 0.95±0.03, EX: 0.94±0.03, Bonferroni *post-hoc*, Figure 4.17 A). However, an age-induced decrease in BDNF mRNA was observed in the cortex of sedentary and exercise mice compared to 3 months old ($p=0.0161$, one-way ANOVA, RQ 3 months old: 1.08±0.11, SED: 0.73±0.08, EX: 0.76±0.06, Bonferroni *post-hoc*, Figure 4.17 B). Statistical analysis of ELISA assay results showed no changes in the concentration of BDNF in the hippocampus in either sedentary or exercise mice ($p=0.3241$, one-way ANOVA, 3 months old: 1530±58.87 pg/mg, SED: 1655±61.88 pg/mg, EX: 1655±76.69 pg/mg, Bonferroni *post-hoc*, Figure 4.17 C).

We also investigated mRNA expression of TrkB and p75 receptors in the hippocampus and cortex, by RT-PCR. Statistical analysis demonstrated no significant changes in TrkB mRNA, in the hippocampus ($p=0.2482$, one-way ANOVA, RQ 3 months old: 1.01±0.06, SED: 1.12±0.06, EX: 1.00±0.03, Bonferroni *post-hoc*, Figure 4.18 A) or cortex ($p=0.2859$, one-way ANOVA, RQ 3 months old: 1.02±0.09, SED: 0.91±0.03, EX: 1.02±0.03, Bonferroni *post-hoc*, Figure 4.18 B). Similarly, statistical analysis showed no significant difference in the mRNA expression of p75 receptor between the groups, neither in the hippocampus ($p=0.3504$, one-way ANOVA, RQ 3 months old: 1.07±0.14, SED: 1.31±0.10, EX: 1.19±0.11, Bonferroni *post-hoc*, Figure 4.18 C) nor in the cortex ($p=0.0912$, one-way ANOVA, RQ 3 months old: 1.46±0.50, SED: 3.86±1.02, EX: 3.24±0.66, Bonferroni *post-hoc*, Figure 4.18 D).

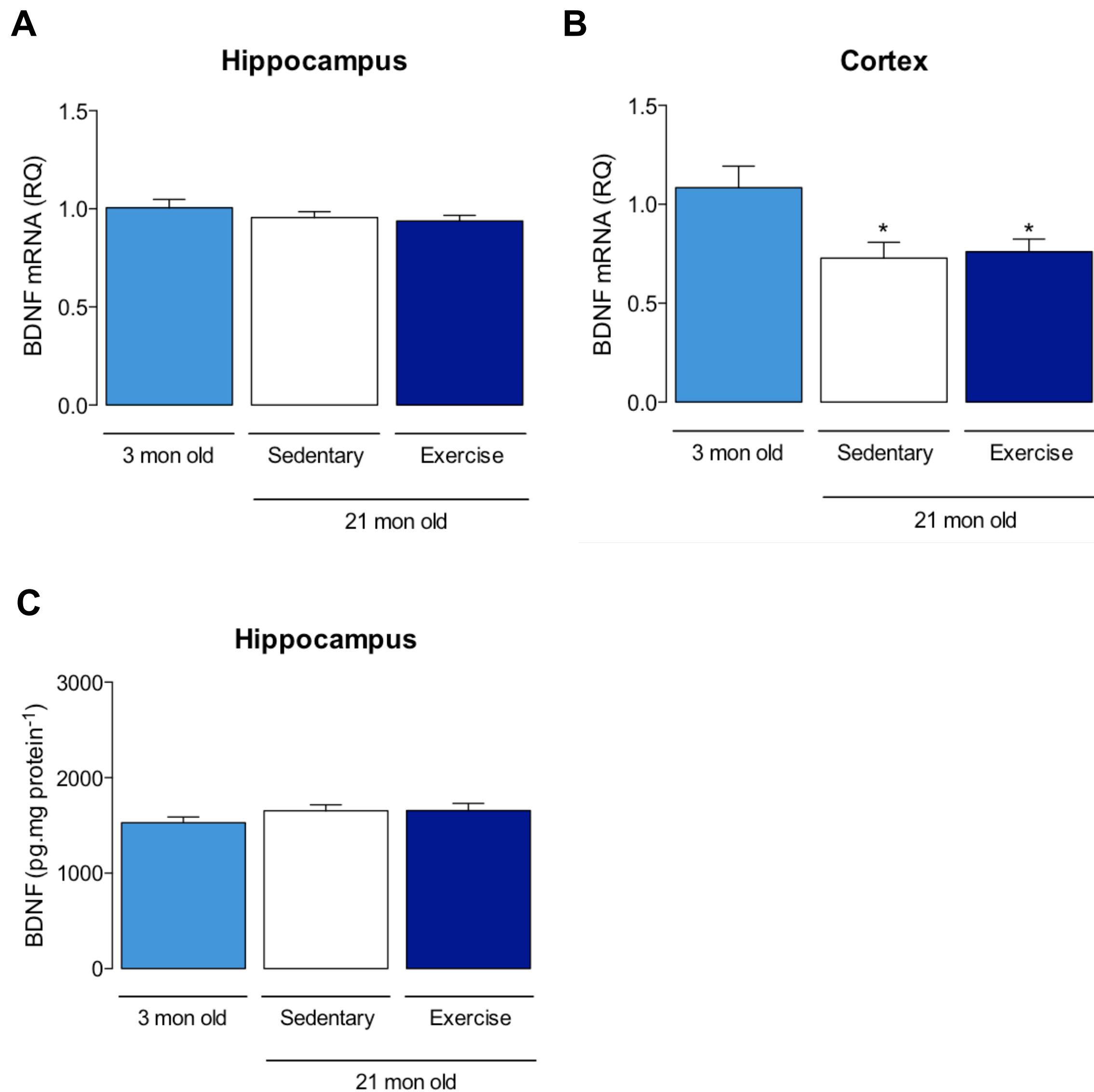


Figure 4.17 - Effects of age and exercise on mRNA expression of BDNF in the hippocampus and in the cortex and in the BDNF protein expression in the hippocampus

Ten months after exercise cessation, hippocampal and cortical mRNA expression of BDNF was assessed by RT-PCR, while BDNF concentration was assessed by ELISA. Expression of BDNF mRNA, in the hippocampus, was not affected either by age or exercise **(A)**. In the cortex, expression of BDNF mRNA was decreased in age-SED and age-EX groups **(B)**. Concentration of BDNF, in the hippocampus, was not affected either by age or exercise. *$p<0.05$, 3 months old vs. SED and EX; one-way ANOVA with Bonferroni *post hoc* analysis.

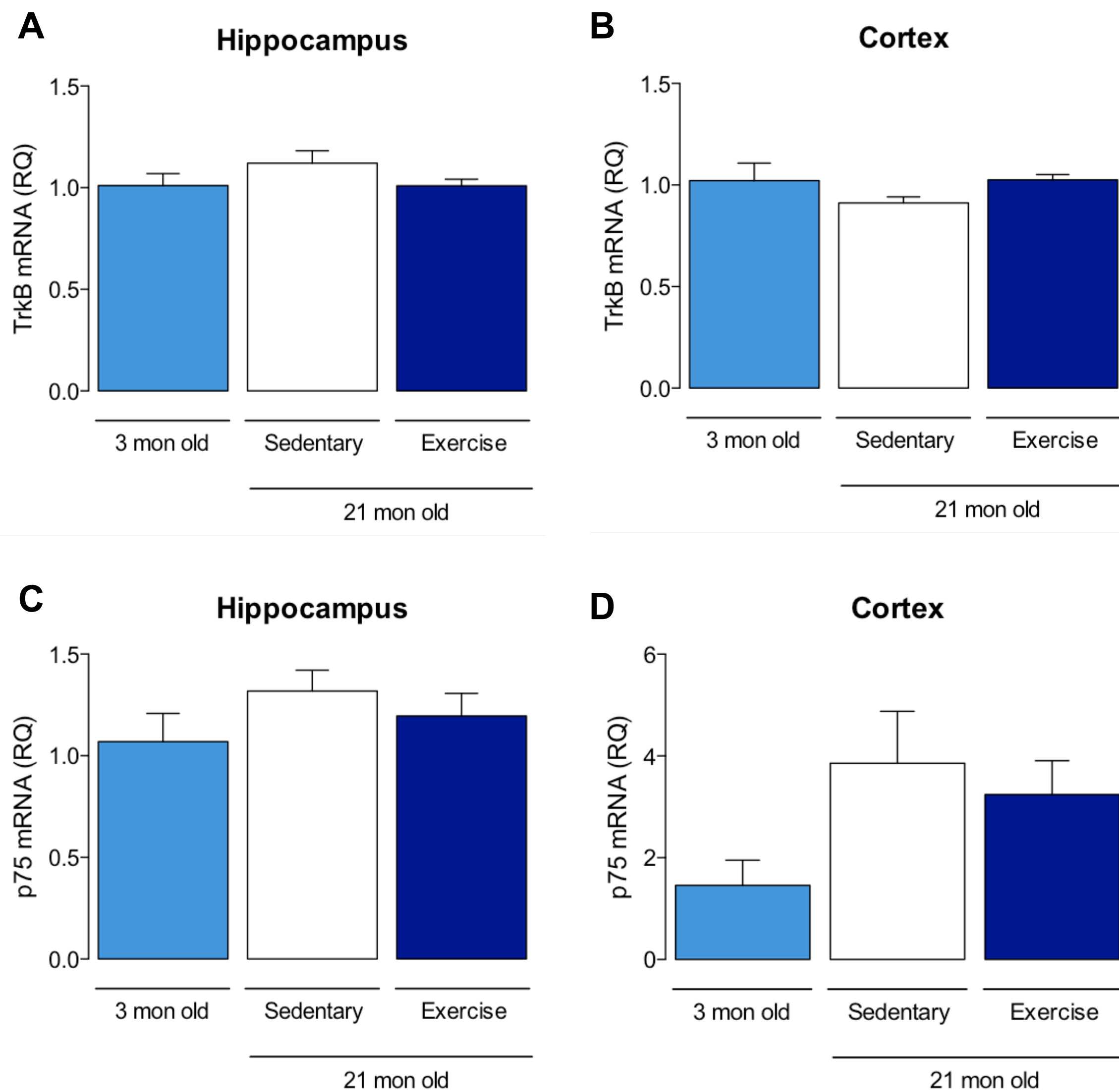


Figure 4.18 - Effects of age and exercise on mRNA expression of TrkB and p75 receptors in the hippocampus and in the cortex

Ten months after exercise cessation, hippocampal and cortical mRNA expression of TrkB and p75 receptors were assessed by RT-PCR. Expression of TrkB receptor, in the hippocampus and cortex, was not affected either by age or exercise **(A, B)**. Similarly, expression of p75 receptor, in the hippocampus and cortex, was not affected either by age or exercise **(C, D)**. Data are presented as mean ± SEM (n=7 to 8); one-way ANOVA with Bonferroni *post hoc* analysis.

4.3.9 Effects of age and exercise on mRNA expression of NGF, Igf-1, VEGF and CREB in the hippocampus and cortex

Expression of NGF, VEGF, Igf-1 and CREB was measured in the hippocampus and cortex by RT-PCR. Statistical analysis demonstrated no significant difference in NGF mRNA expression in the hippocampus (p=0.5807, one-way ANOVA, RQ 3 months old: 1.00±0.05, SED: 1.05±0.07, EX: 0.96±0.06, Bonferroni *post-hoc*, Figure 4.19 A) or cortex (p=0.4421, one-way ANOVA, RQ 3 months old: 1.02±0.06, SED: 0.75±0.05, EX: 0.92±0.06, Bonferroni *post-hoc*, Figure 4.19 B). There was no significant change in the VEGF mRNA in hippocampus (p=0.6990, one-way ANOVA, RQ 3 months: 1.03±0.05, SED: 1.08±0.09, EX: 1.00±0.05, Bonferroni *post-hoc*, Figure 4.19 C) or cortex (p=0.3613, one-way ANOVA, RQ 3 months old: 1.03±0.09, SED: 0.90±0.03, EX: 0.94±0.03, Bonferroni *post-hoc*, Figure 4.19 D).

Moreover, statistical analysis showed no significant difference in Igf-1 mRNA in hippocampus (p=0.2983, one-way ANOVA, RQ 3 months old: 1.01±0.06, SED: 1.08±0.09, 0.93±0.04, Figure 4.20 A) or cortex (p=0.1608, one-way ANOVA, RQ 3 months old: 1.02±0.08, SED: 0.83±0.05, EX: 0.96± 0.06, Figure 4.20 B). CREB mRNA expression was not affected by ageing or exercise in hippocampus (p=0.6567, one-way ANOVA, RQ 3 months old: 1.00±0.04, SED: 0.98±0.07, EX: 0.94±0.03, Figure 4.20 C) or cortex (p=0.1629, one-way ANOVA, RQ 3 months old: 0.99±0.07, SED: 0.87±0.05, EX: 1.01±0.04, Bonferroni *post-hoc*, Figure 4.20 D).

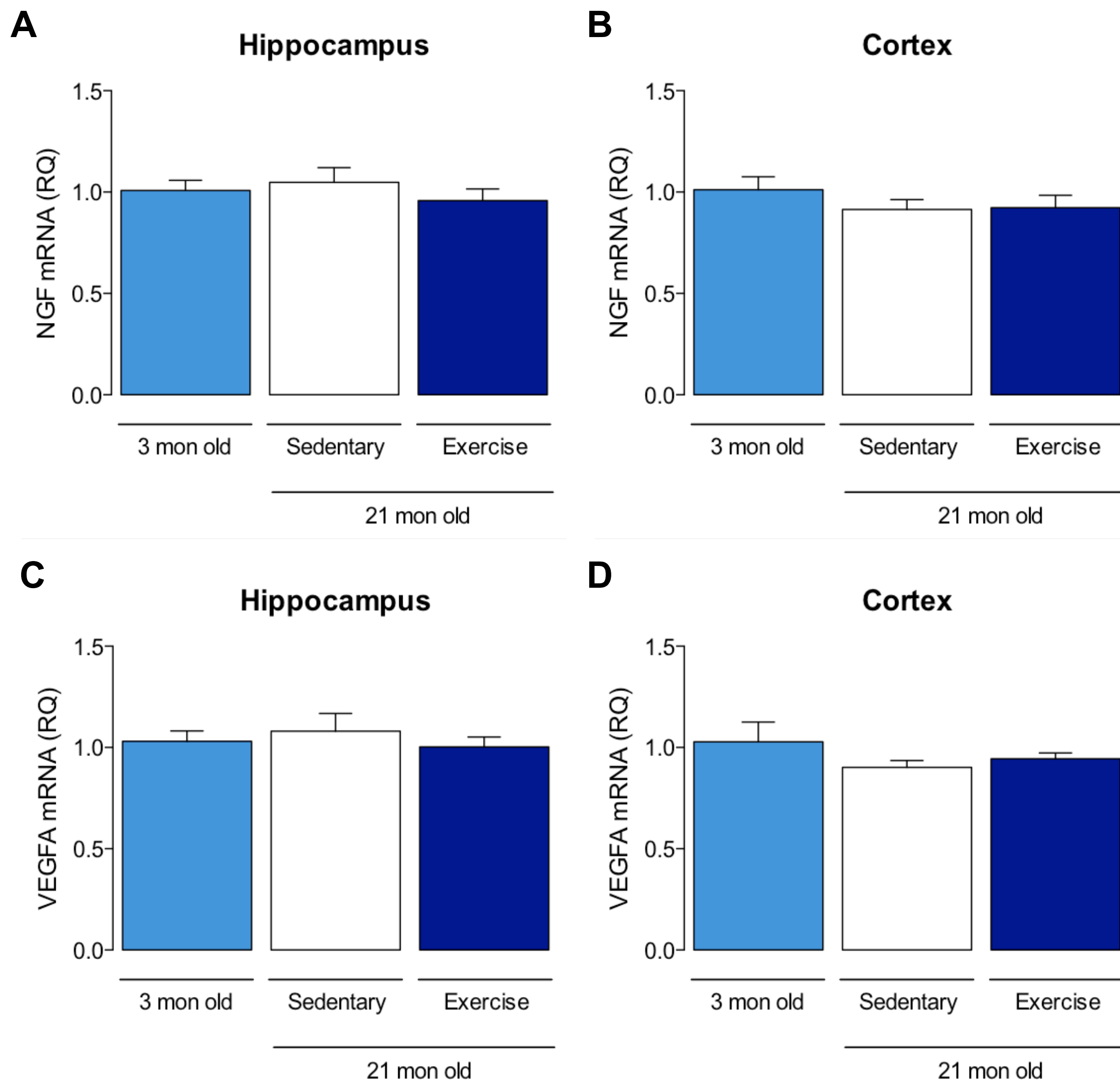


Figure 4.19- Effects of age and exercise on mRNA expression of NGF and VEGF in the hippocampus and in the cortex

Ten months after exercise cessation, hippocampal and cortical mRNA expression of NGF and VEGF were assessed by RT-PCR. Expression of NGF, in the hippocampus and cortex, was not affected either by age or exercise **(A, B)**. Similarly, expression of VEGF, in the hippocampus and cortex, was not affected either by age or exercise **(C, D)**. Data are presented as mean ± SEM (n=7 to 8); one-way ANOVA with Bonferroni *post hoc* analysis.

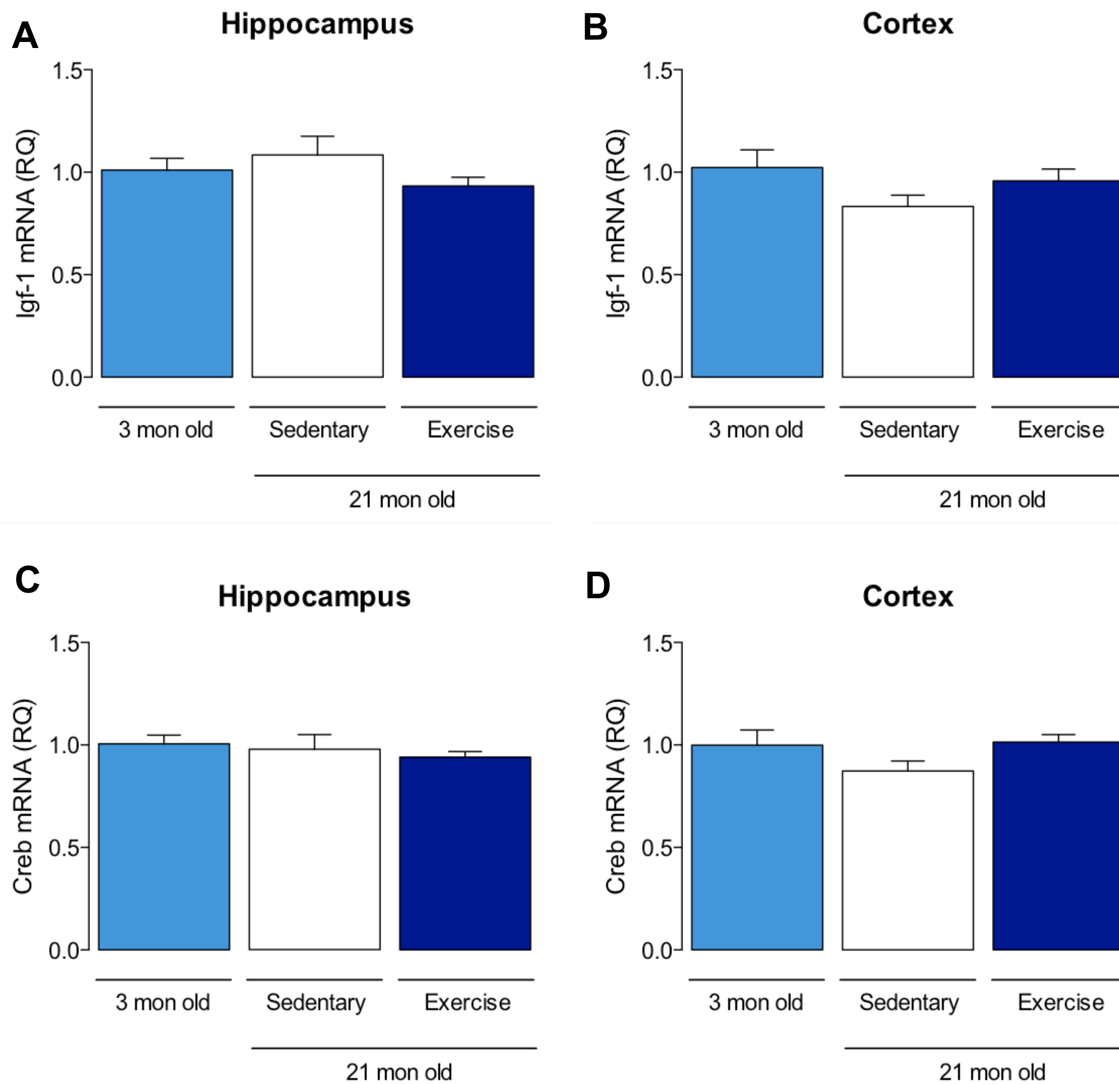


Figure 4.20- Effects of age and exercise on mRNA expression of Igf-1 and CREB in the hippocampus and in the cortex

Ten months after exercise cessation, hippocampal and cortical mRNA expression of Igf-1 and CREB was assessed by RT-PCR. Expression of Igf-1, in the hippocampus and cortex, was not affected either by age or exercise **(A, B)**. Similarly, expression of CREB, in the hippocampus and cortex, was not affected either by age or exercise **(C, D)**. Data are presented as mean ± SEM (n=7 to 8); one-way ANOVA with Bonferroni *post hoc* analysis.

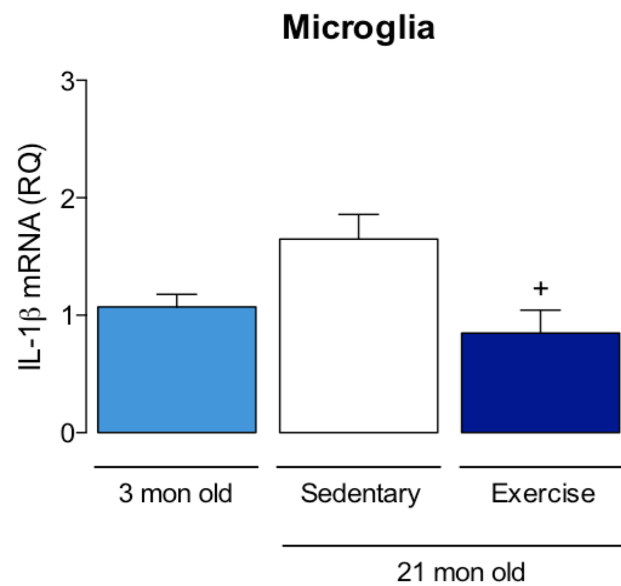
4.3.10 Effects of age and exercise on mRNA expression of M1 phenotype markers in an enriched population of microglia

Following analysis of mRNA expression of cytokines and markers of activation of astrocytes and microglia in tissue prepared from homogenised whole hippocampus and cortex, we decided to investigate, 10 months after exercise cessation, markers of M1 phenotype in an enriched microglial population prepared from brain tissue of these mice. We measured the mRNA expression of IL-1β, TNF-α, iNOS and Iba-1 in microglial cells using RT-PCR.

Statistical analysis demonstrated a significant difference in mRNA expression of IL-1β ($p=0.0297$, Kruskal-Wallis test, Figure 4.21 A). Dunn's *post-hoc* analysis, revealed decrease in mRNA expression of IL-1β in microglia cells from exercise mice, compared to sedentary group (RQ 3 months old: 1.07±0.10, SED: 1.65±0.20, EX: 0.85±0.19, Figure 4.21 A). Similarly, there was a significant difference in the mRNA expression of TNF-α in microglia ($p=0.0102$, Kruskal-Wallis test, Figure 4.21 B). Post-hoc analysis revealed an age-induced increase in TNF-α mRNA in the microglia of sedentary, but not exercise, mice compared to 3 month old mice (RQ 3 months: 1.07±0.10, SED: 2.59±0.66, EX: 1.75±0.24, Dunn's *post-hoc*, Figure 4.21 B).

Although expression of iNOS tends to an increase in microglia of exercised mice, no statistically significant difference was observed in microglial expression of iNOS between the groups ($p=0.3178$, Kruskal-Wallis test, RQ 3 months old: 0.74±0.08, SED: 0.77±0.36, EX: 1.33±0.47, Dunn's *post-hoc*, Figure 4.22 A). However, statistical analysis demonstrated a significant difference in mRNA expression of Iba-1 in microglia cells ($p=0.0127$, Kruskal-Wallis test, Figure 4.22 B). Post-hoc analysis revealed a significant decrease in Iba-1 mRNA expression in the microglia of sedentary mice compared to 3 months old mice (RQ 3 months old: 0.96±0.07, SED: 1.18±0.08, EX: 0.88±0.07, Dunn's *post-hoc*, Figure 4.22 B).

A



B

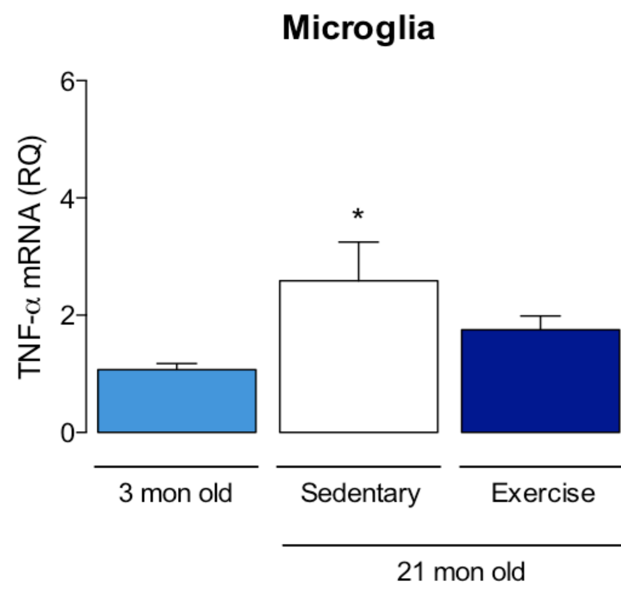


Figure 4.21 - Effects of age and exercise on mRNA expression of M1 phenotype markers IL-1β and TNF-α in an enriched population of microglia

Ten months after exercise cessation, mRNA expression of IL-1β and TNF-α in microglia enriched population was assessed by RT-PCR. Expression of IL-1β was decreased in microglia cells from age-EX group mice compared to microglia cells from age-SED group **(A)**. Expression of TNF-α was increased only in microglia cells from age-SED mice, but not in microglia cells from age-EX mice **(B)**. Data are presented as mean ± SEM (n=7 to 8). *$p<0.05$, 3 months old vs. SED, $^{+}p<0.05$, SED vs. EX; Kruskal Wallis test with Dunn's *post hoc* analysis.

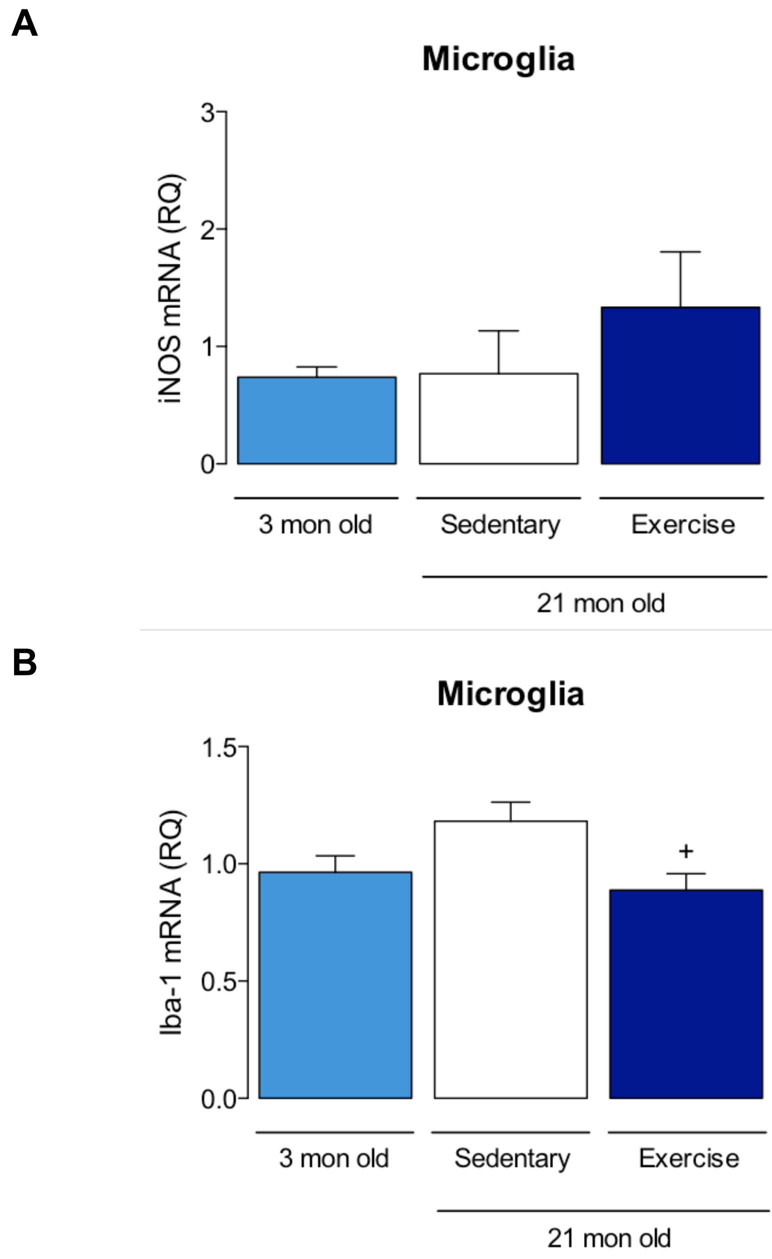


Figure 4.22 - Effects of age and exercise on mRNA expression of M1 phenotype markers iNOS and Iba-1 in an enriched population of microglia

Ten months after exercise cessation, mRNA expression of iNOS and Iba-1 in microglia enriched population was assessed by RT-PCR. Expression of iNOS, in microglia cells, was not affected either by age or exercise **(A)**. Expression of Iba-1 was decreased in microglia cells from age-EX group compared to microglia cells from age-SED group **(B)**. Data are presented as mean ± SEM (n=7 to 8). $^{+}p<0.05$, SED vs. EX; Kruskal Wallis test with Dunn's *post hoc* analysis.

4.3.11 Effects of age and exercise on mRNA expression of M2 phenotype markers in an enriched population of microglia

In order to investigate markers of M2 phenotype in an enriched microglial population prepared from brain tissue of these mice we measured the mRNA expression of Arg-1, Mrc1 and, Ym1 by RT-PCR. Although mRNA expression of Arg-1 tends to decrease in sedentary group, no statistically significant difference was observed between the groups ($p=0.6828$, Kruskal-Wallis test, RQ 3 months old: 1.55 ± 0.43, SED: 0.89 ± 0.25, EX: 1.44 ± 0.63, Dunn's *post-hoc*, Figure 4.23 A).

Similarly, mRNA expression of Mrc1 in microglial cells was not affected either by ageing or exercise ($p=0.0805$, Kruskal-Wallis test, RQ 3 months old: 1.02 ± 0.07, SED: 0.70 ± 0.14, EX: 0.84 ± 0.05, Dunn's *post-hoc*, Figure 4.23 B). Statistical analysis demonstrated a significant difference in mRNA expression of Ym1 in microglia between the groups ($p=0.0481$, Kruskal-Wallis test, Figure 4.23 C). Dunn's *post-hoc* revealed a significant decrease in the mRNA levels of Ym1 in microglia cells from exercise mice compared to 3 month old mice (RQ 3 months old: 1.43 ± 0.39, SED: 0.83 ± 0.30, EX: 0.36 ± 0.09, Figure 4.23 C).

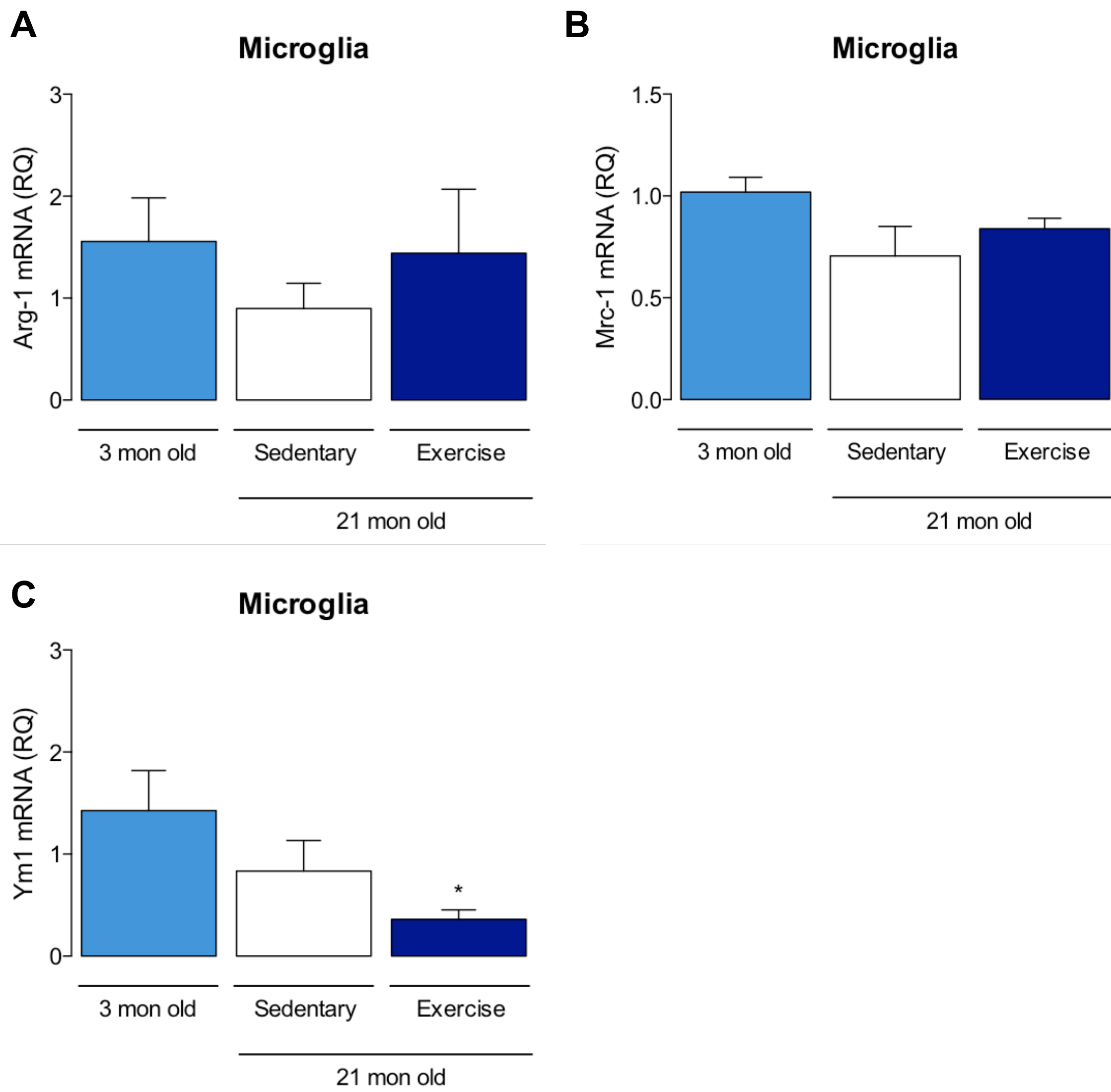


Figure 4.23 - Effects of age and exercise on mRNA expression of M2 phenotype markers Arg-1, Mrc1 and Ym1 in an enriched population of microglia

Ten months after exercise cessation, mRNA expression of Arg-1, Mrc1 and Ym1 in microglia enriched population was assessed by RT-PCR. Expression of Arg-1 and Mrc1, in microglia cells, was not affected either by age or exercise **(A, B)**. Expression of Ym1 was decreased in microglia cells from age-EX group compared to microglia cells from 3 months old group **(C)**. Data are presented as mean ± SEM (n=7 to 8). *$p<0.05$, 3 months old vs. EX; Kruskal Wallis test with Dunn's *post hoc* analysis.

4.3.12 Effects of age and exercise on mRNA expression of A1 phenotype markers in an enriched population of astrocytes

The mRNA expression of A1 phenotype astrocyte activation in an isolated enriched population of astrocytes was measured by RT-PCR analysis of mRNA expression of TNF-α, iNOS and GFAP. Statistical analysis showed a significant difference in mRNA expression of TNF-α in astrocytes ($p=0.0326$, Kruskal-Wallis test, Figure 4.24 A). *Post-hoc* analysis revealed an age-induced increase in TNF-α mRNA in sedentary mice only compared to 3 months old and not in the exercise group (RQ 3 months old: 1.12±0.026, SED: 2.99±0.60, EX: 2.04±0.36, Dunn's *post-hoc*, Figure 4.24 A).

There was a significant change in the mRNA expression of iNOS in the astrocytes, ($p= 0.0307$, Kruskal-Wallis test, Figure 4.24 B). Dunn's *post-hoc* analysis indicated a decrease in iNOS mRNA in astrocytes from the exercised group compared to 3 months old (RQ 3 months old: 1.54±0.39, SED: 0.74±0.23, EX: 0.46±0.14, Figure 4.24 B). Moreover, statistical analysis showed a significant difference in GFAP mRNA in astrocytes ($p=0.0091$, Kruskal-Wallis test, Figure 4.24 C). *Post-hoc* analysis revealed an age-induced increase in GFAP mRNA in astrocytes from the sedentary group that was attenuated by exercise (RQ 3 months old: 1.01±0.15, SED: 1.58±0.15, EX: 1.01±0.07, Dunn's *post-hoc*, Figure 4.24 C).

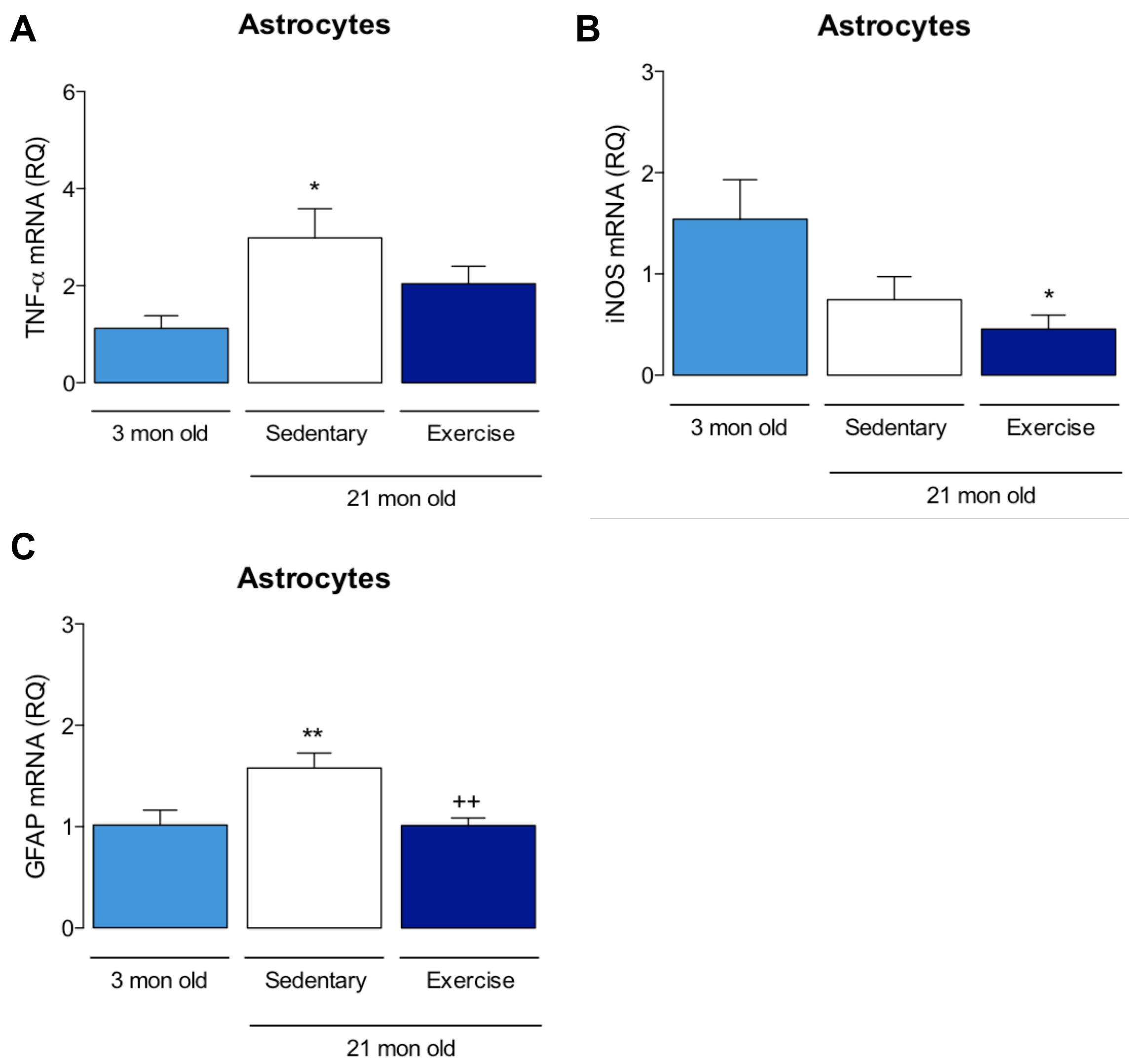


Figure 4.24 - Effects of age and exercise on mRNA expression of A1 phenotype markers TNF-α, iNOS and GFAP in an enriched population of astrocytes

Ten months after exercise cessation, mRNA expression of TNF-α, iNOS and GFAP in astrocytes enriched population was assessed by RT-PCR. Expression of TNF-α, in astrocytes cells, was increased only in age-SED mice, but not in astrocytes cells from age-EX mice **(A)**. Expression of iNOS was decreased in astrocytes cells from age-EX group compared to astrocytes cells from age-SED group **(B)**. Expression of GFAP was increased in astrocytes cells from age-SED, but exercise attenuated this increase **(C)**. Data are presented as mean ± SEM (n=7 to 8). *p<0.05, 3 months old vs. SED and EX, $^{++}$p<0.01, SED vs. EX; Kruskal Wallis test with Dunn's *post hoc* analysis.

4.3.13 Effects of age and exercise on mRNA expression of A2 phenotype markers in an enriched population of astrocytes

We also investigated the mRNA expression of markers of A2 phenotype astrocyte activation in an isolated enriched population of astrocytes. We assessed mRNA expression of IL-10, Arg-1 and Mrc1 using RT-PCR. Although there is a trend toward an increase in IL-10 mRNA expression observed in these astrocytes, the difference was not statistically significant (p=0.110, Kruskal-Wallis test, RQ 3 months old: 0.95±0.50, SED: 1.82±1.51, EX: 5.15±1.30, Dunn's *post-hoc*, Figure 4.25 A).

Moreover, there was no significant difference in mRNA expression of Arg-1 between groups (p=0.7750, Kruskal-Wallis test, RQ 3 months old: 0.90±0.26, SED: 0.88±0.26, EX: 1.55±0.53, Dunn's *post-hoc*, Figure 4.25 B). Similarly, neither age nor exercise affected expression of Mrc1 in astrocytes (p=0.5601, Kruskal-Wallis test, RQ 3 months old: 1.03±0.11, SED: 0.86±0.13, EX: 0.94±0.17, Dunn's *post-hoc*, Figure 4.25 C).

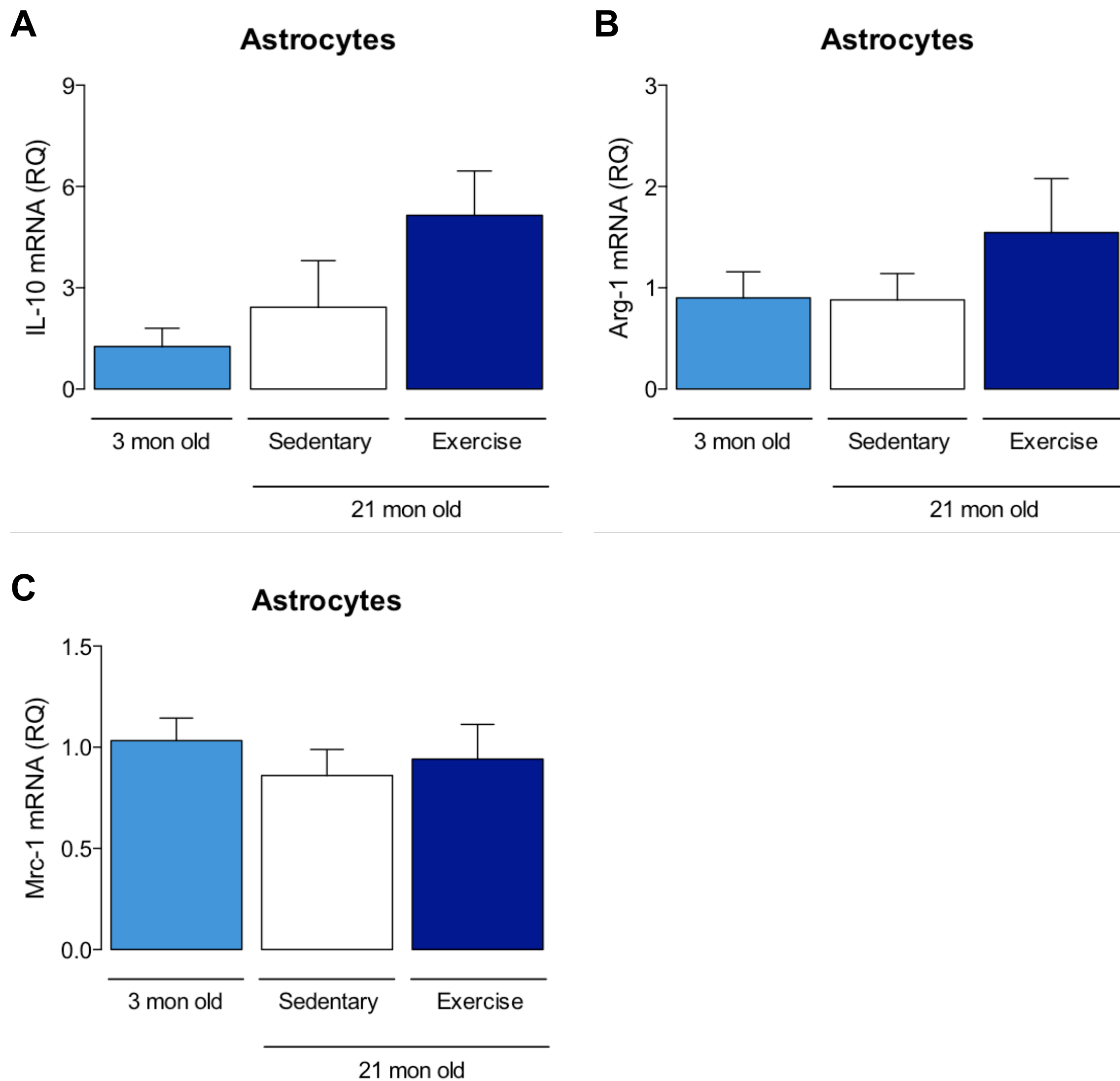


Figure 4.25 - Effects of age and exercise on mRNA expression of A2 phenotype markers IL-10, Arg-1 and Mrc-1 in an enriched population of astrocytes

Ten months after exercise cessation, mRNA expression of IL-10, Arg-1 and Mrc1 in astrocytes enriched population was assessed by RT-PCR. Expression of IL-10, Arg-1 and Mrc1, in astrocytes cells, was not affected either by age or exercise **(A, B and C)**. Data are presented as mean ± SEM (n=7 to 8); Kruskal Wallis test with Dunn's *post hoc* analysis.

4.3.14 Effects of age and exercise on protein content of synaptogenesis markers, PSD-95 and synapsin-1 in the hippocampus

Protein expression of the markers of synaptogenesis PSD-95 and synapsin-1 were assessed by Western immunoblotting. Statistical analysis demonstrated a significant difference in the total content of PSD-95 in the hippocampus between groups ($p=0.0411$, one-way ANOVA, Figure 4.26 A). *Post-hoc* analysis revealed a significant increase in the protein expression of PSD-95 in the hippocampus of exercised mice, compared to 3 months old and sedentary mice (3 months old: 1.00 ± 0.09, SED: 0.83 ± 0.09, EX: 1.97 ± 0.55, Bonferroni *post-hoc*, Figure 4.26 A).

Moreover, a significant difference in the protein content of Synapsin-1 was observed in the hippocampus ($p=0.0426$, one-way ANOVA, Figure 4.26 B). Bonferroni *post-hoc* analysis revealed an increase in protein expression of Synapsin-1 in the hippocampus of exercised mice, compared to 3 month old mice (3 months old: 1.00 ± 0.20, SED: 0.73 ± 0.11, EX: 1.47 ± 0.23, Bonferroni *post-hoc*, Figure 4.26 B).

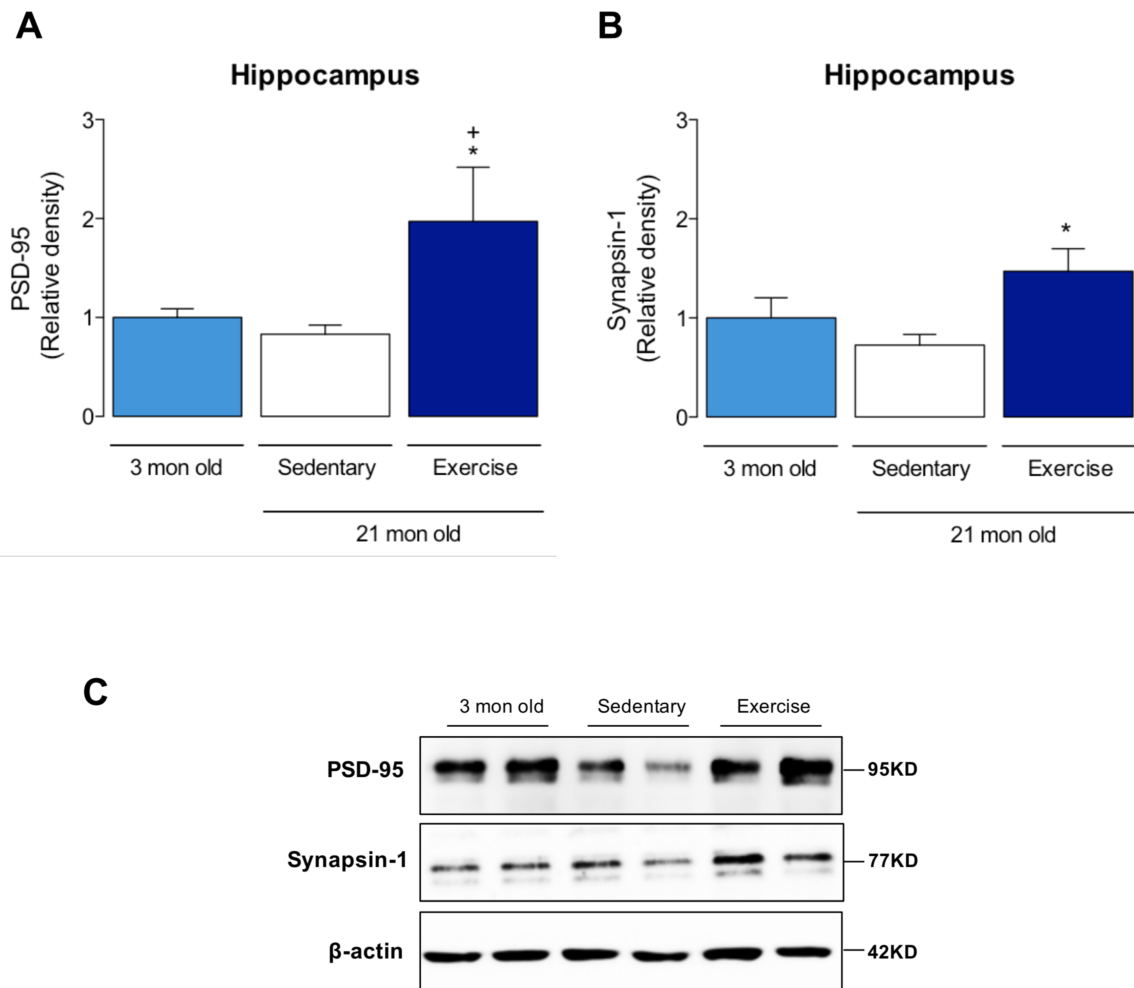


Figure 4.26 - Effects of age and exercise on PSD-95 and synapsin-1 protein expression in the hippocampus

Protein expression of total PSD-95 in hippocampus was measured using Western immunoblotting. Total protein expression of PSD-95 was increased in age-EX mice compared to 3 months old and age-SED mice **(A)**. Total protein expression of Synapsin-1 was increased only in the hippocampus age-EX mice compared to 3 months old mice **(B)**. Representative blots of total PSD-95, Synapsin-1 and β-Actin in hippocampal samples **(C)**. β-Actin was used as loading control of protein content. Data are expressed as relative density and presented as mean ± SEM (n=7 to 8). *$p<0.05$, 3 months old vs. EX, $^{+}p<0.05$, SED vs. EX; one-way ANOVA with Bonferroni *Post hoc* analysis.

4.3.15 Effects of age and exercise on protein expression of TrkB receptor and NF-κB in the hippocampus

Protein expression of total and phosphorylated TrkB receptor and total and phosphorylated NF-κB were measured in the hippocampus by Western immunoblotting. Total TrkB expression in the hippocampus was not affected either by age or exercise (p=0.9003, one-way ANOVA, 3 months old: 1.46±0.16, SED: 1.09±0.26, 1.02±1.02, Bonferroni *post-hoc*, Figure 4.27 A). Similarly, statistical analysis showed no significant difference between the groups in the hippocampal expression of phosphorylated TrkB (p=0.5049, one-way ANOVA, 3 months old: 1.00±0.15, SED: 1.09±0.16, EX: 0.82±0.16, Bonferroni *post-hoc*, Figure 4.27 B). Finally, no changes were observed in the ratio of phosphorylated TrkB and total TrKB (pTrkB/TrkB) in the hippocampus (p=0.6700, one-way ANOVA, 3 months old: 1.07±0.28, SED: 1.34±0.32, EX:1.01±0.19, Bonferroni *post-hoc*, Figure 4.27 C).

Neither was there a statistically significant difference in the total protein expression of NF-κB in the hippocampus between groups (p=0.4390, one-way ANOVA, 3 months old: 0.71±0.22, SED: 0.76±0.23, EX: .45±.11, Bonferroni *post-hoc*, Figure 4.28 A). Similarly, expression of phosphorylated NF-κB was not affected either by age or exercise (p=0.4751, one-way ANOVA, 3 months old: 0.67±0.26, SED: 0.56±0.19, EX: 0.34±0.14, Bonferroni *post-hoc*, Figure 4.28 B) and there was no significant difference in the ratio of phosphorylated NF-κB and total NF-κB protein expression in the hippocampus (p=0.6700, one-way ANOVA, 3 months old: 0.86±0.08, SED: 0.66±0.04, EX: 0.63±0.08, Bonferroni *post-hoc*, Figure 4.28 C).

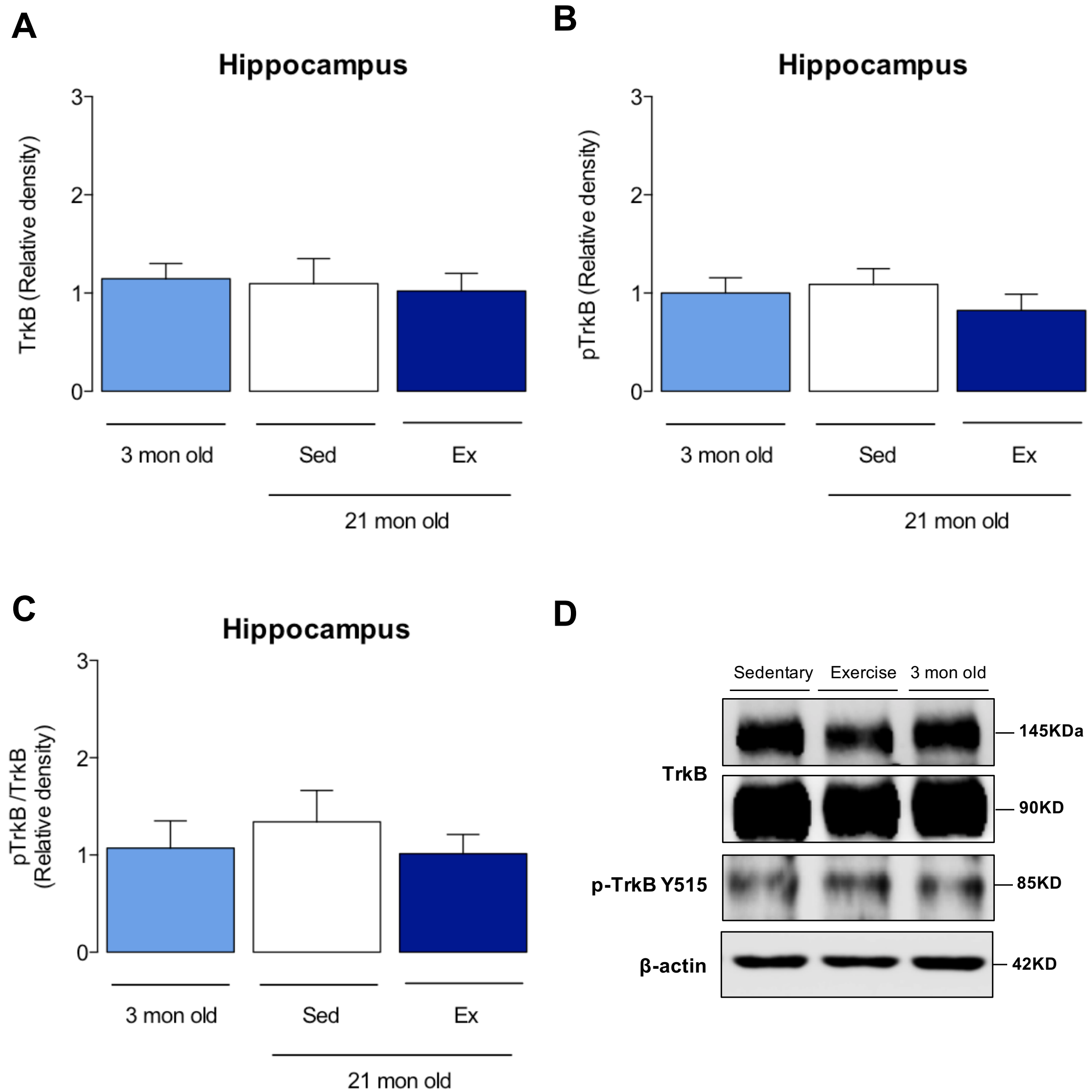


Figure 4.27 - Effects of age and exercise on TrkB receptor protein expression in the hippocampus

Protein expression of total and phosphorylated TrkB receptor, in hippocampus, was measured using Western immunoblotting. In hippocampus, there was no difference in the expression of TrkB receptor **(A)**. Neither age nor exercise altered phosphorylated TrkB receptor expression and the ratio of phosphorylated TrkB by total TrkB protein content in the hippocampus **(B, C)**. Representative blots of total TrkB, phosphorylated TrkB (Y515) and β-Actin in hippocampal samples **(C)**. β-Actin was used as loading control of protein content. Data are expressed as relative density and presented as mean ± SEM (n=7 to 8), one-way ANOVA with Bonferroni *Post hoc* analysis.

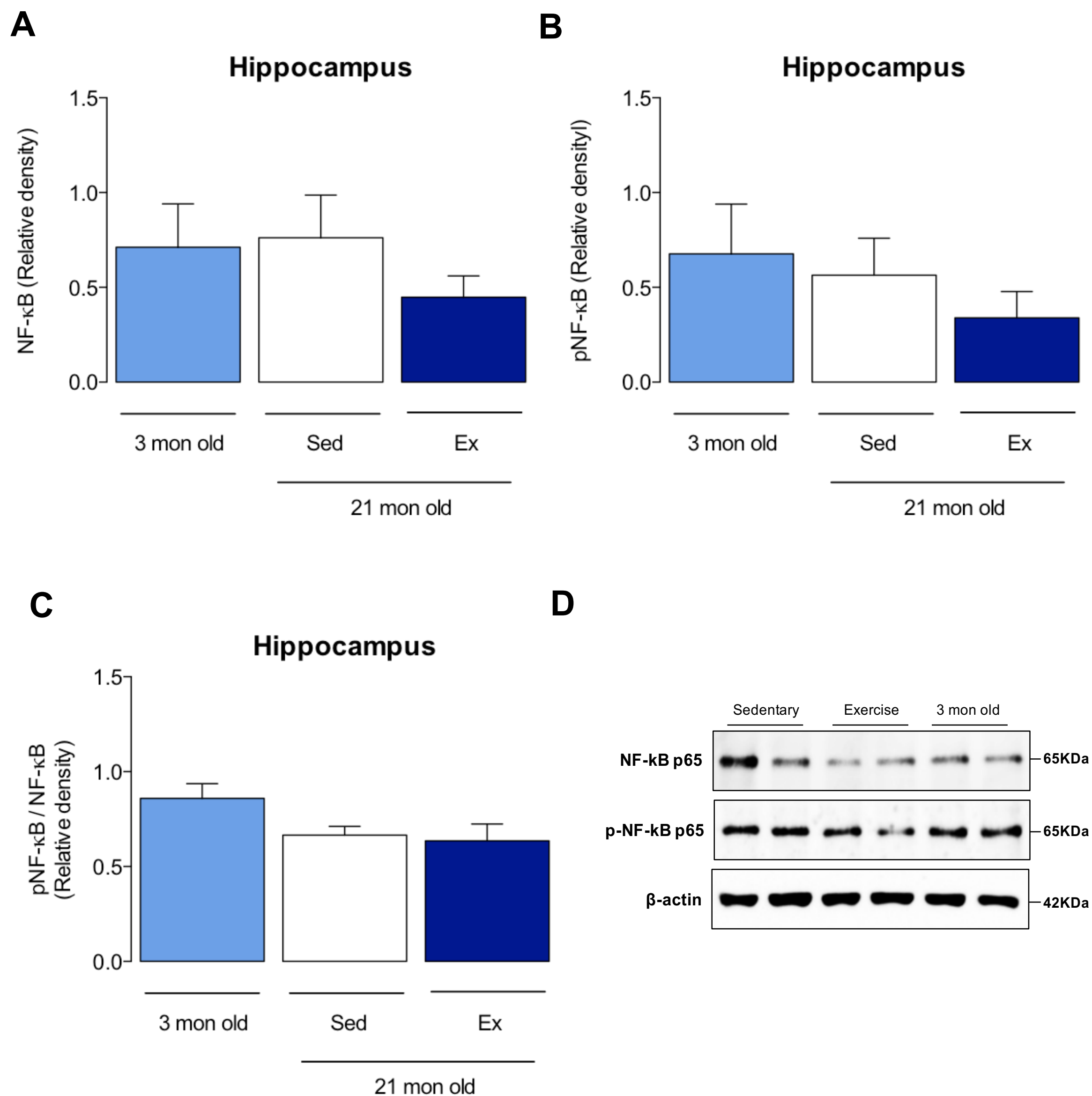


Figure 4.28 - Effects of age and exercise on NF-κB protein expression in the hippocampus
Protein expression of total and phosphorylated NF-κB, in hippocampus, was measured using Western immunoblotting. In hippocampus, there was no difference in the expression of NF-κB **(A)**. Neither age nor exercise altered phosphorylated NF-κB expression and the ratio of phosphorylated NF-κB by total NF-κB protein content in the hippocampus **(B, C)**. Representative blots of total NF-κB, phosphorylated NF-κB and β-Actin in hippocampal samples **(C)**. β-Actin was used as loading control of protein content. Data are expressed as relative density and presented as mean ± SEM (n=7 to 8), one-way ANOVA with Bonferroni *Post hoc* analysis.

4.3.16 Effects of age and exercise on astrocyte reactivity and microglia activation in the CA1 and CA3 regions of hippocampus

After establishing the inflammatory profile in the cortex, hippocampus, microglia and astrocytes of mice, we investigated astrocyte reactivity, labelling GFAP positive cells, and microglia activation, labelling Iba-1 cells, in CA1 and C3 subfields of the hippocampus by immunohistochemistry. In the CA1 subfield of hippocampus, statistical analysis demonstrated a significant difference in the total number of positive GFAP cells between groups (p=0.0032, one-way ANOVA, Figure 4.29 A). Bonferroni *post-hoc* analysis revealed a decrease in the total number of positive GFAP cells in the CA1 subfield of hippocampus of the exercise group compared to the sedentary group (cells 3 months old: 277.4±10.42, SED: 320.5±11.73, EX: 246.6±15.30, Figure 4.29 A).

Similarly, in the CA3 subfield of hippocampus, a significant difference was also observed in the total number of positive GFAP cells (p=0.0096, one-way ANOVA, Figure 4.29 B). The post-hoc analysis showed an age-induced increase in the total number of positive GFAP cells only in the CA3 subfield of hippocampus of sedentary mice and not in exercised mice (cells 3 months old: 247.4±9.65, SED: 307.8±14.23, EX: 248.8±16.08, Bonferroni *post-hoc*, Figure 4.29 B). Moreover, Iba-1 positive cells were labelled to measure microglia activation and statistical analysis demonstrated no significant difference in the total number of Iba-1 positive cells, in either CA1 (p=0.4540, one-way ANOVA, cells 3 month old: 139.7±17.68, SED: 170.5±12.5, EX: 153.7±12.24, Figure 4.30 A) or CA3 (p=0.2755, one-way ANOVA, cells 3 months old: 143.0±13.23, SED:163.5±1.5, EX: 135.3±8.09, Bonferroni *post-hoc*, Figure 4.30 B).

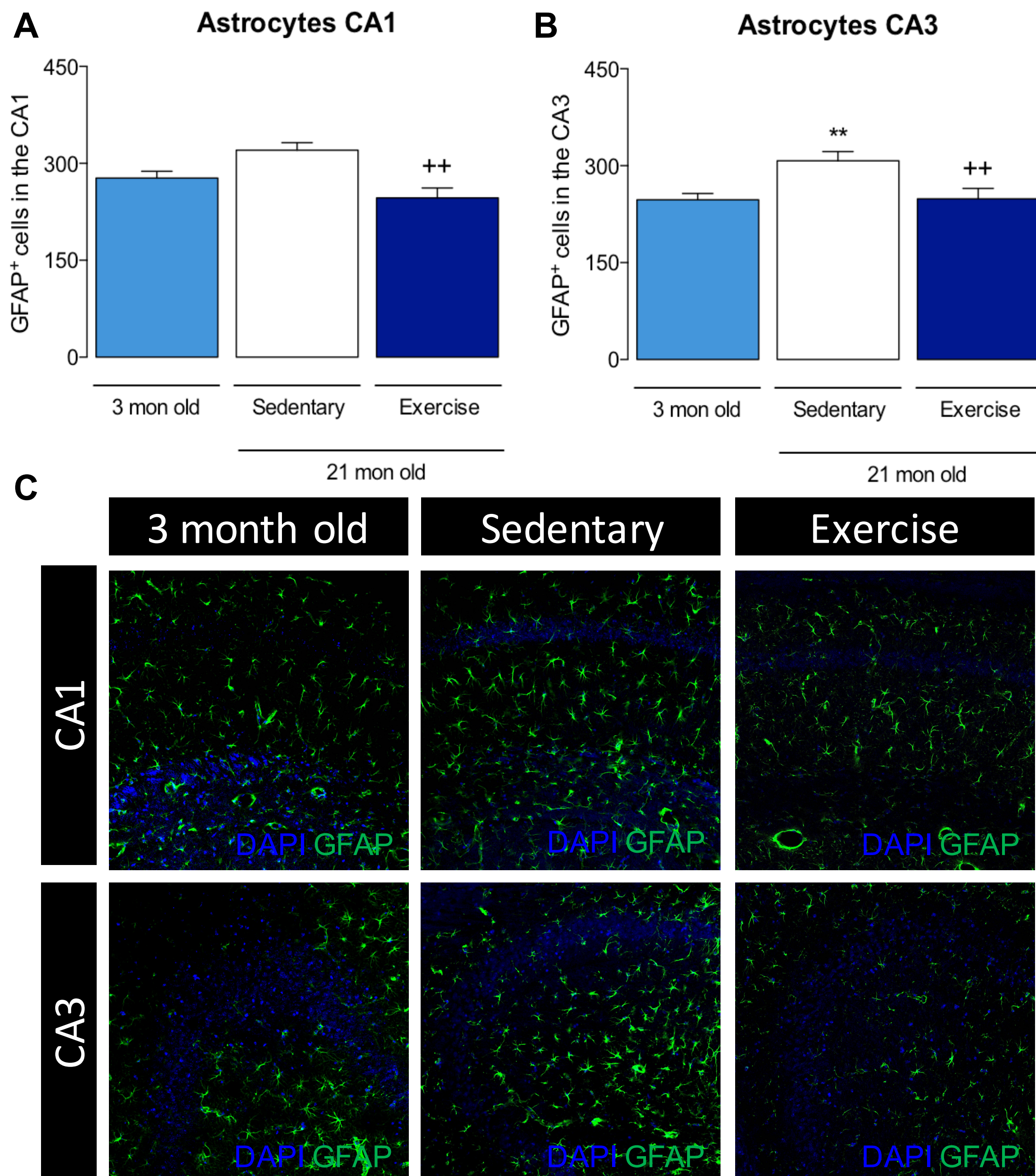


Figure 4.29 - Effects of age and exercise on astrocyte reactivity in CA1 and CA3 subfields of the hippocampus

Ten months after exercise cessation, astrocytes reactivity in CA1 and CA3 subfields of the hippocampus, was assessed by labelling positive GFAP cells using immunohistochemistry. The number of GFAP positive cells, in the CA1 region, was decreased in age-EX mice, but not in CA1 region of age-SED mice **(A)**. In the CA3 region, the number of positive GFAP cells was increased in age-SED group, but exercise attenuated this increase **(B)**. Representative photomicrograph showing positive BrdU cells (red) co-localized with DAPI (blue) and GFAP (green) cells, in CA1 and CA3 subfields **(C)**. Data are presented as mean ± SEM (n=6). $^{**}p<0.01$, 3 months old vs. SED, $^{++}p<0.01$, SED vs. EX; one-way ANOVA with Bonferroni *post hoc* analysis.

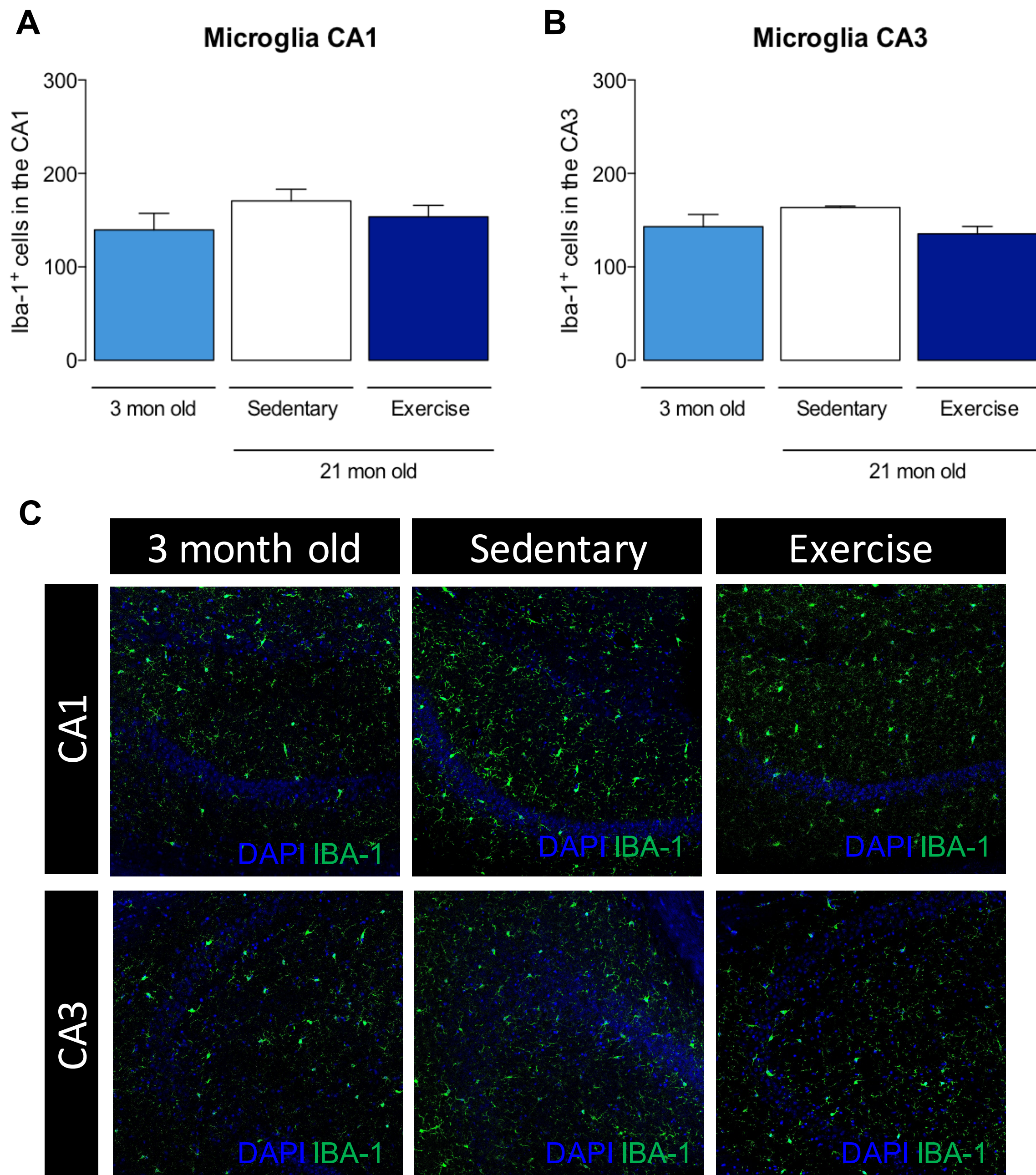


Figure 4.30 - Effects of age and exercise on microglia activation in CA1 and CA3 subfields of the hippocampus

Ten months after exercise cessation, microglia activation in CA1 and CA3 subfields of the hippocampus, was assessed by labelling positive Iba-1 cells using immunohistochemistry. The number of Iba-1 positive cells, in the CA1 and CA3 regions, was not affected either by age or exercise **(A, B)**. Representative photomicrograph showing positive BrdU cells (red) co-localized with DAPI (blue) and Iba-1 (green) cells, in CA1 and CA3 subfields **(C)**. Data are presented as mean ± SEM (n=3); one-way ANOVA with Bonferroni *post hoc* analysis.

4.3.17 Effects of age and exercise on neurogenesis and gliogenesis in the dentate gyrus of hippocampus

Following investigation of markers of neuroinflammation in the brains of mice, we decided to measure neurogenesis and gliogenesis in the DG region of the hippocampus. We assessed the total number of BrdU positive cells and the co-localisation of these cells with NeuN (mature neurons), GFAP (astrocytes) and Iba-1 (microglia) by immunohistochemistry. Statistical analysis revealed a significant difference in the total number of BrdU positive cells in the DG between groups (p=0.0274, one-way ANOVA, Figure 4.31 A). Bonferroni *post-hoc* analysis showed that the total number of BrdU positive cells in the DG was increased in exercise compared to sedentary mice (3 months old: 34.17±7.03 cells/mm^3, SED: 20.59±2.40 cells/mm^3, EX: 46.35±7.13 cells/mm^3, Figure 4.31 A).

Statistical analysis demonstrated a significant difference in the number of BrdU positive cells co-localised with NeuN positive cells (p=0.0352, one-way ANOVA, Figure 4.31 B). Even after 10 months in the absence of exercise, *post-hoc* analysis revealed a significant increase in the number of positive BrdU cells co-localised with NeuN positive cells in exercised mice (3 months old: 26.50±5.90 cells/mm^3, SED: 17.10±3.02 cells/mm^3, EX: 37.5± cells/mm^3, Bonferroni *post-hoc*, Figure 4.31 B), suggesting that exercise promoted the survival of the newborn neurons.

Moreover, we analysed the GFAP positive cells in the DG and no significant change was observed between the groups (p=0.9835, one-way ANOVA, 3 months old: 1301±61.42 cells/mm^3, SED: 1284±97.25 cells/mm^3, EX: 1284±64.64 cells/mm^3, Bonferroni *post-hoc*, Figure 4.32 A). In order to assess gliogenesis, we co-localised the BrdU positive cells with GFAP positive cells in the DG. Although, there is a tendency towards a decrease in the co-localised BrdU positive cells with the GFAP positive cells in the exercise group the difference was not statistically significant between groups (p=0.1621, one-way ANOVA, 3 months old: 10.52±2.68 cells/mm^3, SED: 7.09±0.83 cells/mm^3, EX: 5.66±1.17 cells/mm^3, Bonferroni *post-hoc*, Figure 4.32 B).

Finally, positive Iba-1cells were labelled in the DG and statistical analysis demonstrated no significant difference between the groups ($p=0.4014$, one-way ANOVA, 3 months old: 550.7 ± 70.63 cells/mm^3, SED: 776.9 ± 106.6 cells/mm^3, EX: 463.5 ± 88.28 cells/mm^3, Bonferroni *post-hoc*, Figure 4.33 A). We also co-localised the Iba-1 positive cells with the BrdU positive cells and statistical analysis revealed no changes in the number of co-localised cells $BrdU^+$/Iba-1^+ in the DG ($p=0.5518$, one-way ANOVA, 3 months old: 2.57 ± 1.57 cells/mm^3, SED: 5.78 ± 2.23 cells/mm^3, EX: 2.70 ± 1.86 cells/mm^3, Bonferroni post-hoc, Figure 4.33 B).

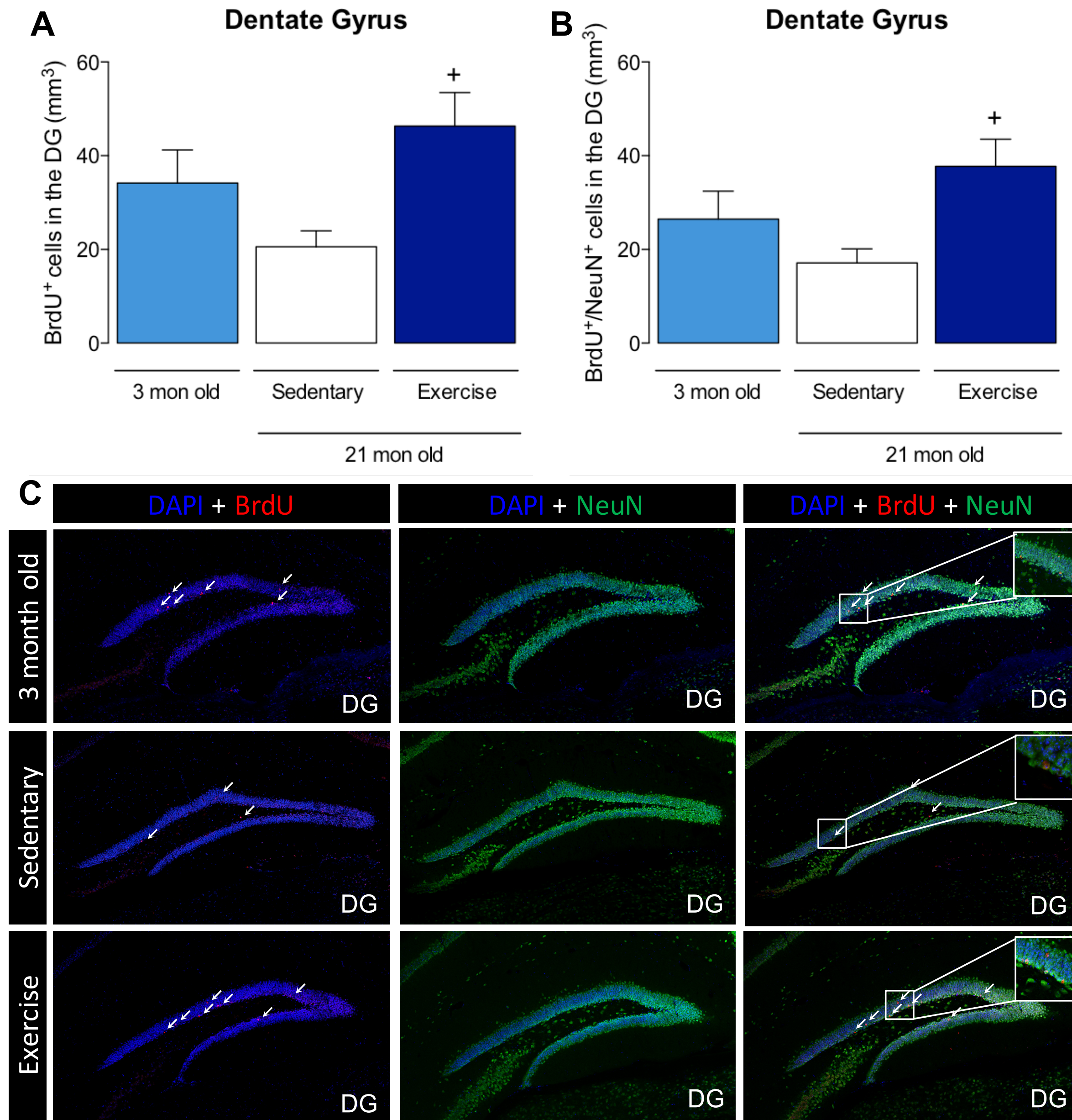


Figure 4.31 - Effects of age and exercise on neurogenesis in the dentate gyrus region of the hippocampus

Ten months after exercise cessation, neurogenesis in the DG region of the hippocampus, was assessed by labelling positive BrdU cells and co-localised positive BrdU cells with positive NeuN cells using immunohistochemistry. The number of BrdU positive cells, in the DG region, was increased in age-EX mice, but not in age-SED mice **(A)**. In the DG region, the number of positive BrdU cells co-localised with positive NeuN cells was also increased only in age-EX group **(B)**. Representative photomicrograph showing positive BrdU cells (red) co-localized with DAPI (blue) and NeuN (green) cells, in the DG region **(C)**. Data are presented as mean ± SEM (n=6). $^{+}p<0.05$, SED vs. EX; one-way ANOVA with Bonferroni *post hoc* analysis.

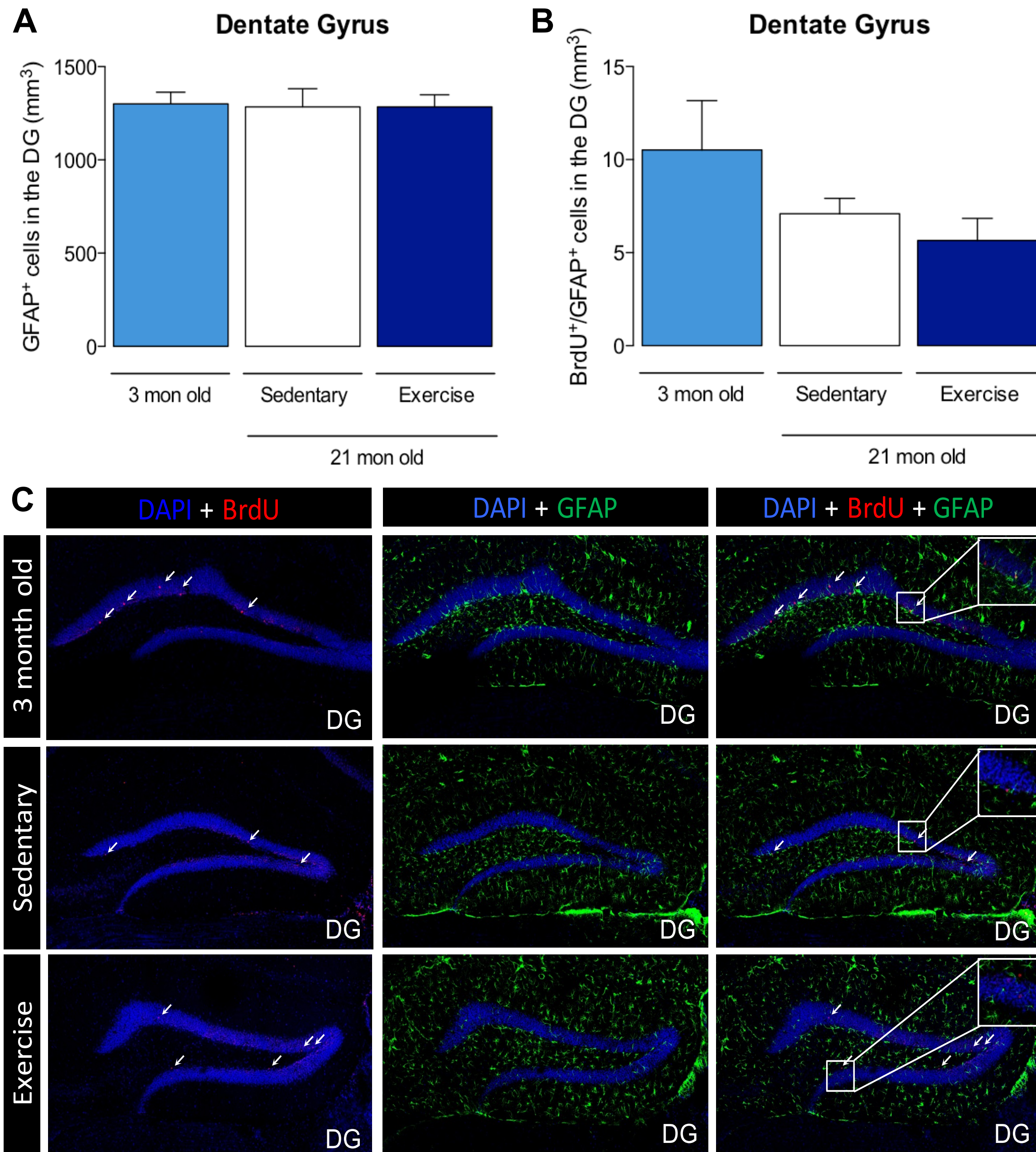


Figure 4.32 - Effects of age and exercise on gliogenesis (astrocytes) in the dentate gyrus region of the hippocampus

Ten months after exercise cessation, gliogenesis in the DG region of the hippocampus, was assessed by labelling positive BrdU cells and co-localised positive BrdU cells with positive GFAP cells using immunohistochemistry. The number of GFAP positive cells and positive GFAP cells co-localised with positive BrdU cells, in the DG region, was not affected either by age or exercise **(A, B)**. Representative photomicrograph showing positive BrdU cells (red) co-localized with DAPI (blue) and GFAP (green) cells, in the DG region **(C)**. Data are presented as mean ± SEM (n=6); one-way ANOVA with Bonferroni *post hoc* analysis.

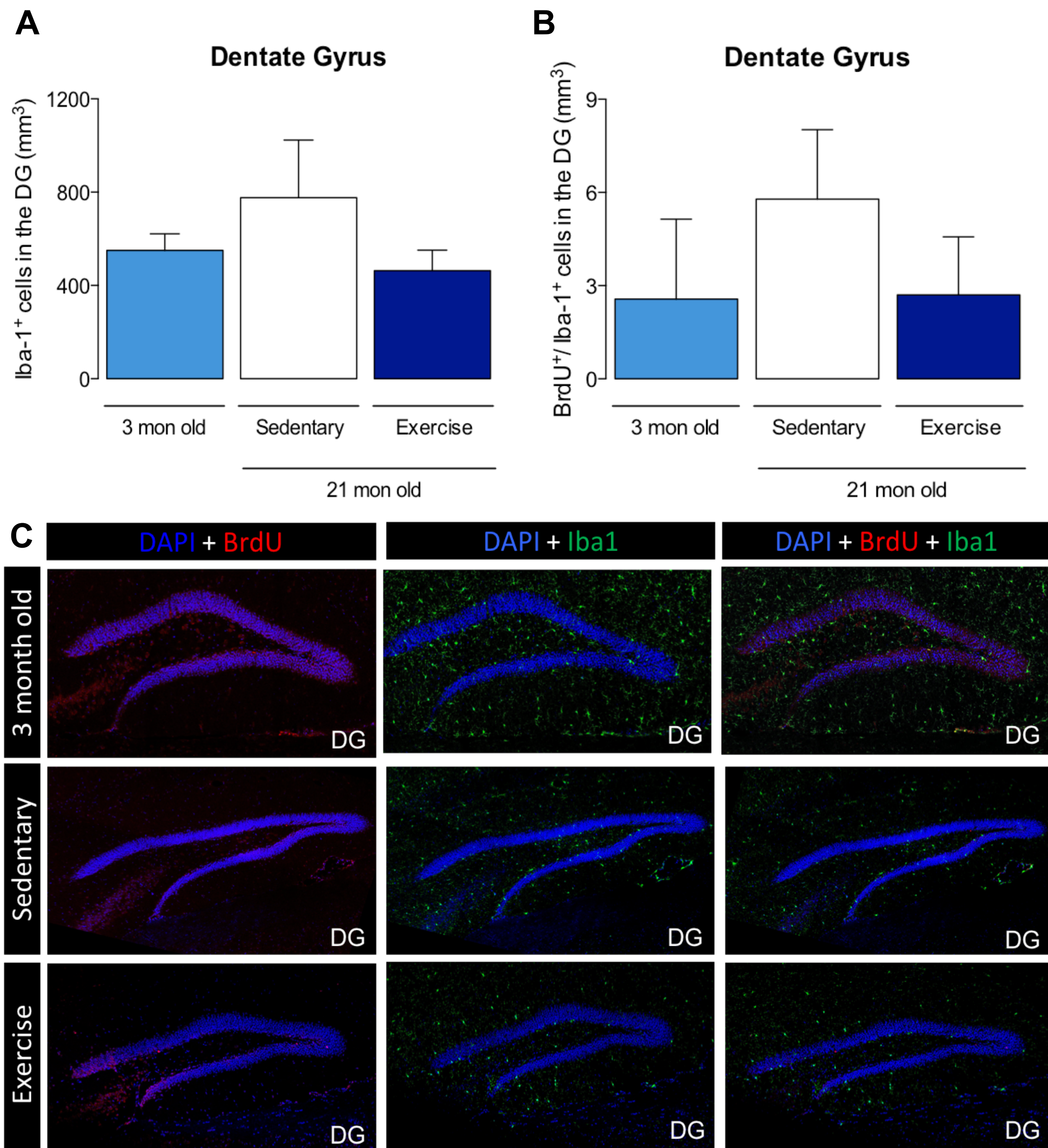


Figure 4.33 - Effects of age and exercise on gliogenesis (microglia) in the dentate gyrus region of the hippocampus

Ten months after exercise cessation, gliogenesis in the DG region of the hippocampus, was assessed by labelling positive BrdU cells and co-localised positive BrdU cells with positive Iba-1 cells using immunohistochemistry. The number of Iba-1 positive cells and positive Iba-1 cells co-localised with positive BrdU cells, in the DG region, was not affected either by age or exercise **(A, B)**. Representative photomicrograph showing positive BrdU cells (red) co-localized with DAPI (blue) and Iba-1 (green) cells, in the DG region **(C)**. Data are presented as mean ± SEM (n=3); one-way ANOVA with Bonferroni *post hoc* analysis.

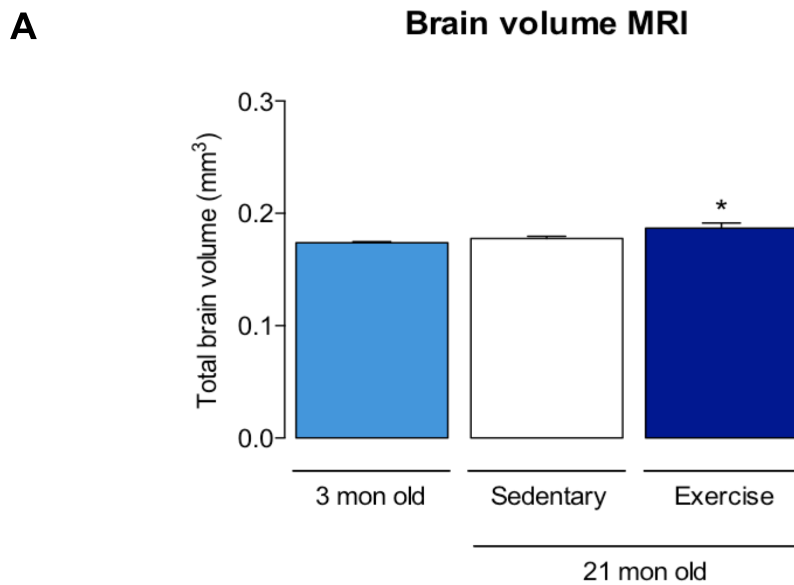
4.3.18 Effects of age and exercise on total brain and hippocampal volumes

The total brain and hippocampal volumes, in order to investigate possible brain morphological changes. These measurements were carried out by analysis of MRI images using the regions of interest method. Statistical analysis demonstrated a significant difference in the total brain volume between groups ($p=0.0147$, one-way ANOVA, Figure 4.34). *Post-hoc* analysis revealed that, 10 months after exercise cessation, total brain volume was increased in exercised mice compared to 3 months old mice, with no effect of ageing (volume 3 months old: 0.173 ± 0.0009 mm^3, SED: 0.177 ± 0.001 mm^3, EX: 0.187 ± 0.001 mm^3, Bonferroni *post-hoc*, Figure 4.34 A), suggesting that exercise induced morphological changes in the brain that persisted even when exercise was stopped.

Moreover, a difference in the right hemisphere hippocampal volume was observed ($p=0.0121$, one-way ANOVA, Figure 4.35 A). Bonferroni *post-hoc* analysis revealed an increase in right hippocampal volume in the exercise compared to 3 months old group (volume 3 months old: 0.0128 ± 0.0002 mm^3, SED: 0.013 ± 0.0003 mm^3, EX: 0.014 ± 0.0002 mm^3, Figure 4.35 A). Also, when left hippocampal volume was analysed, a significant change was observed between groups ($p=0.0164$, one-way ANOVA, Figure 4.35 B). Similar to right hemisphere, *post-hoc* analysis showed an increase in left hippocampal volume of exercise compared to 3 months old mice (volume 3 months old: 0.013 ± 0.0001 mm^3, SED: 0.012 ± 0.0003 mm^3, EX: 0.014 ± 0.0003, Bonferroni *post-hoc*, Figure 4.35 B).

Finally, statistical analysis of total hippocampal volume showed a significant difference in hippocampus size between the groups ($p= 0.0076$, one-way ANOVA, Figure 4.34 C). Bonferroni *post-hoc* analysis revealed a significant increase in the total hippocampal volume of mice from the exercise group, compared to 3 months old mice, with no effect of age (volume 3 months old: 0.025 ± 0.0003 mm^3, SED: 0.026 ± 0.0005 mm^3, EX: 0.029 ± 0.0004 mm^3, Figure 4.34 C), suggesting that an exercise-induced change in hippocampal volume persisted even 10 months after exercise cessation.

A



B

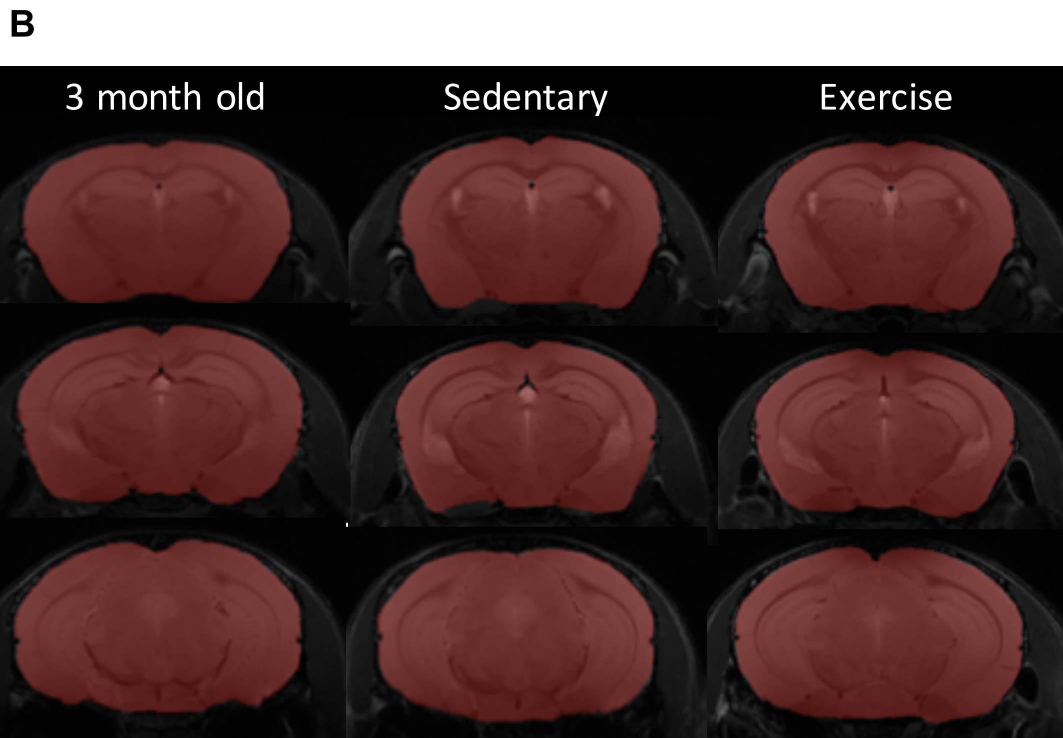
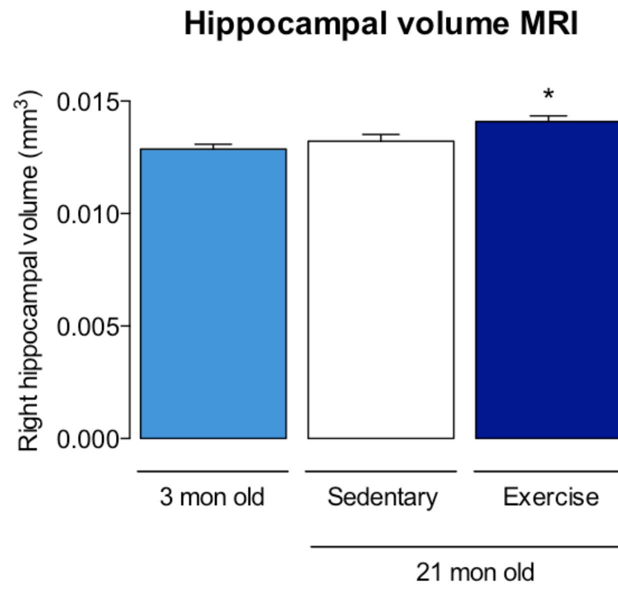


Figure 4.34 - Effects of age and exercise on total brain volume

Ten months after exercise cessation, total brain volume was analysed by measuring brain area from structural MRI images using the region of interest (ROI) method. Total brain volume was increased in age-EX mice, compared to 3 month old mice **(A)**. Representative MRI images of mice from 3 months old, SED and EX groups showing the ROI analysis (red) **(C)**. Data are presented as mean ± SEM (n=6). *p<0.05, 3 month old vs. SED, one-way ANOVA with Bonferroni *post hoc* analysis.

A



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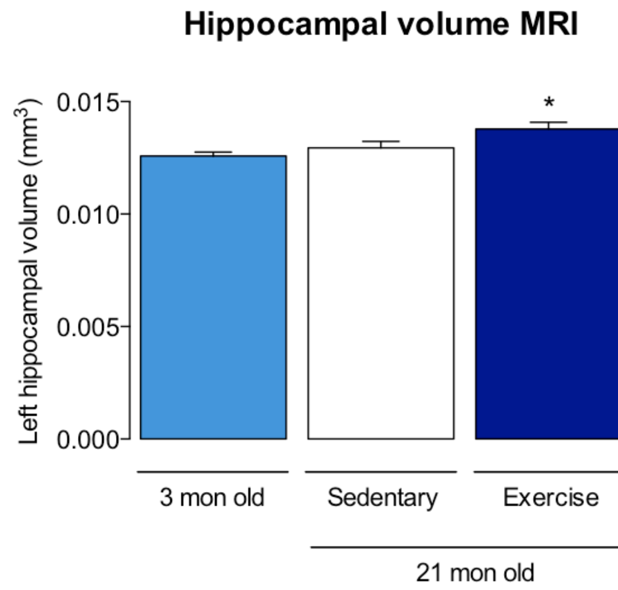
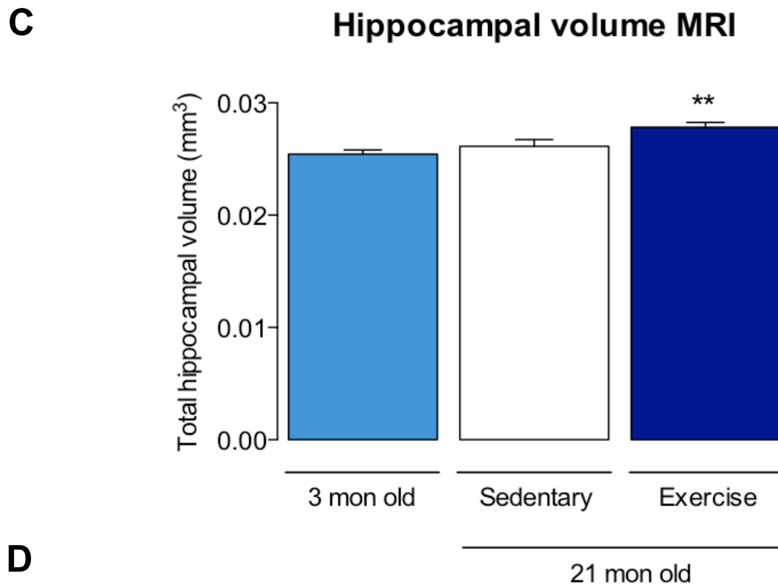


C



D

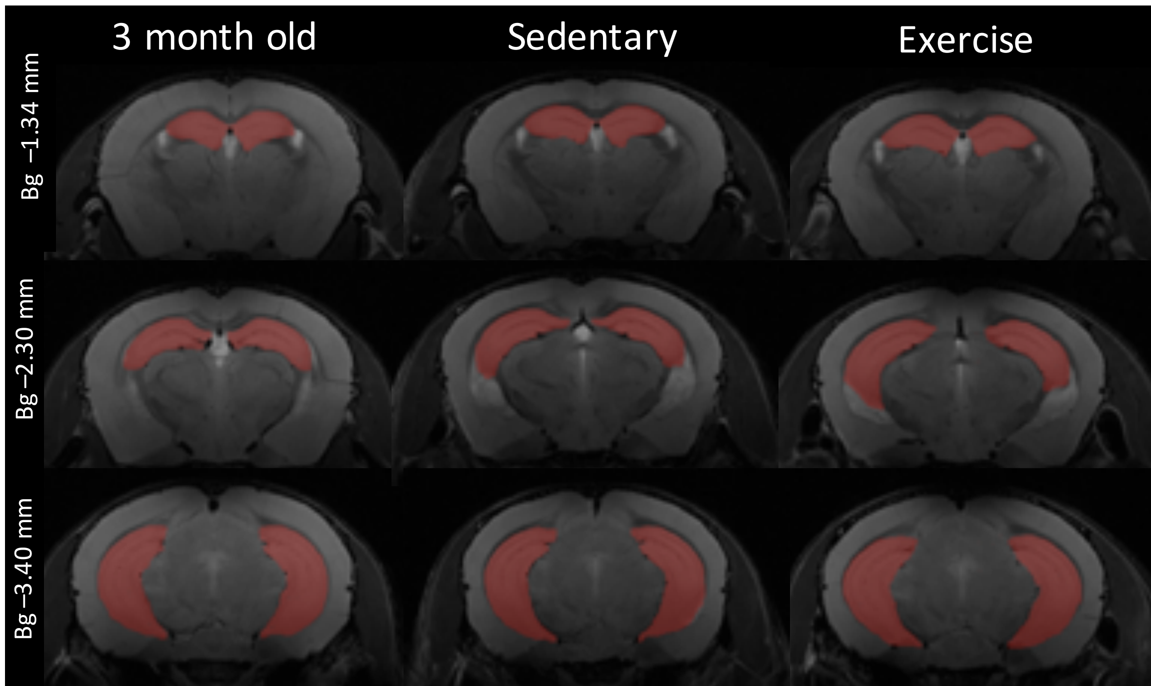


Figure 4.35 - Effects of age and exercise on hippocampal volume

Ten months after exercise cessation, hippocampal was analysed by measuring hippocampus are from structural MRI images using the region of interest (ROI) method. When analysed separated, right and left hemispheres hippocampal volume in both hemispheres was increased in age-EX mice, compared to 3 months old mice **(A, B)**. Also, the total hippocampal volume was increased in age-EX mice, compared to 3 months old mice showing the ROI analysis (red) **(C)**. Representative MRI images of mice from 3 months old, SED and EX groups **(C)**. Data are presented as mean ± SEM (n=6). *p<0.05, 3 months old vs. SED, **p<0.01, 3 months old vs. SED, one-way ANOVA with Bonferroni *post hoc* analysis.

4.3.19 Effects of age and exercise on mRNA expression of inflammatory markers IL-1β, TNF-α, IL-6 and CRP in the liver

As the effects of exercise on peripheral inflammation are well-established, we also investigated the mRNA expression of peripheral inflammatory markers, such as IL-1β, TNF-α, IL-6 and CRP and protein content of IL-1β, TNF-α, in the livers of mice, 10 months after exercise cessation. mRNA expression was assessed by RT-PCR, while protein content was measured using ELISA assay.

Statistical analysis revealed a significant difference between the groups in the IL-1β mRNA expression (p=0.050, Kruskal-Wallis test, Figure 3.36 A). Dunns *post-hoc* analysis showed a decrease in IL-1β mRNA in the liver of exercise compared to sedentary mice (RQ 3 months old: 1.44±0.76, SED: 2.65±1.11, EX: 0.64±0.42, Figure 4.36 A). Moreover, although expression of TNF-α mRNA tends to an increase in the sedentary group, statistical analysis demonstrated no effect of either age or exercise in the liver (p=0.2687, Kruskal-Wallis test, RQ 3 months old: 4.90±1.61, SED: 13.26±3.83, EX: 6.00±2.72, Dunn's *post-hoc*, Figure 3.36 B).

There was a significant difference between the groups in IL-6 mRNA expression in the liver (p=0.0389, Kruskal-Wallis test, Figure 4.36 C)., Dunn's post-hoc analysis revealed a decrease in the IL-6 mRNA in the liver of the exercise group, compared to the sedentary group (RQ 3 months old: 4.62±2.11, SED: 4.23±2.08, EX: 0.99±0.99, Figure 4.36 C). CRP mRNA expression was unaltered either by age or exercise (p=0.2403, Kruskal-Wallis test, RQ 3 months old: 1.01±0.17, SED: 0.93±0.08, EX: 0.88±0.28, Dunn's post-hoc, Figure 4.36 D).

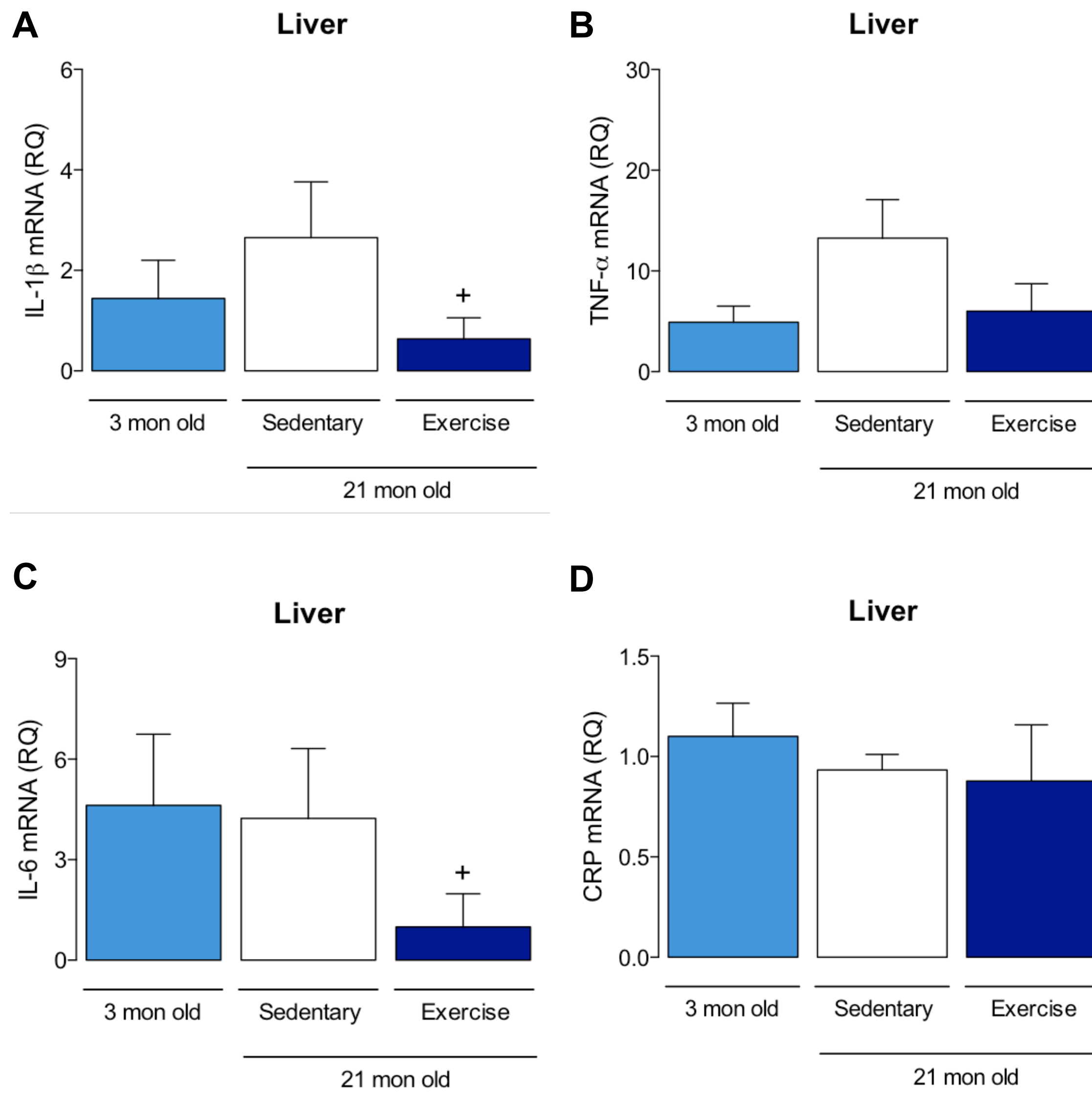


Figure 4.36 - Effects of age and exercise on mRNA expression of peripheral inflammatory markers IL-1β, TNF-α, IL-6 and CRP in the liver

Ten months after exercise cessation, mRNA expression of IL-1β, TNF-α, IL-6 and CRP, in the liver, was assessed by RT-PCR. Expression of IL-1β was decreased in the liver of age-EX group mice compared to age-SED group **(A)**. Expression of TNF-α was, in the liver, was not affected either by age or exercise **(B)**. Also, expression of IL-6 was decreased in the liver of age-EX group mice compared to age-SED group **(C)**, while mRNA expression of CRP, in the liver, was not affected either by age or exercise **(D)**. Data are presented as mean ± SEM (n=7 to 8). $^{+}p<0.05$, SED vs. EX; Kruskal Wallis test with Dunn's *post hoc* analysis.

4.3.20 Effects of age and exercise on protein expression of inflammatory markers IL-1β and TNF-α in the liver and in the gastrocnemius muscle

Finally, we investigated protein concentration of the inflammatory markers IL-1β and TNF-α in the liver and in the gastrocnemius muscle of mice, after 10 months without exercise, by ELISA. In the liver, when protein content of IL-1β was measured, statistical analysis demonstrated that IL-1B concentration was unaffected 10 months after the end of exercise ($p=0.1991$, one-way ANOVA, 3 months old: 3861±237.3 pg/mg, SED: 2976±460.7 pg/mg, EX: 3089±302.8 pg/mg, Bonferroni *post-hoc*, Figure 4.37 A). Similarly, neither age nor exercise affected the TNF-α protein content in the liver ($p=0.6373$, one-way ANOVA, fold change 3 months old: 22988±1435 pg/mg, SED: 21264±3563 pg/mg, EX: 19216±2345 pg/mg, Bonferroni *post-hoc*, Figure 4.37 B).

In the gastrocnemius muscle, statistical analysis demonstrated no significant difference in concentration of IL-1β between the groups ($p=0.8583$, one-way ANOVA, 3 months old: 65.91±9.75 pg/mg, SED: 74.17±14.93 pg/mg, EX: 75.81±17.63 pg/mg, Bonferroni *post-hoc*, Figure 4.37 C). Also, 10 months after exercise cessation, although there is a trend towards a decrease in TNF-a concentration in gastrocnemius muscle of exercise mice, no significant difference was observed between the groups ($p=0.3139$, one-way ANOVA, 3 months old: 129.9±22.56 pg/mg, SED: 125.6±26.89 pg/mg, EX: 87.9±5.29 pg/mg, Bonferroni *post-hoc*, Figure 4.37 D).

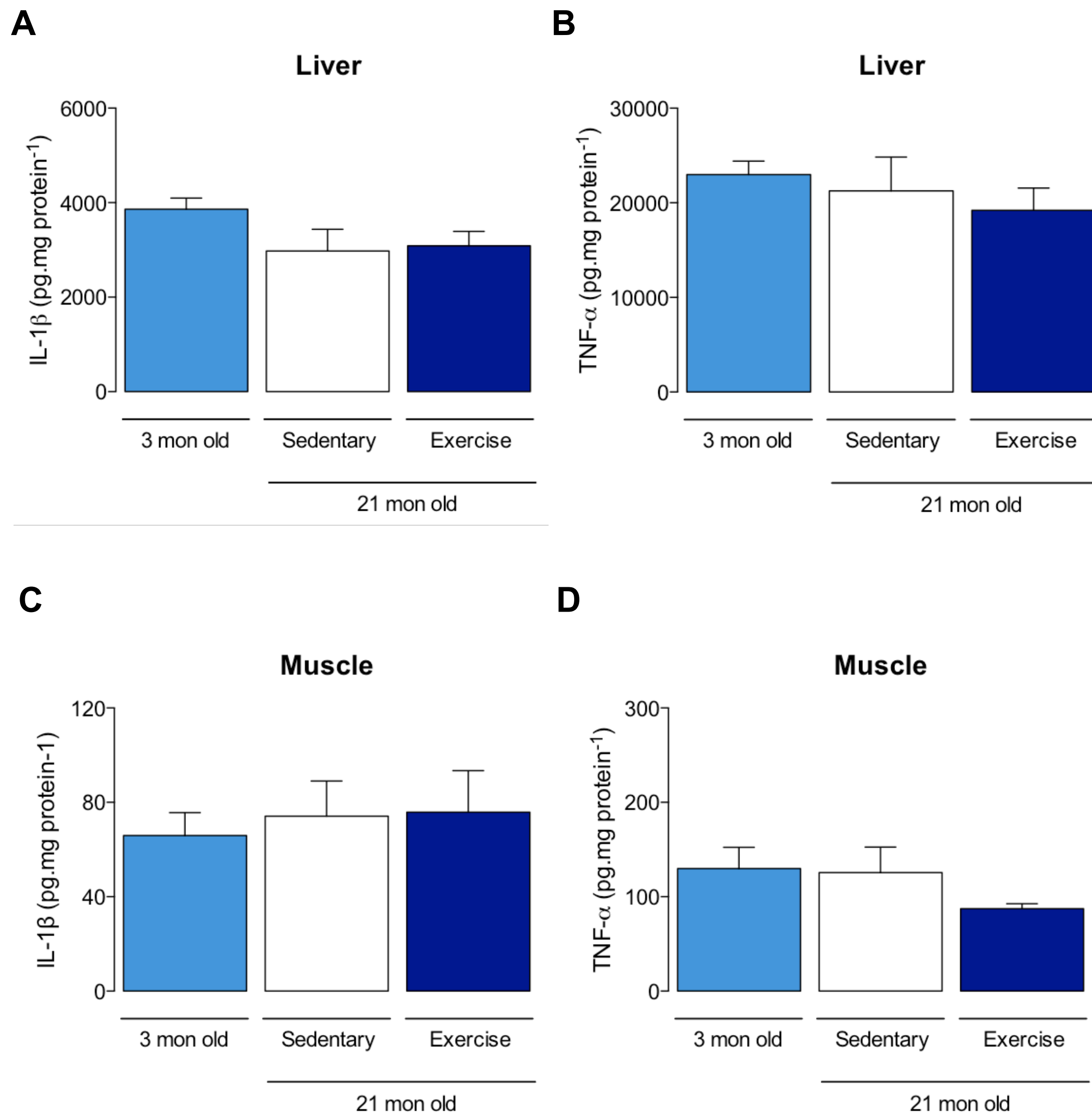


Figure 4.37 - Effects of age and exercise on protein expression of inflammatory markers IL-1β and TNF-α in the liver and in the gastrocnemius muscle

Ten months after exercise cessation, concentrations of IL-1β and TNF-α, in the liver and in the gastrocnemius muscle, were assessed by ELISA. Concentrations of IL-1β and TNF-α, in the liver, were not affected either by age or exercise **(A, B)**. Similarly, concentrations of IL-1β and TNF-α, in the gastrocnemius muscle, were also not affected either by age or exercise **(C, D)**. Data are presented as mean ± SEM (n=7 to 8); one-way ANOVA with Bonferroni *post hoc* analysis.

4.4 Table of results

4.4.1 Cytokines

	Aged-Sed		Aged-Ex	
IL-1β	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↑	↔	ATTENUATED ↑	↔
cortex	↑		PREVENTED ↑	
microglia	↔		ATTENUATED ↑	
Liver	↔	↔	Lower than 3 month old and aged-sed	↔
muscle		↔		↔
TNF-α	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↑	↔	PREVENTED ↑	↔
cortex	↑		PREVENTED ↑	
microglia	↑		PREVENTED ↑	
astrocytes	↑		PREVENTED ↑	
Liver	↔	↔	↔	↔
muscle		↔		↔
IL-6	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↑		PREVENTED ↑	
cortex	↔		↔	
Liver	↔		Lower than 3 month old and aged-sed	
IL-10	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔	↔	↑	↑
cortex	↔		↑	
astrocytes	↔		↔	

Table 4.1 - Effect of exercise on cytokines in age

Illustrative table with the outcome effect of exercise on mRNA and protein expression of cytokines in the hippocampus, cortex, microglia, astrocytes, liver and muscle samples, at 21 months old. (↑) Increased when compared to 3 month old group of mice and aged-exercise group. (↔) No changes compared to compared to 3 month old group of mice and aged-exercise group.

4.4.2 Glial cell markers

	Aged-Sed		Aged-Ex	
GFAP	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↑	↔ in DG ↔ in CA1 ↑ in CA3	↑	↔ in DG PREVENTED ↑ CA1 ATTENUATED ↑CA3
cortex	↑		↑	
microglia	↔		↔	
astrocytes	↑		ATTENUATED ↑	
Iba-1	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↑	↔ in DG ↔ in CA1 ↔ in CA3	↔	↔ in DG ↔ in CA1 ↔ in CA3
cortex	↔		↔	
microglia	↔		PREVENTED ↑	
astrocytes				
CD11b	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↑		↑	
cortex	↔		↔	
NF-κB	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus		↔		↔
iNOS	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		PREVENTED ↑	
cortex	↔		↔	
microglia	↔		↔	
astrocytes	↔		**Lower than 3 month old**	

	Aged-Sed		Aged-Ex	
Arg-1	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↑		ATTENUATED ↑	
cortex	↔		↔	
microglia	↔		↔	
astrocytes	↔		↔	
Mrc1	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔	
cortex	↔		↔	
microglia	↔		↔	
astrocytes	↔		↔	
Ym1	**mRNA**	**protein**	**mRNA**	**protein**
microglia	↔		Lower than 3 month old and aged-Sed	

Table 4.2 - Effect of exercise on glial cells marker in age

Illustrative table with the outcome effect of exercise on mRNA and protein expression of glial cells markers in the hippocampus, cortex, microglia, and astrocytes samples, at 21 months old. (↑) Increased when compared to 3 month old group of mice and aged-exercise group. (↔) No changes compared to compared to 3 month old group of mice and aged-exercise group

4.4.3 Inflammatory and apoptotic markers

	Aged-Sed		**Aged-Ex**	
Cx3cl1	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔	
cortex	Lower than 3 month old		Lower than 3 month old	
Cx3cr1	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	Higher than 3 month old		Higher than 3 month old	
cortex	↔		↔	

	Aged-Sed		Aged-Ex	
CD44	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔	
cortex	↔		↔	
Bax	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔	
cortex	↔		↔	
Bcl-2	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔	
cortex	↔		↑	
CRP	**mRNA**	**protein**	**mRNA**	**protein**
Liver	↔		↔	

Table 4.3 - Effect of exercise on inflammatory and apoptotic markers in age

Illustrative table with the outcome effect of exercise on mRNA and protein expression of inflammatory and apoptotic markers in the hippocampus, cortex, and liver samples, at 21 months old. (↑) Increased when compared to 3 month old group of mice and aged-exercise group. (↔) No changes compared to compared to 3 month old group of mice and aged-exercise group.

4.4.4 Growth factors, neurotrophins and synaptogenesis markers

	Aged-Sed		Aged-Ex	
TrkB	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔	↔	↔	↔
cortex	↔	↔	↔	↔
p75	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔	
cortex	↔		↔	
NGF	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔	
cortex	↔		↔	

	Aged-Sed		Aged-Ex	
VEGF	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔	
cortex	↔		↔	
Igf-1	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔	
cortex	↔		↔	
CREB	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔	↔	↔	↔
cortex	↔		↔	
PSD-95	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus		↔		Higher than 3 month old and aged-Sed
Synapsin-1	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus		↔		Higher than 3 month old

Table 4.4 - Effect of exercise on growth factors, neurotrophins, signalling proteins and synaptogenesis markers in age

Illustrative table with the outcome effect of exercise on mRNA and protein expression of growth factors, neurotrophins, signalling proteins and synaptogenesis markers in the hippocampus and cortex samples, at 21 months old. (↑) Increased when compared to 3 month old group of mice and aged-exercise group. (↔) No changes compared to compared to 3 month old group of mice and aged-exercise group.

4.4.5 Neurogenesis

	Aged-Sed		Aged-Ex	
Ki67	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	Lower than 3 month old		Lower than 3 month old	
cortex	Lower than 3 month old		Lower than 3 month old	
$BrdU^+$	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus		↔ in DG		↑ in DG
$BrdU^+/NeuN^+$	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus		↔ in DG		↔ in DG

Table 4.5 - Effect of exercise on cell proliferation and neurogenesis in age

Illustrative table with the outcome effect of exercise on cell proliferation and neurogenesis at 21 months old. (↑) Increased when compared to 3 month old group of mice and aged-exercise group. (↔) No changes compared to compared to 3 month old group of mice and aged-exercise group.

4.4.6 MRI

MRI	Aged-Sed	Aged-Ex
Total brain volume	↔	↑
Hippocampal volume	↔	↑

Table 4.6 - Effect of exercise on brain and hippocampal volumes in age

Illustrative table with the outcome effect of exercise on brain and hippocampal volumes at 21 months old. (↑) Increased when compared to 3 month old group of mice and aged-exercise group. (↔) No changes compared to compared to 3 month old group of mice and aged-exercise group.

4.5 Discussion

The overall aim of this chapter was to investigate the potential mechanisms that underlie the persistent effects of exercise on cognitive function, anxiety and depression-like behaviour reported in the previous chapter. Firstly, as we know exercise has a primary important role modulating peripheral inflammation, we investigated possible changes induced by age and exercise on inflammatory markers in the gastrocnemius muscle and liver. Secondly, we established the effects of exercise regulating neuroinflammation, focusing on investigating typical inflammatory markers, microglia activation and astrocyte reactivity. Finally, we addressed possible persistent modulation of exercise on neurotrophin expression, apoptosis, neurogenesis and brain morphology.

4.5.1 Persistent effect of exercise on inflammation in age

The presence of low-grade inflammation in ageing has been well-described as well as linked to the age-induced impairment in cognitive function and to age-related neurodegenerative diseases, such as AD (Kohman and Rhodes, 2013)). For this reason, after 8 months undergoing treadmill running, plus 10 months in the absence of this intervention, we investigated possible persistent effects of early-life exposure to exercise on peripheral and central inflammation at old age and compared these results with a 3 month old group of mice to analyse the effects of ageing on these markers. Peripheral inflammation was assessed by analysis of mRNA and protein expression of IL-1β, TNF-α, IL-6 and CRP in the liver and in the gastrocnemius muscle of mice at 21 months old.

Although RNA sequencing has shown an age-induced increase in mRNA expression of pro-inflammatory cytokines, including TNF-α, IL-1β in the mouse liver, we failed to find an increase in these inflammatory markers driven by age. However, interestingly, our results demonstrated that exercise promoted a reduction in the mRNA expression of IL-6 and IL-1β in the liver of the exercise group compared to sedentary aged mice, suggesting exercise effects on peripheral tissues remain persistent even a long time after exercise cessation. Exercise is

well-described to have direct effects in skeletal muscle, inducing IL-6 expression in this tissue and, it seems that the release of IL-6 into the circulation due to exercise is likely to have a hormone-like function, exerting its effect on the liver and adipose tissue (Pedersen et al., 2001); indeed IL-6 is classified as a myokine. IL-6 has a key effect in mediating the acute phase response in the liver and it has been reported to play an important role in restoring normal hepatic function after partial hepatectomy (Michalopoulos, 2007). It is believed that systemic IL-6 can be taken up by the liver and it stimulates the production of acute-phase proteins, such as CRP, meanwhile, during liver injury, Kupffer cells (hepatic macrophages), can also produce IL-6 to stimulate acute-phase proteins (Gauldie et al., 1992). Our results did not demonstrate changes in mRNA expression of CRP, neither by age nor exercise, and this may be explained by the fact that liver IL-6 mRNA levels were not affected by age and were decreased in age-exercised mice.

Even though a decrease in mRNA expression of IL-1β was observed in the liver of exercised mice, we failed to show changes in the protein expression of IL-1β and TNF-α. This dissociation between changes observed in IL-1β mRNA expression and IL-1β synthesis could be explained by the influence of several regulators at the level of gene transcriptional and post-transcriptional modifications (Schindler et al., 1990). Accumulating evidence suggests that post-transcriptional control can play an important role in regulating IL-1β production in humans, as it was demonstrated that prostaglandins supress the synthesis of IL-1β with no effects on mRNA expression (Knudsen et al., 1986). Also, most recently microRNAs, such as miR-155 and miR-146a, have been shown to control cytokine processing at the protein level, including IL-1β synthesis control (Ceppi et al., 2009). Furthermore, we measured protein content of pro-inflammatory IL-1β and TNF-α in the gastrocnemius muscle, however neither age nor exercise affected the expression of these cytokines in the skeletal muscle. Although exercise has been reported to change cytokine expression in the exercised skeletal muscle (Peake et al., 2015), our failure to demonstrate this change could be associated with the long period mice were kept in the absence of exercise. Taken together, our results suggest that, even months after exercise cessation, a discrete, but persistent effect of exercise on peripheral inflammation was observed.

With respect to the brain, positive effects of exercise on CNS inflammatory response have also been widely reported and associated with age-induced cognitive impairments and age-related disease protection (Speisman et al., 2013, Gomes da Silva et al., 2013). However, the possible persistent anti-inflammatory effect of exercise throughout the lifespan and after exercise cessation has not yet been demonstrated. Thus, we decided to analyse the classic pro- and anti-inflammatory cytokines and glial cell activation markers in the cortex and hippocampus of mice, months after exercise cessation, to have an overall view of any changes induced by age and/or exercise in the inflammatory profile of the brain.

Firstly, we investigated age-induced changes in brain inflammatory markers. In fact, ageing is often associated with memory impairment and with neurodegenerative disorders (Mattson and Magnus, 2006) and it is well established that many of these age-related alterations have been linked to changes in cytokine expression (Sparkman and Johnson, 2008), especially in aged rodents. For instance, increased expression of IL-1β and IL-6 has been reported in the hippocampus of aged rodents (Ye and Johnson, 1999, Lynch, 2007) as well as a decrease in the anti-inflammatory cytokine IL-10 (Ye and Johnson, 2001). Our findings regarding expression of cytokines in the aged mouse brain are broadly in accordance with the literature, as we demonstrated an increase in mRNA expression of IL-1β and TNF-α in both hippocampus and cortex, as well as increased expression of IL-6 in the cortex, only in age-sedentary animals.

Moreover, inflammation has also been involved in the pathophysiology of mental and mood disorders, such as depression and anxiety (Miller and Raison, 2016, Vogelzangs et al., 2013). Studies demonstrated that blocking cytokines, such as TNF-α or inflammatory mediators, such as cyclooxygenase 2, result in reduction of depressive symptoms in patients with conditions, such as arthritis and cancer, as well as in patients with major depressive disorder (Abbott et al., 2015). On the other hand, unlikely depression, only few studies have investigated the involvement of inflammation in anxiety disorders. For example, studies have shown a link between anxiety symptoms and elevated cytokine levels and CRP in adults (Pitsavos et al., 2006, Liukkonen et al., 2011). In this context, exercise has been

reported to be a very effective tool as a treatment in mild to moderate depression and anxiety disorders, mainly by exercise modulation of BDNF levels, neurogenesis and inflammation (Carek et al., 2011, Abd El-Kader and Al-Jiffri, 2016). Corroborating with the literature, our results demonstrated that exercise prevented the age-induced changes in depressive and anxiety-related behaviour in mice, 10 months after exercise cessation, accompanied by changes in the cytokines profile in the cortex and hippocampus, suggesting that the anti-inflammatory effect of exercise could alleviate the symptoms of depression and anxiety disorder in aged rodents.

Most interestingly, our results revealed that, even 10 months after exercise cessation, a persistent anti-inflammatory effect of exercise in the hippocampus and cortex was observed. We have demonstrated exercise to attenuate an age-related increase in mRNA expression of IL-1β in the hippocampus and prevent an increase in IL-1β mRNA expression in the cortex, as well as protecting against age-induced increase in the expression of TNF-α in both regions. Also, even months after exercise cessation, exercise protected against age-induced increase in IL-6 mRNA expression in the hippocampus and exercise induced a significant increase in mRNA expression of the anti-inflammatory cytokine IL-10 in both regions, even when compared with young animals. Several studies support our findings regarding exercise attenuating brain inflammation and exercise has been shown not only to attenuate the increase of pro-inflammatory cytokines, but to increase anti-inflammatory cytokines, thereby maintaining a protective balance of cytokines in brain environment. For instance, in support of our results, treadmill running has been demonstrated to decrease gene expression of IL-1β and protein content of TNF-α and IL-6 in the hippocampus and cortex of sleep-deprived mice (Chennaoui et al., 2015). Also, 18 weeks of voluntary exercise has been shown to decrease hippocampal expression of IL-1β in aged rats and this was accompanied by cognitive improvement (Speisman et al., 2013). These effects of exercise on cytokine expression are likely associated with the persistent effects of exercise observed in cognition over the mouse lifespan, even after exercise cessation. The effect of IL-1β on behaviour has been vastly studied and the first report of IL-1β-induced cognitive impairment was demonstrated in rats injected with the cytokine, which exhibited poor performance in the MWM task (Oitzl et al., 1993); since then,

multiple studies have supported this negative effect of IL-1β on cognitive functions (Murray and Lynch, 1998b, Barrientos et al., 2011).

Moreover, our results are in partial agreement with Lovatel and colleagues (2013), who showed that daily forced exercise sustained for 2 weeks decreases pro-inflammatory markers, including IL-1β, TNF-α and NF-κB in the hippocampus of aged rats and ameliorates age-induced cognitive decline in aversive memory (Lovatel et al., 2013). However, we found total and phosphorylated NF-κB protein in the hippocampus to be unaffected by age or exercise. This result is intriguing, since NF-κB is considered one of the major signalling response pathways implicated in ageing (Tilstra et al., 2011). However, it has also been also demonstrated that suppression of IKK2/NF-κB signalling in neurons increases the secondary posttraumatic cascade, worsens the neurological outcome and induces apoptosis, resulting in enhanced expression of pro-inflammatory markers (Mettang et al., 2018). In addition, 10 days of treadmill running have been reported increase protein expression of IL-10 in the hippocampus of aged rats (Gomes da Silva et al., 2013). Interestingly, in the hippocampus, we demonstrated that not only mRNA expression of IL-10 remained increased in exercise mice 10 months after exercise cessation, but we also showed a persistent increase in IL-10 concentration in the hippocampus these mice. Unfortunately, no association between changes in mRNA expression and protein synthesis of IL-1β and TNF-α was observed in the hippocampus.

Having established differences in cytokine expression between our experimental groups, and since glial cells are significant sources of cytokines in the brain, we next assessed the effects of age and exercise on activation of glia in samples from hippocampus and cortex. Our findings demonstrate age-induced changes in GFAP expression, a marker of astrocyte activity, in the brain, even 10 months after exercise cessation. We observed that age decreased mRNA expression of GFAP, a marker of astrocyte reactivity, in the hippocampus and cortex of both sedentary and exercise mice. Interestingly, when a more specific analysis of GFAP expression was carried out using immunohistochemistry, analysis of the total number of GFAP positive cells in the CA1 and CA3 subfields of the hippocampus demonstrated age-induced increase of GFAP positive cells in the CA3 region.

However, 10 months after exercise cessation, a persistent effect of exercise was observed, shown by a decrease in GFAP positive cells in both CA1 and CA3. The literature strongly supports our findings, since age-induced increases in GFAP immunoreactivity and GFAP mRNA expression has been widely reported in different brain regions, including the hippocampus of rodents (Goss et al., 1990, Cotrina and Nedergaard, 2002, Hayakawa et al., 2007). Ablation of GFAP in astrocytes has been shown to promote neural survival and enhance LTP (McCall et al., 1996, Menet et al., 2000), while overexpression of GFAP is associated with fatal encephalopathy (Messing et al., 1998), however, the exact functional consequences of the increase in GFAP expression associated with ageing, remain unclear. Here, we demonstrated a persistent effect of exercise to decrease GFAP immunoreactivity in the CA1 and CA3 subfields of the hippocampus, even 10 months after exercise cessation, which is associated with a persistent enhancement in cognition. These results are also in agreement with the literature, since chronic exercise has been shown to decrease the number of activated astrocytes, demonstrated by reduced hippocampal number of GFAP positive cells in an AD mouse models (Leem et al., 2011, Zhang et al., 2017).

Age-induced microglia activation was also observed in hippocampal homogenate of sedentary mice at 21 months old, demonstrated by an increase in mRNA level of Iba-1. However, more targeted immunohistochemical analysis showed that neither age nor exercise induced changes in Iba-1 protein expression in the CA1 and CA3 regions of the hippocampus. Moreover, CD11b mRNA expression was upregulated in both aged sedentary and exercise mice. It is generally agreed in the literature that age impacts microglia activation (Lynch et al., 2010). In this context, our data are in accordance with several studies that reported age-induced increase in expression of inflammatory markers including MHC II, CD11b and Iba-1 in the aged brain in different species (Streit et al., 2008, Godbout et al., 2005, Henry et al., 2009). Here we show that exercise protected against the increase in mRNA expression of Iba-1 induced by age, in the hippocampus of mice, even 10 months after cessation, agreeing with studies that demonstrated exercise to reduce Iba-1 expression in ageing (Gebara et al., 2013). However, our results failed to show a significant difference in the number of positive Iba-1 cells between the groups, perhaps, because a small number of tissue was analysed due to time constraints

(n=3) Future tissue analysis of more samples per group might also reveal significant changes in Iba-1 protein content modulated by age and exercise.

We expanded our analysis to include other molecules implicated in age-related neuroinflammation. The chemokine fractalkine (Cx3cl1) is expressed primarily on neurons and its receptor (Cx3cr1) is expressed mainly on microglia, and the interaction Cx3cl1-Cx3cr1 has been shown to downregulate microglial activation *in vitro* and *in vivo.* Fractalkine signalling has been reported to play an important role in maintaining microglia in a resting state and also attenuating microglial activation following the removal of inflammatory stimulus (Ransohoff, 2007, Lyons et al., 2009). In this study, age increased mRNA expression of Cx3cl1 in the cortex, whereas age induced a decrease in Cx3cr1 in the hippocampus of both sedentary and exercise mice. The lack of difference between sedentary and exercise mice in age-induced changes of mRNA levels of Cx3cx1-Cx3cr1 was surprising, however analysis of protein expression of fractalkine and its microglial receptors might help to explain these findings. Furthermore, in the hippocampus, mRNA expression of iNOS was decreased in aged exercise mice, supporting the literature that reports exercise to decrease iNOS expression and promote functional recovery after focal cerebral ischemia in rats (Park et al., 2012), a condition associated with inflammation. Surprisingly, Arg-1 mRNA levels were increased in the hippocampus of age sedentary mice compared to 3 months old mice. Arg-1 is reported to have anti-inflammatory effects and to contribute to restore inflammation-associated damage (Hunt et al., 2015), suggesting this result might be associate with a compensatory mechanism to counteract the exacerbated inflammatory response we have demonstrated in the hippocampus of sedentary mice.

The results reported above were obtained from analysis of homogenate prepared from hippocampus or cortex, or from immunohistochemical analysis of whole brain sections. In order to probe further the observed changes in the inflammatory profile of brain tissue and to obtain more specific information relating to glial cell activation, we investigated the activation of specific microglia and astrocyte cell populations prepared from the brains of our 3 experimental groups to assess the effect of age and exercise in modulating these cells. Similar to the findings in the hippocampus and cortex, even after 10 months of exercise cessation, classical M1 state markers

in microglia cell population were modulated and attenuated by exercise. Early-life exposure to long-term exercise decreased microglia mRNA expression of IL-1β and Iba-1 in age. Furthermore, exercise protected against age-induced increase in mRNA expression of TNF-α. Surprisingly, considering its proposed anti-inflammatory effects, exercise had no significant effect on M2 state polarisation, observed by decreased mRNA expression of Ym1 in microglia cells, with no changes in Arg-1 and Mrc1 mRNA expression. Although the microglial response to pro- and anti-inflammatory stimuli has been established, very few studies have demonstrated the effect of exercise directly on glia isolated from the adult rodent brain and the present results extend the existing literature findings. For instance, Barrientos and colleagues (2011), demonstrated that microglia isolated from the hippocampus of aged exercising rats showed decreased expression of IL-1β, TNF-α, and IL-6 following LPS administration compared to cells isolated from control aged rats and this was accompanied by protection against age-induced cognitive impairment in contextual fear conditioning in rats (Barrientos et al., 2011). Moreover, voluntary wheel-running in aged mice alters microglia activation as evidenced by changes in the expression of MHC II and CD86 in isolated microglia cells (Kohman and Rhodes, 2013). Nevertheless, still supporting our results, a previous study has suggested that voluntary wheel running had minimal effect on M2 markers, such as Arg-1, IL-1ra, TGF-β, and CD206, in the hippocampus of adults and aged mice following IL-4/IL-13 administration (Littlefield and Kohman, 2017).

In isolated astrocytes, exercise also had a significant effect, attenuating their modulation towards a pro-inflammatory state. Our data demonstrate exercise to decrease mRNA expression of iNOS as well as to protect against age-induced increase in TNF-α mRNA and to attenuate age-related increase in GFAP mRNA expression in astrocytes. Although effects of age and exercise were observed in astrocyte A1 state markers, neither age nor exercise affected the markers of A2 state analysed in astrocytes. Our results therefore support a recent study, that reported astrocytes to be primed toward an A1 state of reactivity in age (Clarke et al., 2018). However, although exercise has been previously reported to modulate astrocyte reactivity as measured by changes in the expression of GFAP (Zhang et al., 2017), the effects of exercise directly in isolated astrocytes have not yet been

investigated. Taken together, our results suggest a persistent effect of exercise in regulating peripheral and central inflammation, attenuating glia cell activation towards a pro-inflammatory state, with a consequent decrease in the expression of pro-inflammatory cytokines and upregulation of the important anti-inflammatory cytokine, IL-10.

4.5.2 Lack of effect of exercise on neurotrophin and growth factor expression in age

Neurotrophins and growth factors have been demonstrated to be crucial for the promotion of neurogenesis and brain plasticity and consequently for promotion of cognitive enhancement (Vilar and Mira, 2016). Age has been described to negatively affect the expression of neurotrophic factors (Neeper et al., 1995), while exercise has been shown repeatedly to upregulate neurotrophins and growth factors, events associated with the beneficial effects of exercise in improving neurogenesis, LTP and cognitive function (Voss et al., 2013). Undoubtedly, BDNF has been the main neurotrophin studied regarding the mechanism of exercise enhancing memory (Cotman and Berchtold, 2002). Our results demonstrated an age-induced decrease in BDNF mRNA expression in the cortex of sedentary and exercise mice while, in the hippocampus, no effect of either age or exercise was observed in mRNA or protein expression of BDNF. Although increased mRNA and protein expression of BDNF in these brain regions has been reported immediately following exercise (Ding et al., 2006. Griffin et al., 2011), evidence suggests that alterations in BDNF mRNA expression do not always positively correlate with changes in protein expression (Tropea et al., 2001). Also, an inverse relationship between exercise intensity and BDNF expression has been described (Lou et al., 2008). It must be considered that our analysis of BDNF expression was carried out 10 months after exercise cessation, thus we did not expect to observe significant changes in BDNF and BDNF signalling, as the majority of studies have shown BDNF upregulation immediately after exercise cessation. Also, BDNF concentration has been shown to return to baseline levels after a delay of only 10–60 min in the periphery (Etnier et al., 2016) and after 4 hours in the hippocampus (Wu et al., 2007), suggesting that ability of exercise to increasing BNDF is transient and certainly unlikely to persist for 10 months without exercise.

Also, we demonstrated no difference between the groups in mRNA expression of BDNF receptor, TrkB, in either hippocampus or cortex, and in TrKB protein expression in the hippocampus. Similarly, p75 mRNA expression was unaffected in hippocampus and cortex. Moreover, mRNA of NGF, VEGF, Igf-1 and transcriptional factor CREB were also unaltered in the hippocampus and cortex of mice after 10 months in the absence of exercise, suggesting early-life exposure to exercise does not result in persistent modulation of these molecules. Although exercise has been reported to upregulate TrkB receptor (Jeong et al., 2014) and trophic factors, such as Igf-1 (Carro et al., 2000) and VEGF (Yasuhara and Date, 2007), as mentioned before, these effects might be only observed immediately after exercise cessation and they might not persist for such a long time in the absence of exercise. Together, our results suggest that exercise has no persistent effect on neurotrophin and growth factor expression in the aged brain, suggesting that the continuous effects of exercise in enhancing memory throughout the mouse lifespan are mediated through other mechanisms, perhaps associated with permanent changes in brain structure, as was assessed in subsequent experiments.

4.5.3 Persistent effect of exercise on synaptogenesis and neurogenesis in age

Inflammation and glial cell responses can exert either a supportive or a detrimental effect on adult hippocampal neurogenesis and synaptic plasticity (Kohman and Rhodes, 2013). We observed exercise to exert positive effects in modulating brain inflammatory profile and glial cell activation, even after months without exercise. For this reason, we investigated synaptogenesis and hippocampal neurogenesis in aged mice, at 21 months old. Even 10 months after exercise cessation, Synapsin-1 and PSD-95 protein expression were increased in the hippocampus of exercised mice. Our results are in agreement with studies that have demonstrated exercise to play an important role in modulating synaptogenesis. For example, voluntary running can increase spine synapse density in the DG and CA1 regions of the hippocampus (Dietrich et al., 2008), while, a single bout of resistance

exercise increases expression of Synapsin, Synaptophysin and PSD-95 in the hippocampus of rats (Fernandes et al., 2013).

Although neurogenesis continues throughout the lifespan, the rate declines with increasing age (Galvan and Jin, 2007). Accordingly, our results demonstrate an age-induced decrease in mRNA levels of the marker of proliferation ki67 in the hippocampus and cortex of sedentary and exercise mice. Age-induced decrease in cell proliferation in the DG has been demonstrated in both rodents (Amrein et al., 2004) and humans (Sorrells et al., 2018). For example, in the SGZ of the DG an *in vivo* study demonstrated a decrease of about 75% in the number of proliferating progenitors at middle-age to old age compared with young mice (Lugert et al., 2010). Running is well known to increase NSC proliferation, increasing ki67 expression, and to promote neurogenesis in the SGZ and DG (Wu et al., 2007) and although we did not observed a persistent effect of exercise in cell proliferation in our study, interestingly we observed an increase in the number of positive BrdU cells in the DG, even 10 months after exercise cessation. The discrepancy between the lack of Ki67 mRNA expression induced by exercise and the BrdU positive cells increased observed, could be associated with the different quantification methods used in the assessments.

Most interestingly, our findings demonstrated an increase in the number positive BrdU cells co-localised with NeuN, a marker of mature neuron, in the DG, demonstrating exercise is promoting survival of newborn neurons. In extension to our finding, several studies have shown either voluntary or forced running to increase neurogenesis in the adult and aged hippocampus in rodents (van Praag et al., 1999, van Praag et al., 2005, Marlatt et al., 2012) and most recently, resistance exercise was also reported to promote adult hippocampal neurogenesis (Nokia et al., 2016). It has been suggested that an increase in adult hippocampal neurogenesis promoted by running is due to an increase in the number of surviving neuronal precursor cells (type-2 cells) rather than the shortening of the cell cycle (Fischer et al., 2014). Also, some studies demonstrated that running increases the survival, incorporation and also reorganization of the circuitry of newborn hippocampal cells, generated days before commencing training, to increase net neurogenesis (Sah et al., 2016). Our findings showing the maintenance of

hippocampal neurogenesis in the DG of aged mice, suggest that early-life exposure to aerobic exercise can promote structural brain changes that persist even months after exercise cessation.

Simultaneous with generation of neurons, generation of new glial cells, through the process called gliogenesis, constantly occurs in the DG (Cameron and Rakic, 1991). However, the balance between these processes is important, and a dysfunction in this balance has been associated with some neurological and psychiatric diseases (Frisen, 2016). In order to verify this balance between neurogenesis and gliogenesis in ageing, and the possible effects of exercise on this balance, the positive BrdU cells were also co-localised with positive GFAP and Iba-1 cells in the DG. However, although there is a trend toward a decrease in positive BrdU cells co-localised with glial cell markers in the DG of exercise mice, no significant changes in the number of these co-localised cells was observed. The failure to demonstrate changes modulated by age or exercise in the number of positive BrdU/Iba-1 cells in the DG, might be in part due to the small number of mice analysed (n=3 per group); these numbers should be increased in order to provide a conclusive answer to this question of altered balance of newborn cells.

Our approach using GFAP as a marker of newborn astrocytes has some limitations and might be not the best method for this assessment, mainly because embryonic and adult neural precursors express proteins, including GFAP (Miller and Gauthier, 2007). For instance, embryonic radial-glia-cells have been shown to be neural precursors that could generate neurons and astrocytes (Gotz and Barde, 2005). Our lack of results demonstrating changes in the generation of newborn astrocytes, might be explained by the caveats of the used method. Therefore, our results suggest that exercise does not alter gliogenesis, however exercise not only affected the survival of newborn cells, but also drove these newborn cells to be integrated as newborn neurons.

Age-related changes in brain and hippocampal volume have been described, but some discrepancies exist in the literature. For instance, imaging studies in humans and animals have reported atrophy of the hippocampus with age (Resnick et al., 2003, Quallo et al., 2009). Moreover, studies have demonstrated reduced hippocampal volume in older depressive and AD patients that is associated with

cognitive impairments (Hickie et al., 2005). However, other studies have failed to show such changes in brain structures in aged individuals (Schuff et al., 1999)), similar to our MRI results, that did not show age-induced changes in the total brain and hippocampal volume of sedentary mice at 21 months old. On the other hand, strong evidence in the literature indicates exercise can induce volumetric brain changes. For example, aerobic exercise has been shown to increase grey and white matter volume in older adults (Colcombe et al., 2006) and is associated with sparing of prefrontal and temporal brain regions over a 9-year period, which was linked to reduced risk of cognitive impairment (Erickson et al., 2010).

In support of our results of a persistent effect of exercise on neurogenesis, MRI analyses of region of interest revealed that, even after 10 months in the absence of exercise, total brain volume and right and left hippocampal volume of exercised mice were increased compared to 3 months old mice. Supporting our results, Erickson and collaborators (2011) have shown a high association between physical activity, increased hippocampal volume and better cognitive function in older humans (Erickson et al., 2011). Most recently, a study conducted in mice revealed exercise to induce changes in the volume of hippocampal grey matter and that this change was strongly correlated with increased number of hippocampal DCX positive cells, suggesting a larger hippocampal volume might be, in part, associated with enhanced hippocampal neurogenesis (Biedermann et al., 2016). In this way, our findings strongly extend those presently in the literature, adding the novel aspect of a permanent and persistent exercise-induced change in brain structure, even months after exercise cessation. Together, our data suggest that early exposure to exercise induced lasting changes in brain structure as well as function.

4.5.4 Summary

In summary, our results demonstrated that, even 10 months after exercise cessation, mRNA expression of pro-inflammatory markers was decreased in the liver of exercised mice, whereas no changes were observed in the gastrocnemius muscle. Moreover, regarding neuroinflammation, our findings suggest a persistent anti-inflammatory effect of exercise on the hippocampus and cortex, demonstrated

by a persistent increase in IL-10 expression. We also demonstrated exercise to decrease and to attenuate age-induced increases in pro-inflammatory cytokines and glial cell activation in both hippocampus and cortex. Furthermore, our findings in enriched populations of microglia and astrocytes suggest that exercise induced a persistent effect on controlling activation of M1 and A1 phenotypes, with minimal changes in M2 and A2 state markers. Exercise also showed a persistent effect of reducing astrocyte reactivity, with no effect on microglia activation, in CA1 and CA3 subfields of the hippocampus. However, exercise had no persistent effects on expression of neurotrophins and growth factors in the hippocampus and cortex nor did it have an effect on expression of NF-κB protein in the hippocampus. Nevertheless, even 10 months after exercise cessation, an increase in the expression of synaptogenesis protein markers in the hippocampus, and in promotion of the survival of newborn neurons and maintenance of brain and hippocampus volume was observed when compared with mice that remained sedentary throughout life.

These results together suggest that, early life exposure to physical exercise, induced persistent change in brain inflammatory profile until old age, promoting a positive brain microenvironment. This primed anti-inflammatory brain environment could be contributing to the lasting effects of exercise on synaptogenesis and neurogenesis observed in our study. Furthermore, as structural factors, such as increased neurogenesis and synaptic plasticity are associated with a greater cognitive reserve, we can suggest the early life exposure to exercise built-up such a reserve, rendering the brain resilient to age-induced changes.

Chapter 5

The effect of exercise on memory in a mouse model of neuroinflammation: the role of glial cells

Chapter 5: The effect of exercise on memory in a mouse model of neuroinflammation: the role of glial cells

5.1 Introduction

Ageing is strongly associated with the presence of low-grade chronic inflammation (Singh and Newman, 2011, Heneka et al., 2015). Also, a wide body of evidence supports an important role of resident glial cells in the process of age-induced neuroinflammation. Microglia cells can assume an inflammatory activation (M1) state (Sierra et al, 2007), characterised by an increase in the expression of pro-inflammatory cytokines IL-1β, IL-6 and tumor necrosis factor alpha (TNF-α) (Hanisch and Kettenmann, 2007). However, they can also assume the M2 state, characterised by an anti-inflammatory modulation. The M2 phenotype features an increase in the production of anti-inflammatory cytokines, as well as upregulation in the levels of neurotropic factors, such as BDNF (Mosser, 2003) In addition, it has been shown that when the M2 state is activated, there is an increase in the expression of several markers, such as Arg-1, chitinase-like 3 (Ym1) and the Mrc1, which contribute to repair of inflammation-induced damage (Cherry et al., 2014, Pepe et al., 2014).

Astrocytes have also been found to contribute to inflammation during ageing (Nichols et al., 1993). Most recently, it has been suggested that astrocytes can assume a pro- and an anti-inflammatory phenotype (A1 and A2), similar to microglia cells, supporting evidence of the role of astrocytes in the inflammatory process (Roybon et al, 2013). The presence of glial cells primed towards an inflammatory phenotype is tightly linked with altered brain plasticity and cognitive decline in ageing (Ryan et al., 2014). Likewise, systemic inflammation also may induce neuroinflammation and glial cell polarisation (Jeong et al., 2010). In this context, systemic administration of lipopolysaccharide (LPS), has been widely used as a model of age-related neuroinflammtion, glial cell activation and cognitive impairment (Cunningham et al, 2005).

Previous studies have demonstrated that, when exposed to anti-inflammatory agents, microglia can shift from M1 towards an M2 phenotype and mediate resolution of inflammation (Fenn et al., 2012). In this context, physical exercise is a potentially potent tool to attenuate age-related neuroinflammation and cognitive deficit, since many studies have demonstrated the anti-inflammatory effects of physical exercise in the healthy brain and in several neurodegenerative diseases (Kelly, 2015, Ryan and Kelly, 2016).

Although exercise has been shown to modulate inflammation and glial cell activation, its potential to prime microglia and astrocyte polarisation towards an anti-inflammatory-inducing signal remains unclear. In the previous chapter, we showed that long term-exercise during youth and middle-age modulates the inflammatory profile affecting microglia and astrocyte activation, even 10 months after exercise cessation. For this reason, the objective of this chapter is to investigate whether prior short-term exercise can affect inflammation and the polarization of isolated enriched population of microglia and astrocytes and protect mice against cognitive impairment, following an immune challenge with a sub-septic dose of LPS.

5.2 Methods

5.2.1 Animals

Six-month-old male C57BL/6 mice (20-30g, n=47) obtained from Comparative Medicine Unit, Trinity College Dublin were used in these experiments. Mice were given four weeks of acclimation to the facility and were handled daily for 5 minutes during this period. All experiments were performed in accordance with National and European directives on the protection of animals European Union and were approved from the Animal Ethics Committee, Trinity College Dublin. Animals were group-housed, 4 to 5 per cage, under a 12:12-hour light-dark cycle with food and water available *ad libitum* and with controlled temperature and humidity.

5.2.2 Experimental design

Mice were randomly assigned to either sedentary (SED, n=23) or exercise (EX, n=24) group. EX mice underwent one hour per day of moderate exercise on a motorised treadmill for a period of nine consecutive days (Figure 5.1). Twenty-four hours after the last session of exercise EX and SED mice received a single intraperitoneal (i.p.) injection of saline (0.89% NaCl (w/v)) or a sub-septic dose of lipopolysaccharide (LPS) *from Escherichia coli* (100µg/kg). Four hours after the injection, mice were trained in the Object Displacement (OD) task and tested thirty minutes after training. Mice were euthanised immediately following behavioural testing by sodium pentobarbital overdose and transcardial perfusion with sterile phosphate-buffered saline (PBS).

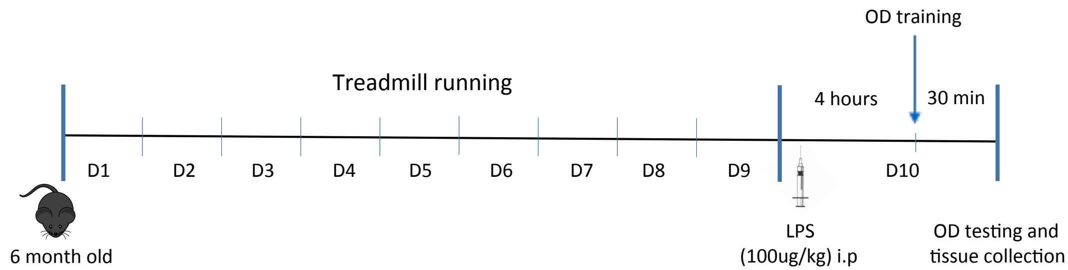


Figure 5.1- Experiment timeline

Timeline showing the physical exercise protocol, LPS injection, and Object Displacement (OD) task employed.

5.2.3 Exercise protocol

Mice were familiarised to motorised rodent treadmills (Exercise 3/6 treadmill, Columbus Instruments, USA) daily for five days prior to the commencement of the exercise protocol by walking for 15 to 20 min at a speed of 4 to 6m/min. The exercise protocol consisted of nine consecutive days of running for one hour/day (belt speed 8-14 m/min, at zero inclination). The treadmills are equipped with wire loops at one end of the belt through which a mild electric shock can be delivered. In this experiment, mice ran in the absence of electric shock, just a gentle hand prodding was used to motivate animals to run. SED mice were placed on stationary treadmills for the same duration, to control for the effects of handling and exposure to a new environment.

5.2.4 LPS administration

One single intraperitoneal injection was administered four hours prior to the object displacement task. SED and EX mice were randomly assigned to receive either saline (0.89% (w/v) NaCl) or a subseptic dose of *Escherichia coli* LPS (100μg/kg) in saline.

5.2.5 Object displacement (OD) task

Spatial memory was assessed using the object displacement (OD) task, described in detail in section 2.7. Briefly, mice were individually habituated to the open field in the absence of objects for five minutes for two consecutive days prior to training. The apparatus consisted of a black circular open field (diameter, 0.5 m; height, 0.48 m). Objects were constructed from plastic toy blocks and were fixed to the floor of the open field. Four hours after Saline or *E. coli* LPS injection, mice were trained in the OD task (Figure 5.2). For training, mice were placed individually in the open field with three different objects (Objects A, B and C) and were allowed to explore the objects for five minutes. Thirty minutes after training, one of the three objects (Object C) was displaced to a different part of the open field and animals were allowed to explore the three objects for five minutes. The time spent exploring each object was recorded during training and testing and results were calculated and expressed as a percentage of the total exploration time.

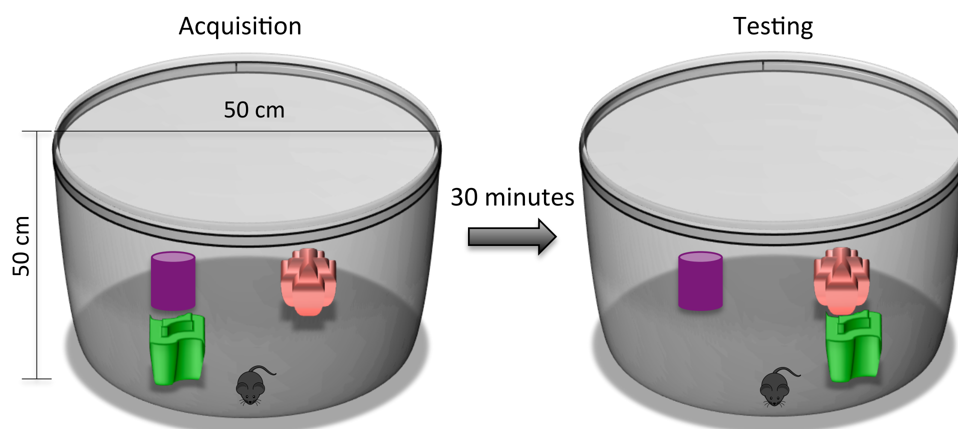


Figure 5.2 - Object displacement task (OD)

Representative image of the arena, the layout of the objects and delay for testing of Object displacement task (OD).

5.2.6 Tissue preparation and isolation of microglia and astrocytes

After the behaviour assessment, one set of mice (n=23) was sacrificed by cervical dislocation; hippocampus and prefrontal cortex samples were dissected and were either homogenized in 500µl of lysis buffer for Western blotting analysis or placed

in RNAse-free tubes for PCR assay, as described in section 2.11.1. Concentration of total protein in the tissue homogenates was measured using the BCA assay, as described in section 2.10. Another set of mice (n=24) was anaesthetised, a blood sample was collected by cardiac puncture and animals were perfused intracardially with sterile ice-cold PBS. The brain was removed, and hippocampus and prefrontal cortex samples were dissected and stored at -80°C. The remaining brain tissue (except cerebellum) was placed in 1× Hank's Balanced Salt Solution (HBSS; Invitrogen, UK), cross-chopped and homogenised for the isolation of an enriched population of microglia and astrocytes, as described in detail in section 2.11.3.

5.2.7 Analysis of protein expression by western immunoblotting

To assess the effect of prior exercise after a single inflammatory challenge on signaling proteins downstream of TrkB receptor activation, expression of phosphorylated and total, Akt, CREB, ERK1/2 and TrkB (for dilutions, see table 2.2.1 in section 2.14.3) was measured in hippocampal and cortical tissue using western blot technique, as described in detail in section 2.12. β-Actin was used as an endogenous control for total protein concentration.

5.2.8 Analysis of mRNA expression by polymerase chain reaction (RT-PCR)

RNA was isolated from hippocampus, cortex, astrocytes and microglial cells using a Nucleospin RNAII kit (Macherey-Nagel, Germany) and samples were reverse transcribed into complementary DNA (cDNA) using the ABI High Capacity cDNA archive kit (Applied Biosystems, Ireland) as described in detail in section 2.13. Gene expression of several targets was assessed using "Taqman gene expression assays" (Applied Biosystems, Ireland; table 2.3 in section 2.15.4). RT-PCR was performed using Step One Plus TM Software (Applied Biosystems), as described in detail in section 2.13, and data were quantified using the ΔΔCT method and expressed as relative quotient (RQ) values.

5.2.9 Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). All data were analysed using two-way analysis of variance (two-way ANOVA) with Bonferroni *post-hoc* tests (GraphPad Prism software). Statistical significance was accepted at $p<0.05$.

5.3 Results

5.3.1 Effects of LPS and exercise on spatial learning and memory

The effect of LPS injection and prior exercise on spatial learning and memory was assessed using the OD task (Figure 5.3 A). Four hours after LPS injection, no difference in total exploration was found during the training and testing phases, indicating no effect of exercise or LPS on general exploratory behaviour. In the training phase, there was no difference in the exploration time of the three objects in all groups, indicating no preference for any single object (p=0.3338, SED-SAL: Obj. A: 35.72±2.25%, Obj. B: 32.22±1.46%, Obj. C: 32.05±1.39%; SED-LPS: Obj. A: 31.15±1.94%, Obj. B: 35.16±2.32%, Obj. C: 33.69±1.78%; EX-SAL: Obj. A: 34.43±0.99%, Obj. B: 31.07±1.47%, Obj. C: 34.49±1.27%; EX-LPS: Obj. A: 33.31±1.35%, Obj. B: 31.15±0.98%, Obj. C: 35.57±1.17%, two-way ANOVA, Bonferroni post-hoc, Figure 5.3 B).

During testing, when object C was displaced to a different quadrant in the arena, SED-LPS mice explored the displaced object less than the stationary objects, while all other groups spent significantly more time exploring the displaced object (p<0.001, SED-SAL: Obj. A: 24.78±1.01%, Obj. B: 29.18±2.06%, Obj. C: 46.03±2.17%; SED-LPS: Obj. A: 37.03±3.32%, Obj. B: 36.07±4.48%, Obj. C: 26.90±2.18%; EX-SAL: Obj. A: 19.62±1.40%, Obj. B: 23.93±1.34%, Obj. C: 56.45±1.85%; EX-LPS: Obj. A: 22.86±1.38%, Obj. B: 25.74±1.12%, Object C: 51.40±1.84%, two-way ANOVA Object A and B vs. Object C, Bonferroni *post-hoc*, Figure 5.3 C).

Furthermore, mice from the EX-LPS group spent significantly more time exploring the displaced object C when compared with SED-LPS group (p<0.001, two-way ANOVA, Object C exploration EX-LPS vs. SED-LPS, Bonferroni *post-hoc*, Figure 5.3 C), indicating that exercise counteracted the impairment of LPS injection on spatial memory.

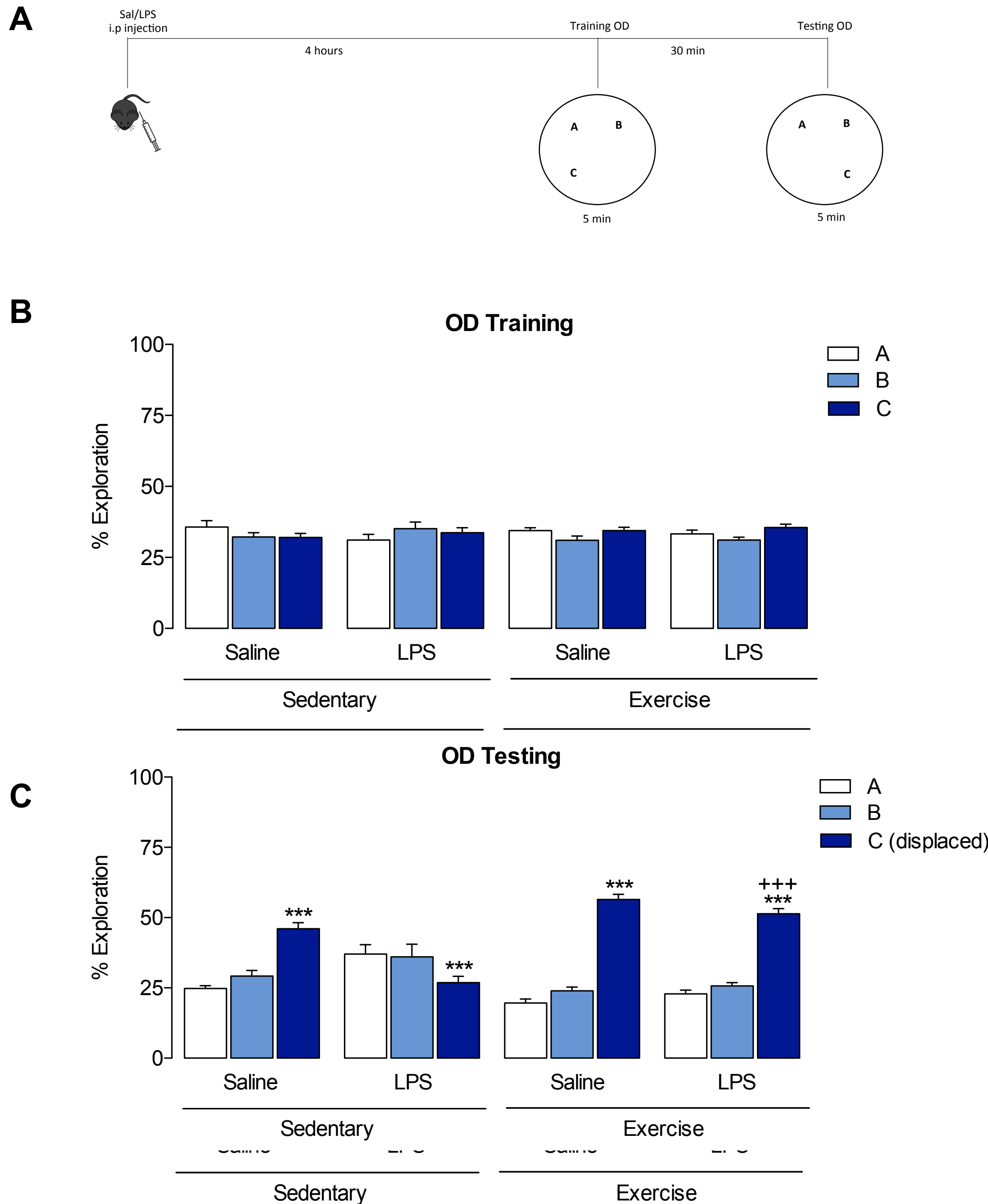


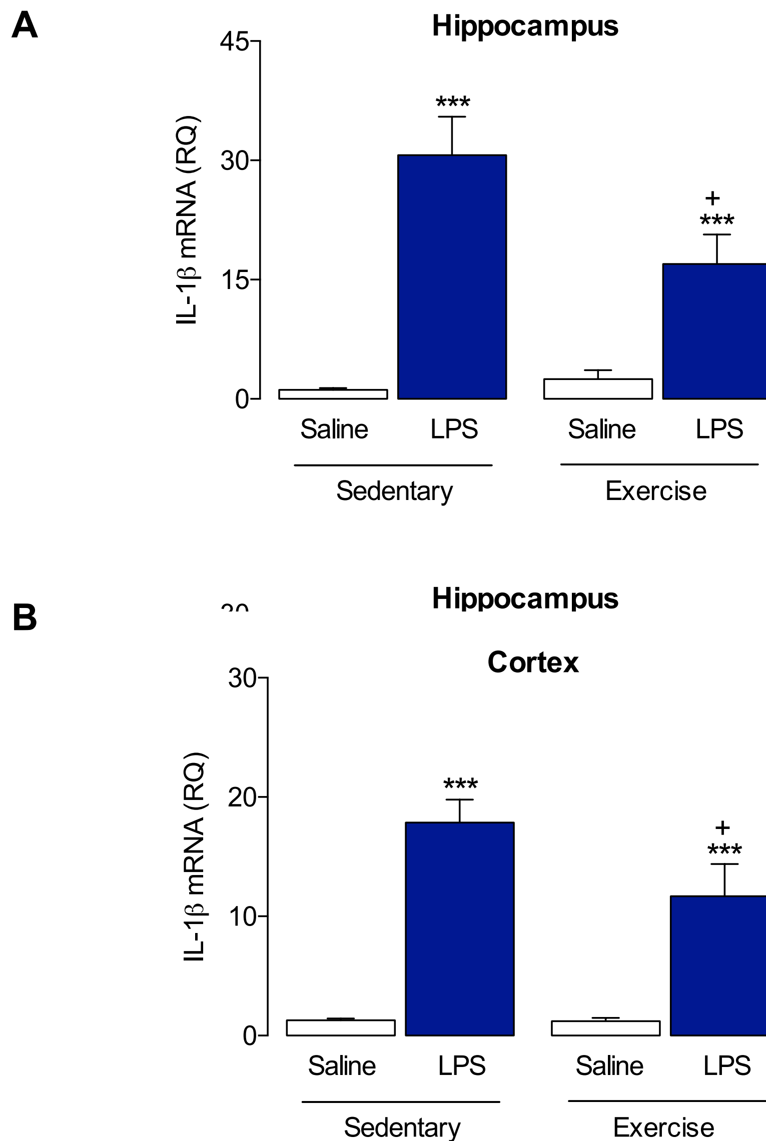
Figure 5.3 - Effect of LPS injection and prior short-term exercise on memory
Experimental timeline LPS injection and OD testing **(A)**. There was no difference on exploration of objects between the groups during training phase of OD **(B)**. In the testing phase, SED-LPS failed to explore the displaced object more than the other objects **(C)**. All data are expressed as mean ± SEM (n=11 to 12). ***p<0.001, Object A and B exploration vs. Object C exploration in the same group; +++p<0.001, Object C exploration EX-LPS vs. SED-LPS; two-way ANOVA with Bonferroni *post hoc* analysis.

5.3.2 Effects of LPS and exercise on mRNA expression of pro-inflammatory cytokines, IL-1β, TNF-α and IL-6 in the hippocampus and cortex

In order to investigate mRNA expression of pro-inflammatory cytokines in hippocampal and prefrontal cortical tissue, mRNA levels of IL-β, TNF-α and IL-6 were analysed by RT-PCR. Four hours after LPS injection, statistical analysis demonstrated a significant difference in hippocampal and cortical mRNA levels of pro-inflammatory cytokines, IL-1β ($p<0.001$, two-way ANOVA, Figure 5.4 A and B), TNF-α ($p<0.001$, two-way ANOVA, Figure 5.5 A and B).

Bonferroni post-hoc analysis revealed, in the hippocampus and cortex, a significant LPS-induced increase in IL-1β mRNA expression (Hippocampus fold change SED-SAL: 1.15±0.23; SED-LPS: 30.65±4.87, EX-SAL: 2.49±1.13, EX-LPS: 16.98±3.68, Figure 5.5 A; Cortex fold change SED-SAL: 1.30±0.13, SED-LPS: 17.86±1.91, EX-SAL: 1.22±0.26; two-way ANOVA, LPS vs. Saline, Figure 5.5 B) and TNF-α mRNA expression (Hippocampus fold change SED-SAL: 1.03±0.09, SED-LPS: 15.66±2.14, EX-SAL: 0.94±0.16, EX-LPS: 9.40±1.28, Figure 5.6 A; Cortex fold change SED-SAL: 1.64±0.19, SED-LPS: 19.45±1.95, EX-SAL: 1.72±0.31, EX-LPS: 14.14±0.99, two-way ANOVA, LPS vs. Saline, Figure 5.6 B) in both SED and EX mice. However, the LPS-induced increase in IL-1β and TNF-α in the hippocampus and cortex was attenuated in mice that had undergone prior exercise ($p<0.05$, SED-LPS vs. EX-LPS, Bonferroni *post-hoc*, Figure 5.4 A and B and 5.5 A and B, respectively).

Moreover, statistical analysis showed a significant difference in the mRNA expression of IL-6 in the hippocampus ($P<0.001$, two-way ANOVA, Figure 5.5 C) and post-hoc analysis also revealed LPS-induced increase in mRNA levels of IL-6 in the hippocampus of SED and EX mice (fold change SED-SAL: 1.10±0.16, SED-LPS: 5.37±0.66, EX-SAL: 1.27±0.26, EX-LPS: 3.55±0.96, two-way ANOVA LPS vs. Saline, Bonferroni *post-hoc*, Figure 5.5 C), but no effect of exercise on expression of IL-6 in the hippocampus was observed ($p=0.090$, two-way ANOVA, Figure 5.5 C).



5.4 - Effect of L … **ssion of IL-1β in the**
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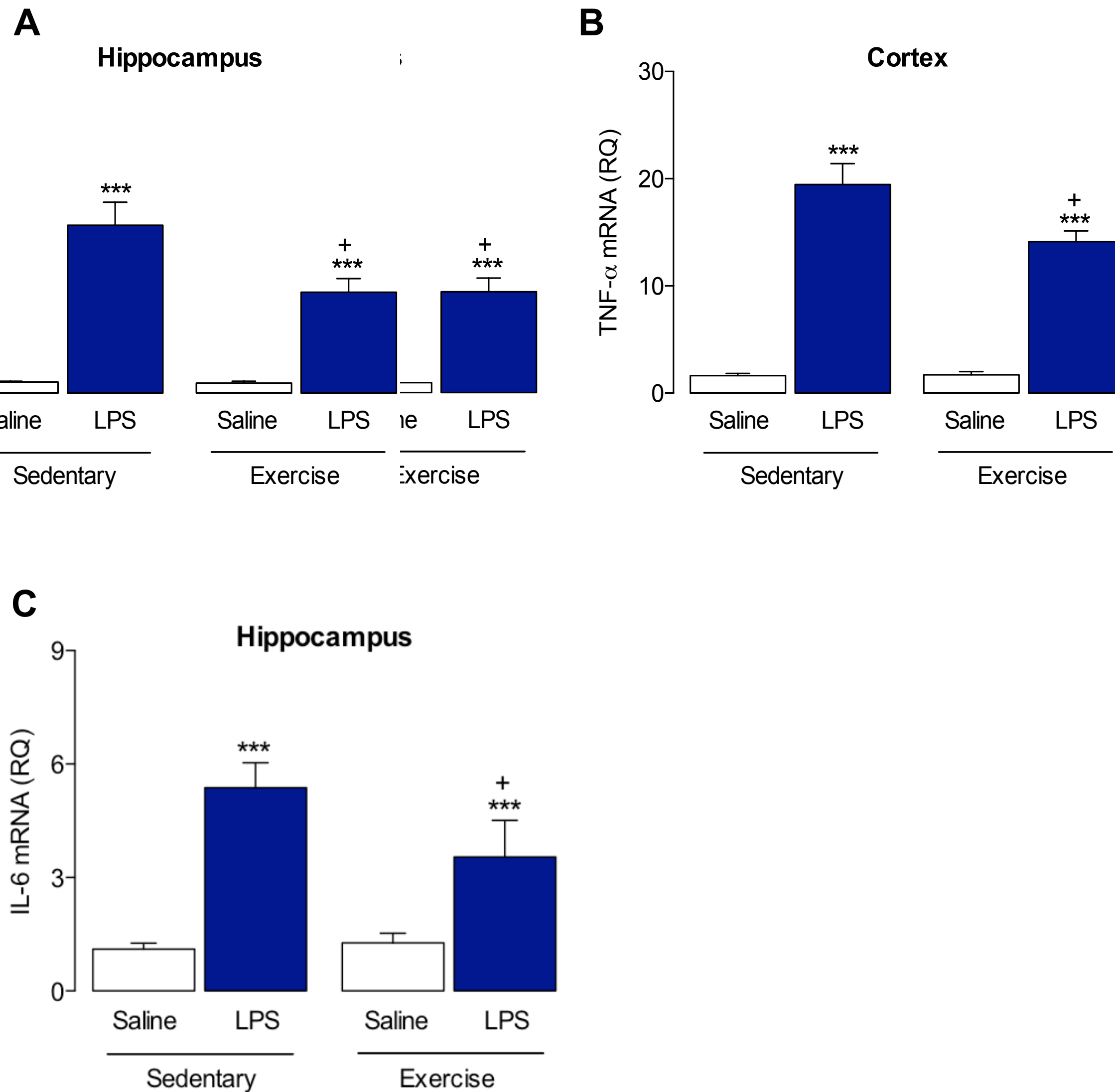


Figure 5.5 - Effect of LPS injection and prior exercise on mRNA expression of TNF-α and IL-6 in the hippocampus and cortex

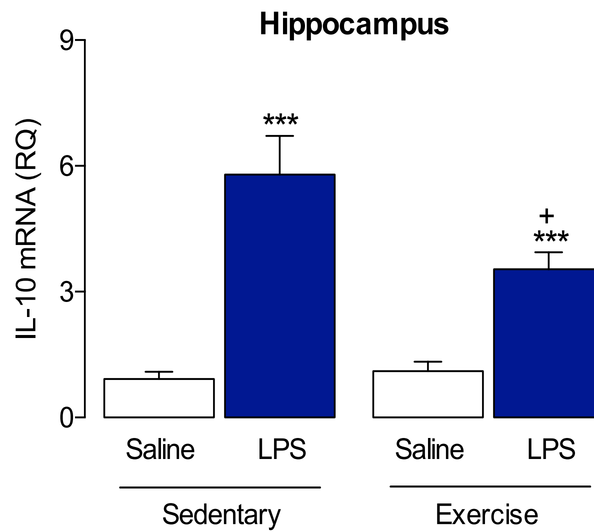
Four hours after i.p. injection of LPS, hippocampal and cortical mRNA expression of TNF-α and hippocampal mRNA expression of IL-6 were assessed by RT-PCR. Expression of TNF-α was increased in hippocampus and cortex of SED and EX mice injected with LPS, but exercise attenuated this increase in both regions **(A, B)**. In the hippocampus, mRNA expression of IL-6 was increased in both SED and EX LPS-injected mice, however exercise had no effect on the LPS-induced increase in expression of IL-6 mRNA **(C)**. Data are presented as mean ± SEM (n=11 to 12). ***$p<0.001$, Saline vs. LPS; $^{+}p<0.05$, SED-LPS vs. EX-LPS; two-way ANOVA with Bonferroni *post hoc* analysis.

5.3.3 Effects of LPS and exercise on mRNA expression of anti-inflammatory cytokines, IL-10 and IL-4 in the hippocampus and cortex

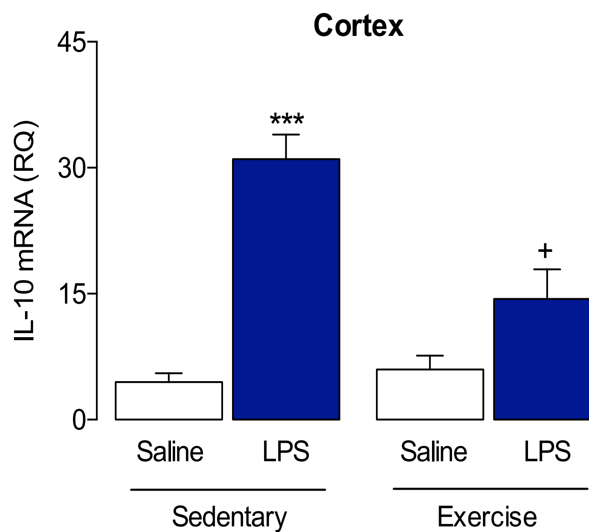
mRNA expression of anti-inflammatory cytokines IL-10 and IL-4 in hippocampal and prefrontal cortical tissue was analysed by RT-PCR. LPS induced an increase in mRNA expression of the anti-inflammatory cytokine IL-10 in the hippocampus of SED and EX mice and ($p<0.001$, two-way ANOVA LPS vs. Saline, fold change SED-SAL: 0.92±0.17; SED-LPS: 5.79±0.92; EX-SAL: 1.11±0.22; EX-LPS: 3.53±0.40, Bonferroni *post-hoc*, Figure 5.6 A). In the cortex, IL-10 was increased only in LPS-injected SED mice ($p<0.001$ two-way ANOVA LPS vs. Saline, fold change SED-SAL: 4.47±1.06; SED-LPS: 31.01±2.93; EX-SAL: 5.99±1.65; EX-LPS: 14.39±3.50, Bonferroni *post-hoc*, Figure 5.6 B).

Interestingly, prior exercise attenuated the effect of LPS on IL-10 mRNA expression in the hippocampus ($p=0.0372$, two-way ANOVA SED-LPS vs. EX-LPS, Figure 5.6 A) and completely blocked the effect of LPS on expression of IL-10 in the cortex ($p=0.0038$, SED-LPS vs. EX-LPS, Figure 5.6 B). The expression of another anti-inflammatory cytokine, IL-4, was also increased only in the hippocampus of SED mice injected with LPS ($p=0.0005$, two-way ANOVA LPS vs. Saline, fold change SED-SAL: 0.85±0.16; SED-LPS: 2.59±0.56. EX-SAL: 0.85±0.13; EX-LPS: 1.14±0.18, Bonferroni *post-hoc*, Figure 5.7 A) and prior exercise prevented this increase ($p=0.0073$, two-way ANOVA SED-LPS vs. EX-LPS, Figure 5.7 A). However, neither LPS nor prior exercise affected the expression of IL-4 in the cortex ($p=0.6591$, two-way ANOVA, fold change SED-SAL: 2.67±0.74; SED-LPS: 1.83±0.50. EX-SAL: 1.56±0.70; EX-LPS: 1.31±0.50, Figure 5.7 B).

A



B



5.6 - Effect of LI **on of IL-10 in the**
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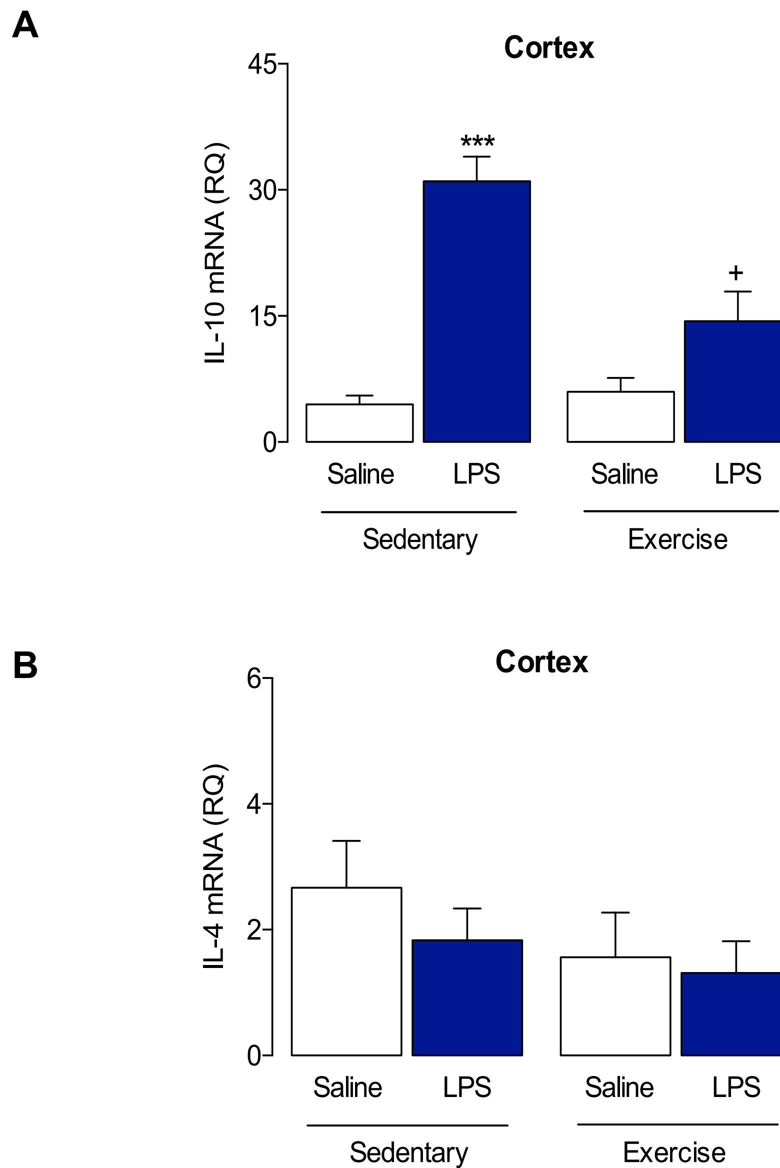


Figure 5.7 - Effect of LPS injection and prior exercise on mRNA expression of IL-4 in the hippocampus and cortex

Four hours after i.p. injection of LPS, hippocampal and cortical mRNA expression of IL-4 was assessed by RT-PCR. In hippocampus, expression of IL-4 was increased only in SED LPS-injected mice and this increase was prevented by prior exercise **(A)**. In cortex, neither LPS nor exercise altered IL-4 mRNA expression **(B)**. Data are presented as mean ± SEM (n=11 to 12). $^{**}p<0.01$, Saline vs. LPS; $^{++}p<0.01$, SED-LPS vs. EX-LPS; two-way ANOVA with Bonferroni *post hoc* analysis.

5.3.4 Effects of LPS and exercise on mRNA expression of markers of activation of glial cells in the hippocampus and cortex

Having established the modulatory effect of prior exercise on LPS-induced changes in hippocampal and cortical cytokine expression, we investigated the modulation by prior exercise of mRNA expression of glial cell activation markers in the hippocampus and cortex of LPS-injected mice. Neither LPS nor exercise affected Iba-1 mRNA expression in either, hippocampus ($p=0.2840$, two-way ANOVA, fold change SED-SAL: 1.00±0.03; SED-LPS: 1.00±0.15; EX-SAL: 1.08±0.13; EX-LPS: 0.83±0.06, Bonferroni *post-hoc*, Figure 5.8 A) or cortex ($p=0.7392$, two-way ANOVA fold change SED-SAL: 1.09±0.11; SED-LPS: 0.94±0.04; EX-SAL: 0.95±0.03; EX-LPS: 0.85±0.04, Bonferroni *post-hoc*, Figure 5.8 B). However, in the hippocampus, CD11b mRNA expression was increased in SED-LPS only ($p=0.0023$, two-way ANOVA LPS vs. Saline, fold change SED-SAL: 1.01±0.06; SED-LPS: 1.38±0.13; EX-SAL: 0.98±0.05; EX-LPS: 1.12±0.04, Bonferroni *post-hoc*, Figure 5.8 C) with no changes in the cortex ($p=0.6084$, two-way ANOVA, fold change SED-SAL: 1.04±0.06; SED-LPS: 1.43±0.24; EX-SAL: 1.29±0.26; EX-LPS: 1.47±0.23, Bonferroni *post-hoc,* Figure 5.8 D).

Also, four hours after LPS injection, statistical analysis demonstrated significant difference in the expression of GFAP, in the hippocampus ($p<0.001$, two-way ANOVA, Figure 5.9 A) and cortex ($p<0.001$, two-way ANOVA, Figure 5.9 B) Bonferroni *post-hoc* analysis revealed significant increase in GFAP mRNA expression in the hippocampus of SED group ($p<0.001$, two-way ANOVA LPS vs. Saline, fold change SED-SAL: 1.00±0.05; SED-LPS: 2.28±0.27; EX-SAL: 1.13±0.09; EX-LPS: 1.65±0.08, Figure 5.9 A) and in cortex of SED mice ($p<0.001$, two-way ANOVA LPS vs. Saline, fold change SED-SAL: 1.50±0.30; SED-LPS: 3.80±0.64; EX-SAL: 1.46±0.25; EX-LPS: 2.90±0.48, Bonferroni *post-hoc*, Figure 5.9 B), while this effect was significantly attenuated only in the hippocampus of EX group ($p=0.0158$, two-way ANOVA SED-LPS vs. EX-LPS, Figure 5.9 A).

Similarly, in the hippocampus, statistical analysis showed significant changes in the expression of iNOS and *post-hoc* analysis revealed an increase in the expression of iNOS only in SED-LPS mice ($p=0.0305$, two-way ANOVA LPS vs.

Saline, fold change SED-SAL: 0.65±0.16; SED-LPS: 2.09±1.67; EX-SAL: 0.54±0.12; EX-LPS: 0.70±0.20, Bonferroni *post-hoc*, Figure 5.10 A), and this increase was attenuated by exercise (p=0.0142, two-way ANOVA SED-LPS vs. EX-LPS, Figure 5.10 A). Also, in the hippocampus, a difference was observed in the expression Arg-1 (p<0.001, two-way ANOVA, Figure 5.10 B) and Bonferroni *post-hoc* analysis showed LPS-induced increase in the expression of Arg-1 in the hippocampus of SED mice only (p<0.001, two-way ANOVA LPS vs. Saline, fold change SED-SAL: 1.07±0.15; SED-LPS: 1.80±0.20; EX-SAL: 0.92±0.11; EX-LPS: 1.32±0.13, Figure 5.10 B). However, expression of Mrc1 was not affected by either LPS or EX (p=0.2182, two-way ANOVA, fold change SED-SAL: 3.12±0.62; SED-LPS: 2.60±0.25; EX-SAL: 1.95±0.22; EX-LPS: 2.33±0.26, Bonferroni *post-hoc*, Figure 5.10 C).

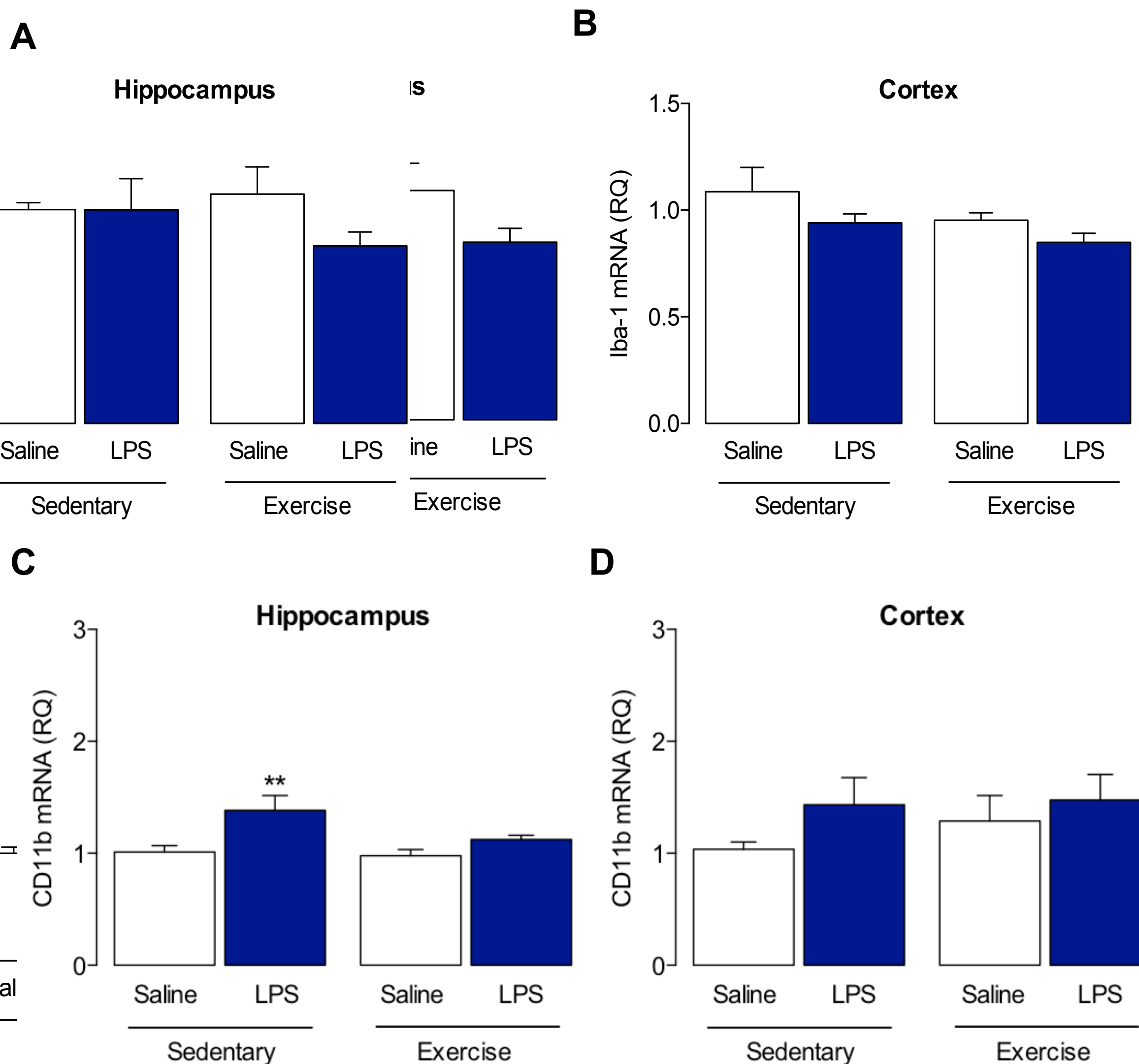


Figure 5.8 - Effect of LPS injection and prior exercise on mRNA expression of Iba-1 and CD11b in the hippocampus and cortex

Four hours after i.p. injection of LPS, hippocampal and cortical mRNA expression of Iba-1 was assessed by RT-PCR. In both hippocampus and cortex, the expression of Iba-1 was not affected either by LPS or prior exercise **(A, B)**. In hippocampus, an increase in mRNA expression of CD11B was observed only in SED mice injected with LPS and this was completely blocked by exercise **(C)**. In cortex, CS11b mRNA expression was not altered by LPS administration or prior exercise **(D)**. Data are presented as mean ± SEM (n=11 to 12). **p<0.01, Saline vs. LPS; two-way ANOVA with Bonferroni *post hoc* analysis.

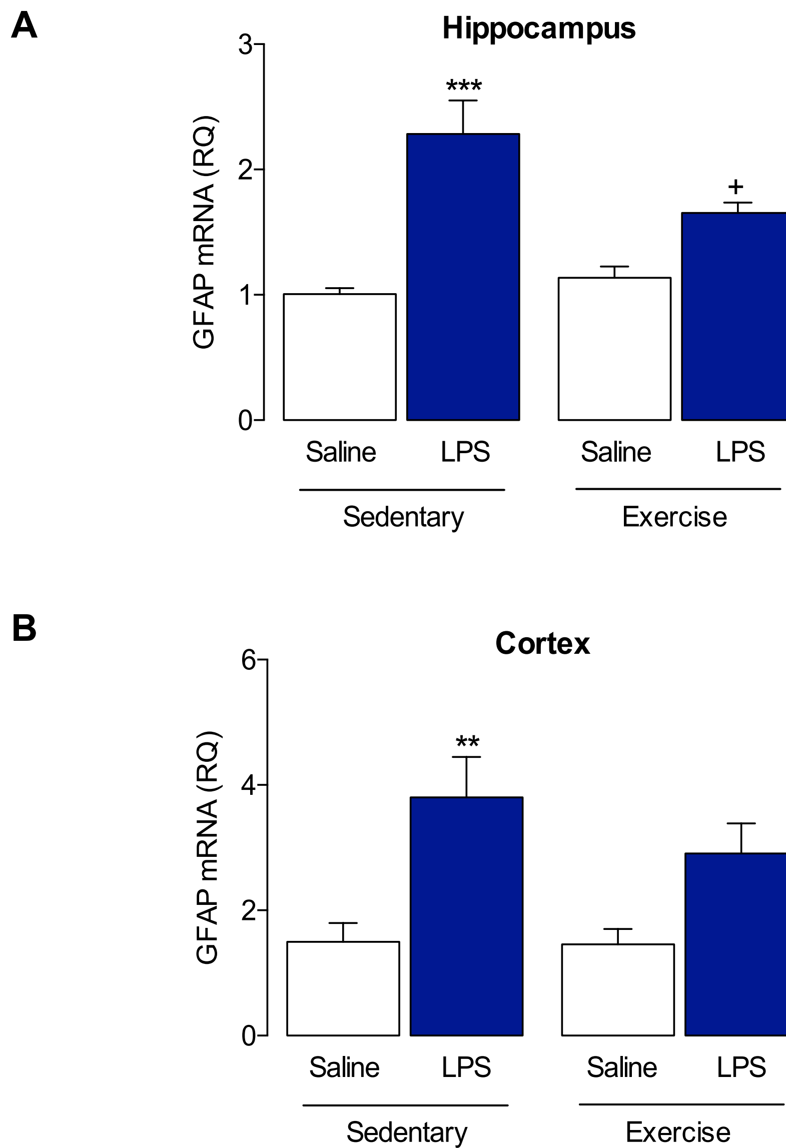


Figure 5.9- Effect of LPS injection and prior exercise on mRNA expression of GFAP in the hippocampus and cortex

Four hours after i.p. injection of LPS, hippocampal and cortical mRNA expression of GFAP was assessed by RT-PCR. In hippocampus, LPS injection induced an increase in the mRNA expression of GFAP in SED mice and this increase was completely blocked in EX mice **(A)**. In cortex, GFAP mRNA expression was up regulated only in SED-LPS injected mice **(B)**. Data are presented as mean ± SEM (n=11 to 12). ***$p<0.001$, Saline vs. LPS; **$p<0.01$, Saline vs. LPS; $^{+}p<0.05$, SED-LPS vs. EX-LPS; two-way ANOVA with Bonferroni *post hoc* analysis.

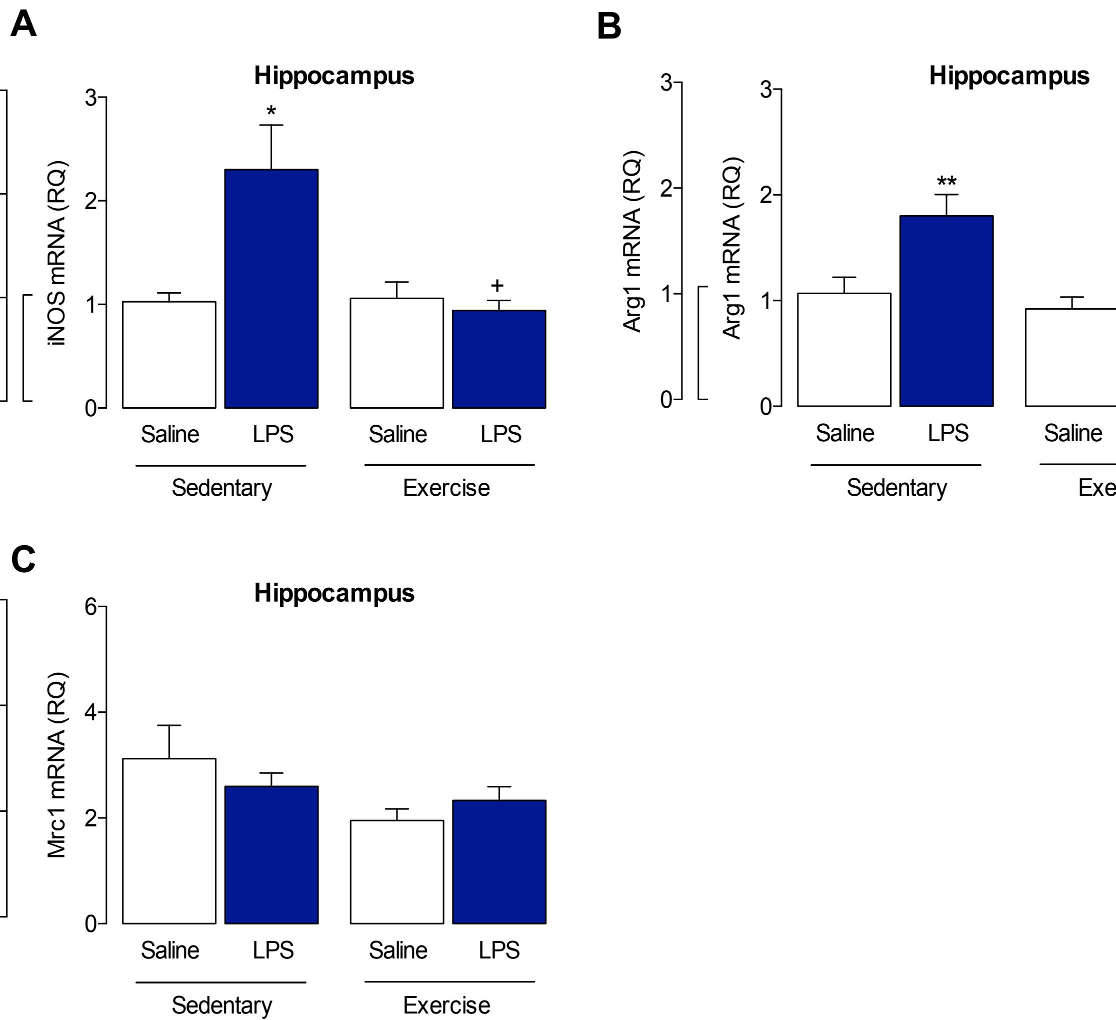


Figure 5.10 - Effect of LPS injection and prior exercise on mRNA expression of iNOS, Arg-1 and Mrc-1 in the hippocampus

Four hours after i.p. injection of LPS, hippocampal mRNA expression of iNOS, Arg-1 and Mrc1 was assessed by RT-PCR. An increase in mRNA expression of iNOS was observed only in SED-LPS mice and this effect was completely blocked by exercise **(A)**. mRNA expression of Arg-1 was upregulated in SED mice treated with LPS, but no effect of exercise was observed **(B)**. mRNA expression of Mrc1 was not altered in hippocampus, by LPS or prior exercise **(C)**. Data are presented as mean ± SEM (n=11 to 12). ***p<0.001, Saline vs. LPS; **p<0.01, Saline vs. LPS; ⁺p<0.05, SED-LPS vs. EX-LPS; two-way ANOVA with Bonferroni *post hoc* analysis.

5.3.5 Effects of LPS and exercise on mRNA expression of fractalkine and cell-surface glycoprotein CD44 in the hippocampus and cortex

We investigated whether LPS injection and prior short-term exercise modulate mRNA expression of Cx3cl1, Cx3cr1 and the adhesion molecule CD44 in the hippocampus and cortex. Neither LPS nor prior exercise affected mRNA expression of Cx3cl1 n the hippocampus ($p=0.3395$, two-way ANOVA, fold change SED-SAL: 1.00 ± 0.02; SED-LPS: 1.04 ± 0.12; EX-SAL: 1.03 ± 0.07; EX-LPS: 0.91 ± 0.04, Bonferroni post-hoc, Figure 5.11 A) and cortex ($p=0.9062$, two-way ANOVA, fold change SED-SAL: 0.96 ± 0.04; SED-LPS: 0.94 ± 0.04; EX-SAL: 0.94 ± 0.06; EX-LPS: 0.90 ± 0.06, Bonferroni post-hoc, Figure 5.11 B). Similarly, Cx3cr1 mRNA expression was not altered either by LPS or prior exercise in both regions, hippocampus ($p=0.4613$, two-way ANOVA, fold change SED-SAL: 1.02 ± 0.08; SED-LPS: 0.95 ± 0.09; EX-SAL: 0.94 ± 0.03; EX-LPS: 0.70 ± 0.05, Bonferroni post-hoc, Figure 5.11 C) and cortex ($p=0.5299$, two-way ANOVA, fold change SED-SAL: 0.88 ± 0.05; SED-LPS: 0.85 ± 0.05; EX-SAL: 0.89 ± 0.07; EX-LPS:0.78 ± 0.09, Bonferroni *post-hoc*, 5.11 D).

Moreover, statistical analysis demonstrated significant difference in mRNA expression of the cell surface glycoprotein CD44 in the hippocampus ($p<0.001$, two-way ANOVA, Figure 5.12 A). *Post-hoc* analysis revealed an increased in mRNA expression of CD44 in the hippocampus of SED-LPS mice ($p <0.001$ two-way ANOVA LPS vs. Saline, fold change SED-SAL: 1.08 ± 0.14; SED-LPS: 3.32 ± 0.46; EX-SAL: 0.92 ± 0.13; EX-LPS: 1.67 ± 0.14, Bonferroni *post-hoc*, Figure 5.12 A), while prior exercise blocked this increase ($p<0.001$, two-way ANOVA SED-LPS vs. EX-LPS, Figure 5.12 A). Similarly, in the cortex, significant difference was observed in mRNA expression of CD44 ($p<0.001$, two-way ANOVA, Figure 5.12 B). Bonferroni *post-hoc* analysis revealed an increased in expression of CD44 in the cortex of SED-LPS mice ($p<0.001$, two-way ANOVA LPS vs. Saline, fold change SED-SAL: 1.23 ± 0.25; SED-LPS: 3.65 ± 0.36; EX-SAL: 1.31 ± 0.16; EX-LPS: 2.71 ± 0.32, Figure 5.12 B) and this increase was attenuated by prior exercise ($p<0.001$, two-way ANOVA SED-LPS vs. EX-LPS, Figure 5.12 B).

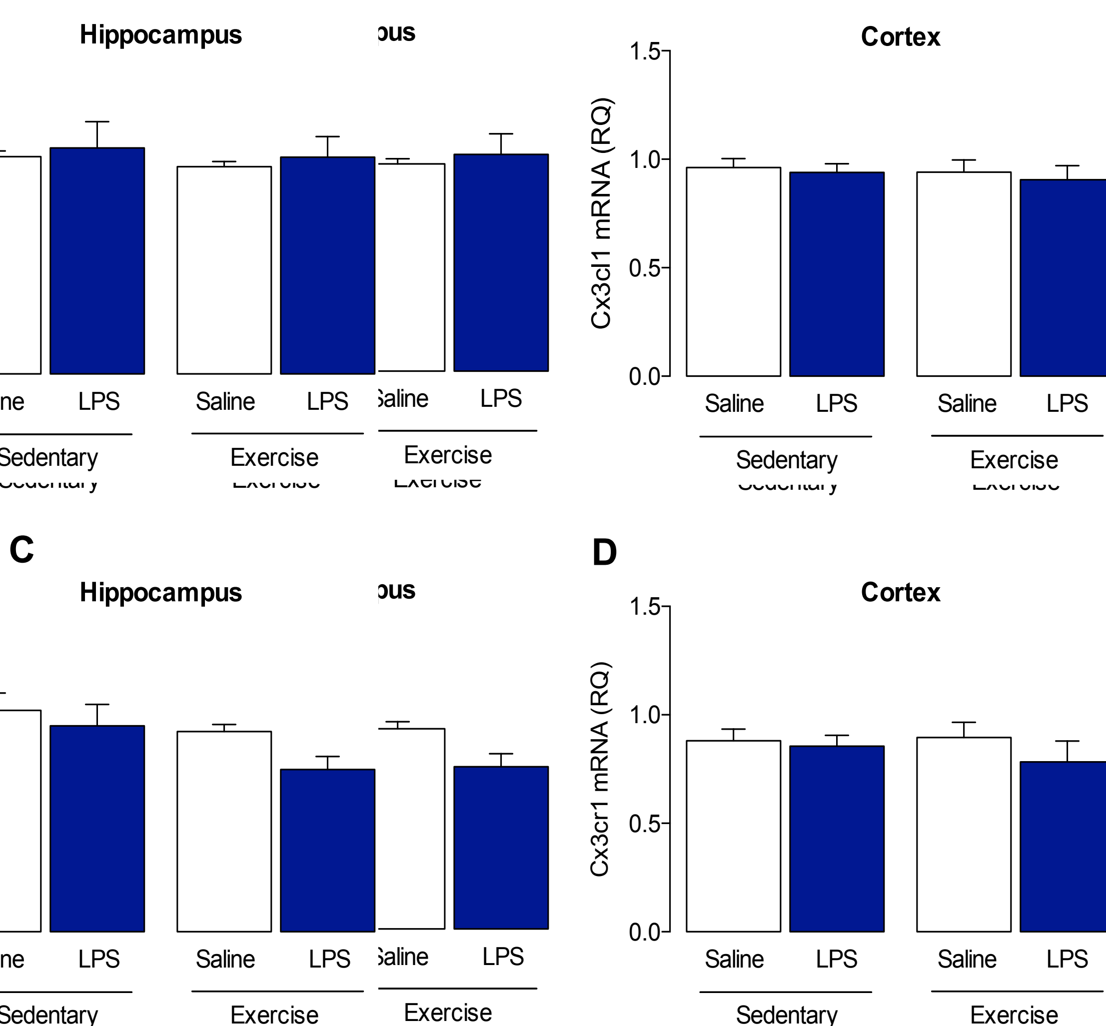


Figure 5.11 - Effect of LPS injection and prior exercise on mRNA expression of Cx3cl1 and Cx3cr1 in the hippocampus and cortex

Four hours after i.p. injection of LPS, hippocampal and cortical mRNA expression of Cx3cl1 was assessed by RT-PCR. In the hippocampus and cortex, mRNA expression of both Cx3cl1 and Cx3cr1 were not altered by LPS administration or by prior exercise **(A-D)**. Data are presented as mean ± SEM (n=11 to 12); two-way ANOVA with Bonferroni *post hoc* analysis.

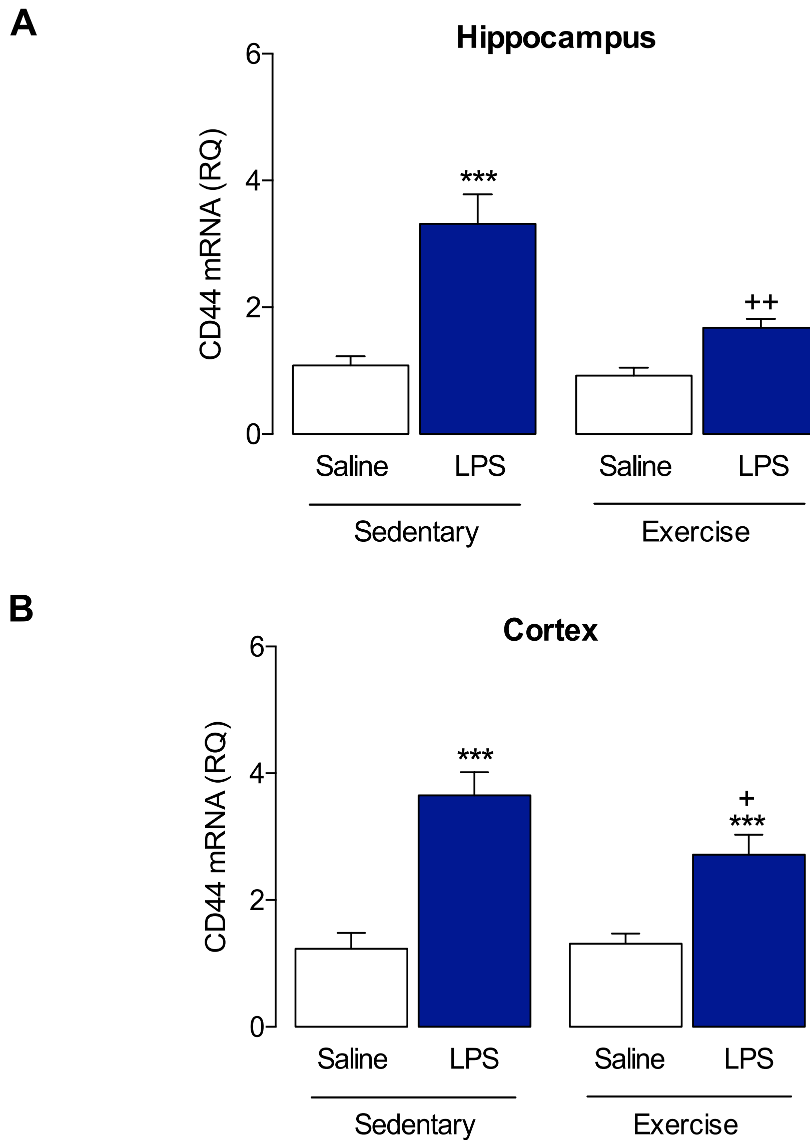


Figure 5.12 - Effect of LPS injection and prior exercise on mRNA expression of CD44 in the hippocampus and cortex

Four hours after i.p. injection of LPS, hippocampal and cortical mRNA expression of CD44 was assessed by RT-PCR. In hippocampus, mRNA expression of CD44 was increased in SED-LPS mice and this effect was blocked in EX-LPS mice **(A)**. In cortex, CD44 mRNA expression was upregulated of SED and EX mice treated with LPS and exercise attenuated this effect **(B)**. Data are presented as mean ± SEM (n=11 to 12). ***p<0.001, Saline vs. LPS; $^{+}$p<0.05, SED-LPS vs. EX-LPS; $^{++}$p<0.01, SED-LPS vs. EX-LPS; two-way ANOVA with Bonferroni *post hoc* analysis.

5.3.6 Effects of LPS and exercise on mRNA expression of neurotrophins, growth factors and markers of apoptosis in the hippocampus and cortex

Inflammation can affect the expression of neurotrophins and growth factors in the brain. Thus, having established the effects of LPS and prior exercise on mRNA expression of cytokines and glia cell markers, we decided to investigate whether LPS and/or short-term exercise would affect mRNA expression of several growth factors and neurotrophins in the hippocampus and cortex. In both regions, 4 hours following LPS injection, neither LPS nor prior exercise affected mRNA expression of BDNF (Hippocampus p=0.7982, fold change SED-SAL: 1.01±0.05; SED-LPS: 0.86±0.07; EX-SAL: 1.01±0.07; EX-LPS: 0.81±0.06, Figure 5.13 A; and Cortex p=0.8556, fold change SED-SAL: 0.86±0.09; SED-LPS: 0.59±0.08; EX-SAL: 0.83±0.08; EX-LPS: 0.59±0.06, two-way ANOVA, Bonferroni post-hoc, Figure 5.13 B), TrkB receptor (Hippocampus p=0.2208, fold change SED-SAL: 1.00±0.03; SED-LPS: 1.14±0.15; EX-SAL: 1.05±0.08; EX-LPS: 0.93±0.03, Figure 5.13 C; and Cortex p=0.4987, fold change SED-SAL: 1.35±0.11; SED-LPS: 1.61±0.09; EX-SAL: 1.18±0.07; SED-LPS: 1.07±0.11, two-way ANOVA, Bonferroni *post-hoc* Figure 5.13 D), p75 receptor (Hippocampus p=0.2510, fold change SED-SAL: 1.03±0.10; SED-LPS: 1.10±0.14; EX-SAL: 1.09±0.14; EX-LPS: 0.88±0.07, Figure 5.14 A; and Cortex p=0.3995, fold change SED-SAL: 2.02±0.47; SED-LPS: 2.67±0.58; EX-SAL: 3.68±0.65; EX-LPS: 3.12±0.94, two-way ANOVA, Bonferroni *post-hoc*, Figure 5.14 B), NGF (Hippocampus p=0.8270, fold change SED-SAL: 1.00±0.05; SED-LPS: 0.91±0.03; EX-SAL: 0.98±0.04; SED-LPS: 0.90±0.03, Figure 5.14 C; and Cortex p=0.4284, fold change SED-SAL: 1.12±0.34; SED-LPS: 0.99±0.13; EX-SAL: 0.88±0.06; EX-LPS: 1.12±0.21, two-way ANOVA, Figure 5.14 D), Igf-1 (Hippocampus p=0.7961, fold change SED-SAL: 1.02±0.07; SED-LPS: 0.92±0.07; EX-SAL: 1.08±0.22; EX-LPS: 0.90±0.09, Figure 5.15 A; and Cortex p=0.8254, fold change SED-SAL: 1.61±0.35; SED-LPS: 1.19±0.13; EX-SAL: 1.46±0.23; EX-LPS: 1.15±0.20, two-way ANOVA, Bonferroni *post-hoc*, Figure 5.15 B), or VEGFA (Hippocampus p= 0.1028, fold change SED-SAL: 1.03±0.10; SED-LPS: 1.08±0.17; EX-SAL: 1.23±0.17; EX-LPS: 0.80±0.04, Figure 5.15 C; and Cortex p=0.7544, fold change SED-SAL: 1.01±0.13; SED-LPS: 0.85±0.06; EX-

SAL: 1.05±0.09; EX-LPS: 0.95±0.15, two-way ANOVA, Bonferroni *post-hoc*,Figure 5.15 D).

Furthermore, it has been shown that pro-inflammatory cytokines can modulate proteins that regulate apoptosis. For this reason, we also decided to address whether LPS injection and prior physical exercise would alter the expression of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2. However, 4 hours after LPS injection, mRNA expression of Bax proteins was unaffected in the hippocampus ($p=0.0587$, two-way ANOVA, fold change SED-SAL: 1.01±0.06; SED-LPS: 1.12±0.11; EX-SAL: 1.07±0.05; EX-LPS: 0.89±0.04, Bonferroni post-hoc, Figure 5.16 A) and cortex ($p=0.5886$, two-way ANOVA, SED-SAL: 1.38±0.21; SED-LPS: 1.32±0.11; EX-SAL: 1.34±0.11; EX-LPS: 1.25±0.08, Bonferroni *post-hoc*, Figure 5.16 B). Similarly, no changes in mRNA expression of Bcl-2 was observed in the hippocampus ($p=0.7462$, two-way ANOVA, fold change SED-SAL: 1.03±0.11; SED-LPS: 0.90±0.08; EX-SAL: 0.88±0.05; EX-LPS: 0.80±0.05, Bonferroni post-hoc, Figure 5.16 C) and cortex ($p=0.7707$, two-way ANOVA, fold change SED-SAL: 1.39±0.31; SED-LPS: 1.23±0.12; EX-SAL: 1.27±0.15; EX-LPS: 1.24±0.25, Bonferroni *post-hoc*, Figure 5.16 D). Also, there was no effect of prior exercise in the mRNA expression of Bax and Bcl-2 in either brain region analysed.

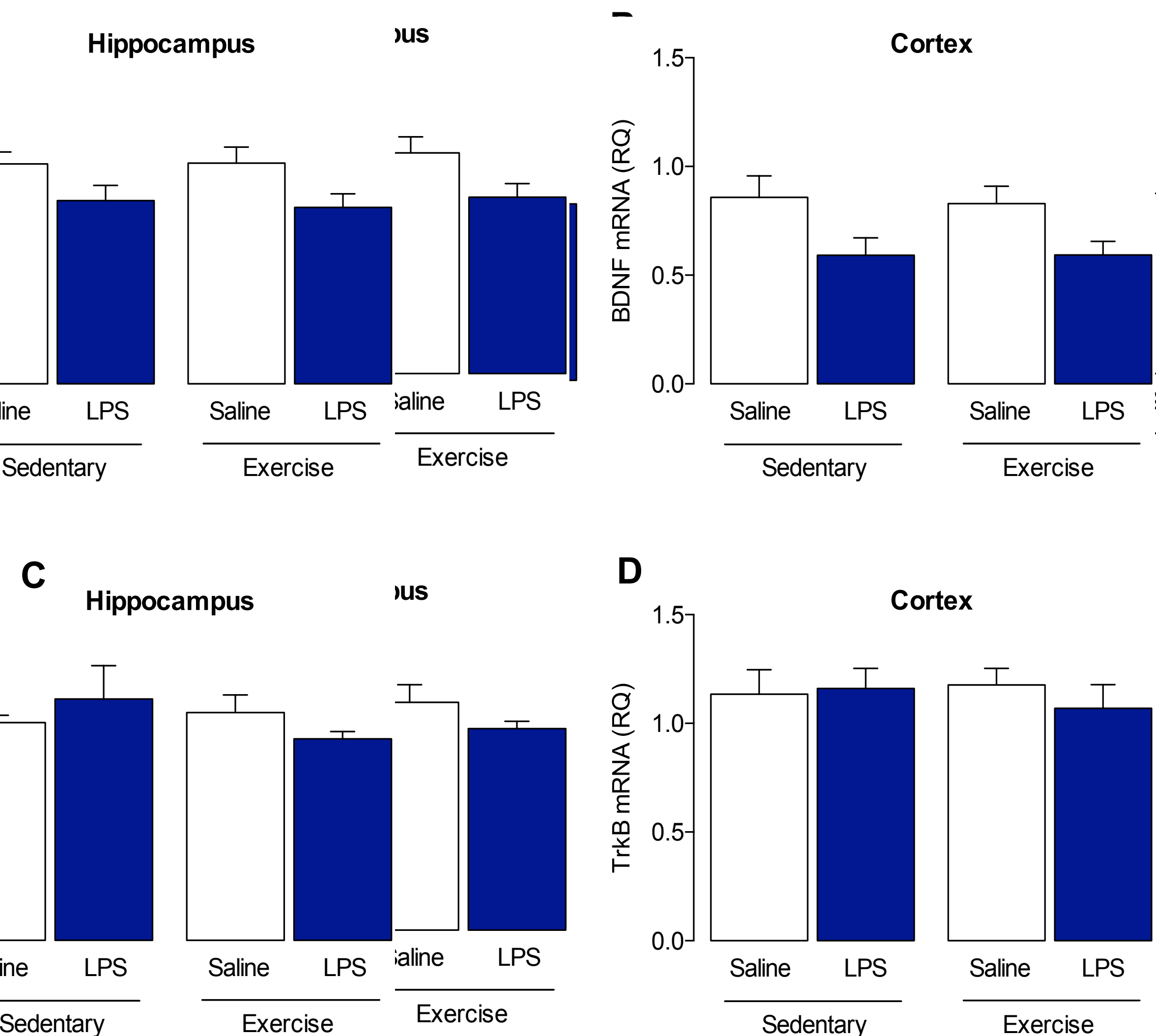


Figure 5.13 - Effect of LPS injection and prior exercise on mRNA expression of BDNF and TrkB receptor in the hippocampus and cortex

Four hours after i.p. injection of LPS, hippocampal and cortical mRNA expression of BDNF and TrkB receptor was assessed by RT-PCR. In both hippocampus and cortex, there was no difference in mRNA expression of BDNF after LPS injection or prior exercise **(A, B)**. Similarly, neither LPS injection nor prior short-term exercise affected mRNA expression of TrkB receptor in hippocampus and cortex **(C, D)**. Data are presented as mean ± SEM (n=11 to 12), two-way ANOVA with Bonferroni *post hoc* analysis.

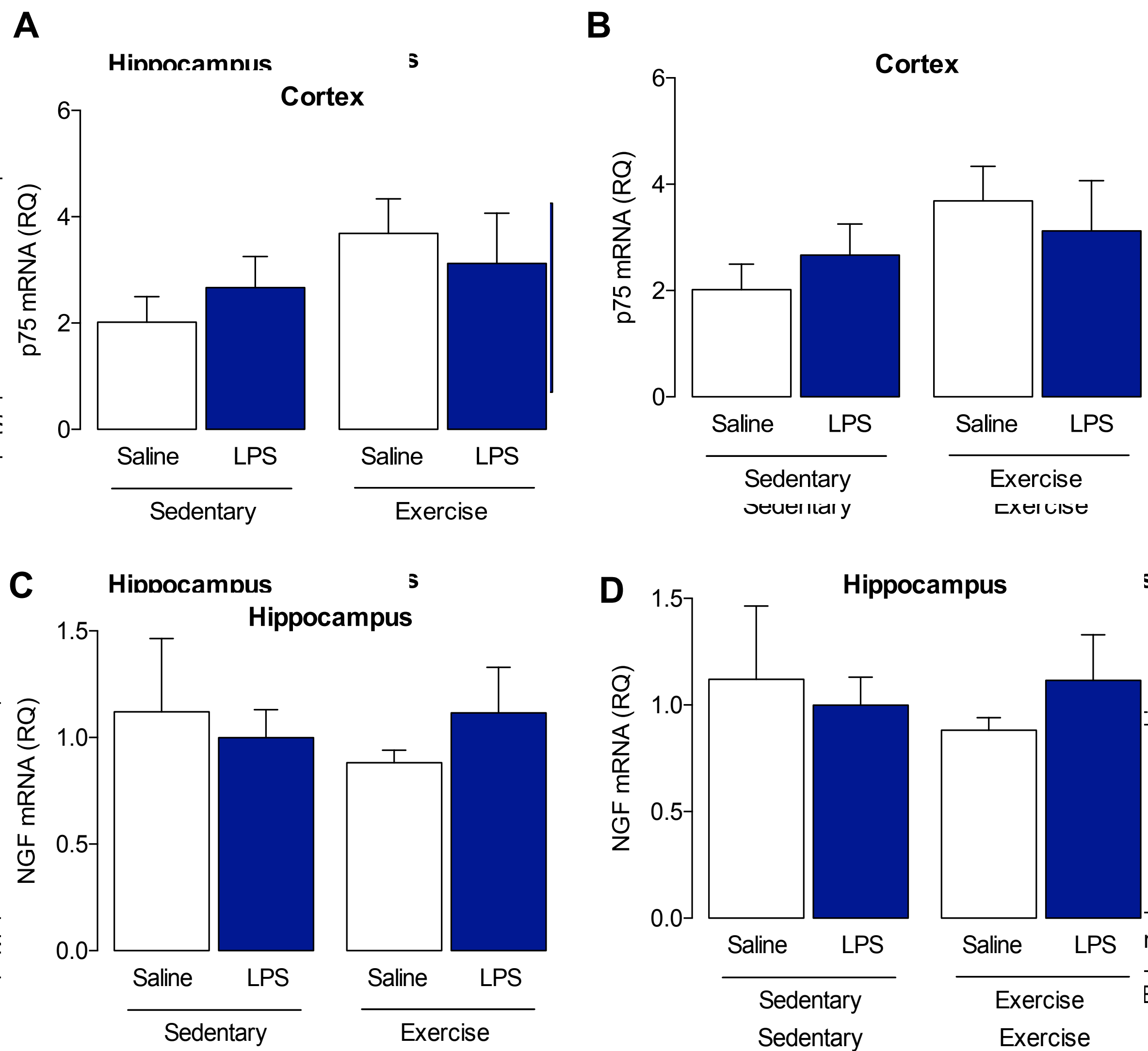
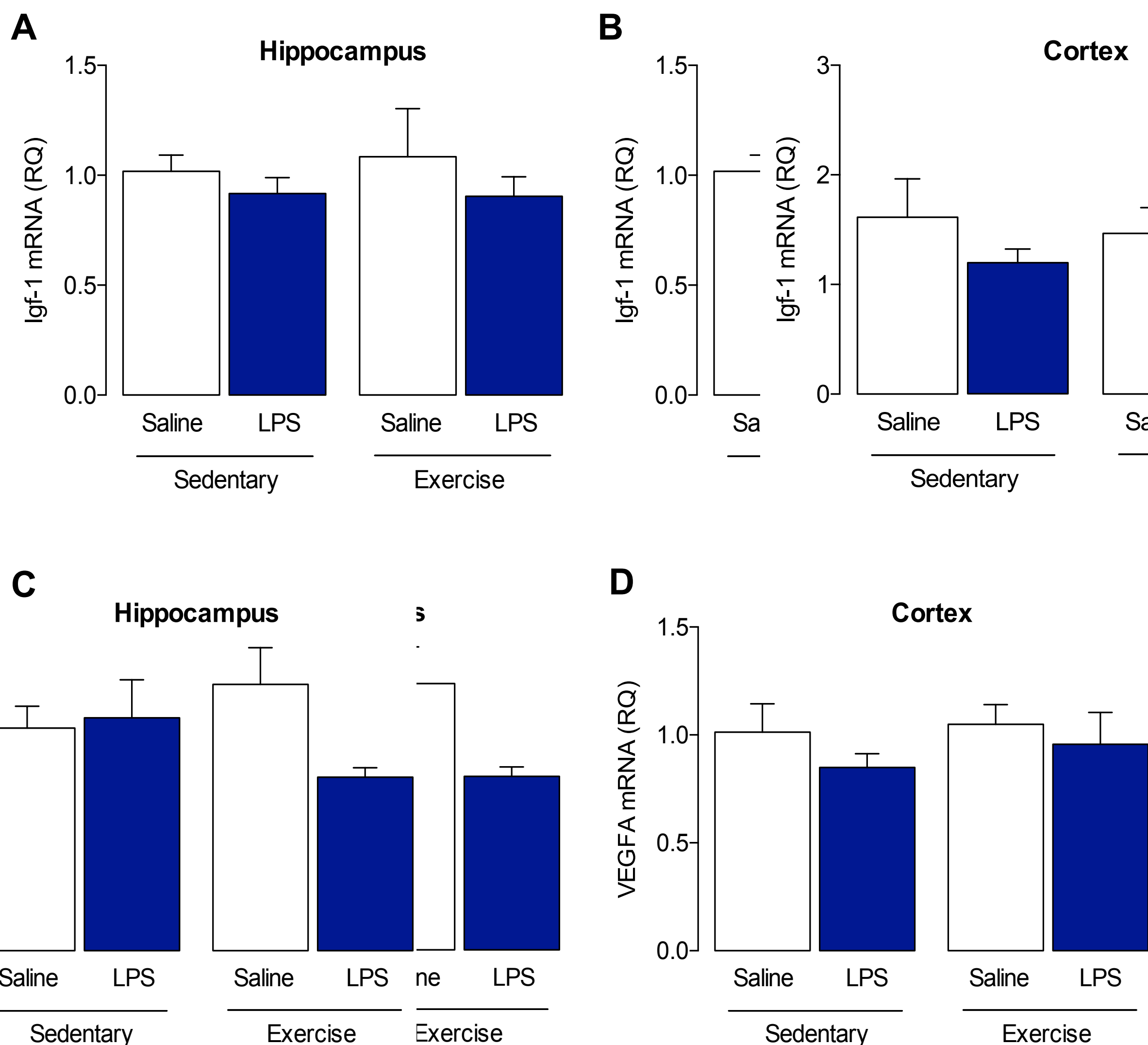
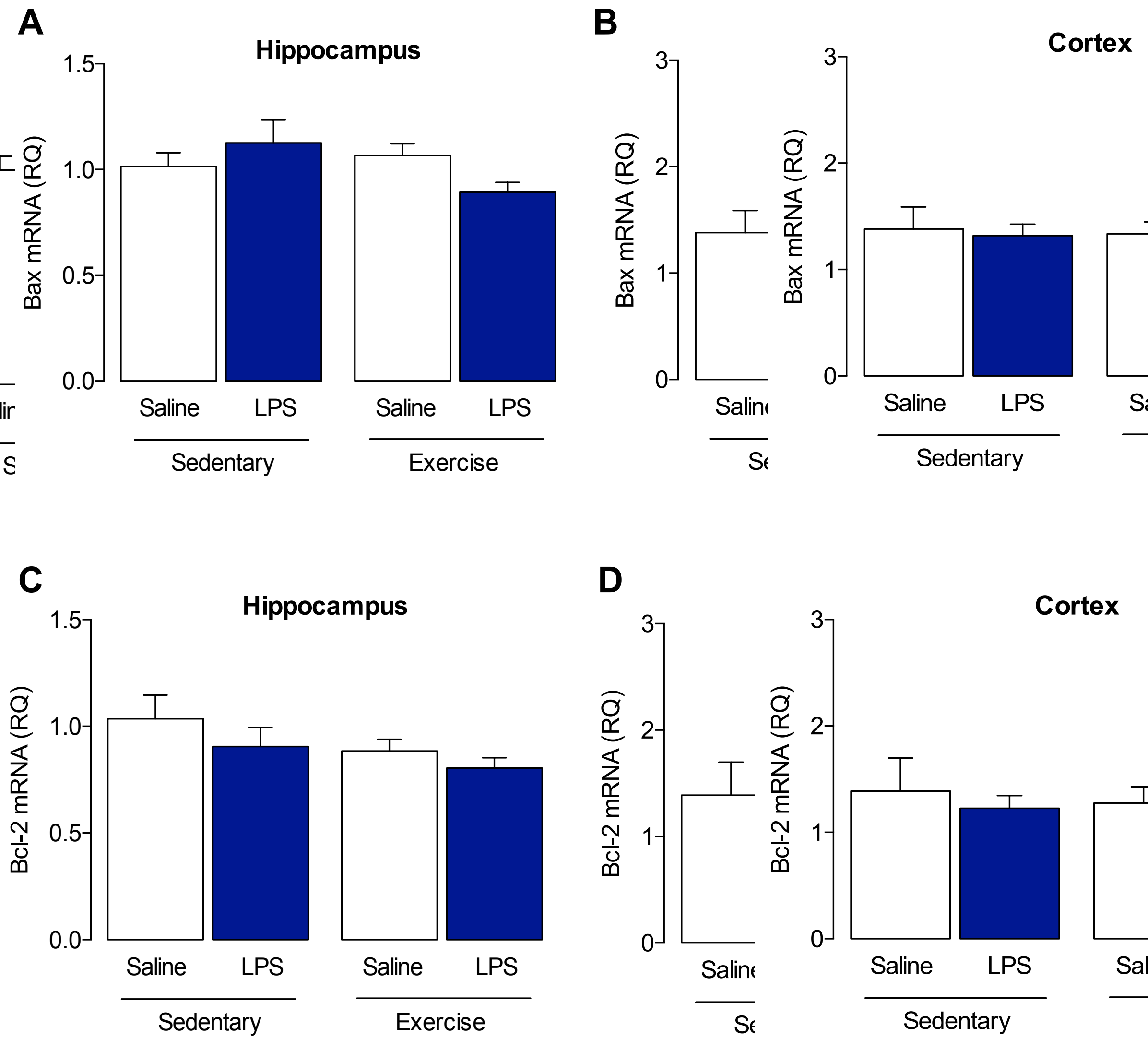


Figure 5.14 - Effect of LPS injection and prior exercise on mRNA expression of p75 receptor and NGF in the hippocampus and cortex

Four hours after i.p. injection of LPS, hippocampal and cortical mRNA expression of p75 receptor and NGF was assessed by RT-PCR. Hippocampal and cortical mRNA expression of p75 receptor was not altered by LPS or by prior exercise **(A, B)**. Similarly, neither LPS injection nor prior short-term exercise affected mRNA expression of NGF in hippocampus and cortex **(C, D)**. Data are presented as mean ± SEM (n=11 to 12), two-way ANOVA with Bonferroni *post hoc* analysis.



A
Hippocampus
Igf-1 mRNA (RQ)
Saline
LPS
Sedentary
Exercise
B
Cortex
Igf-1 mRNA (RQ)
C
Hippocampus
VEGFA mRNA (RQ)
D
Cortex
VEGFA mRNA (RQ)



A
Hippocampus
Bax mRNA (RQ)
1.5
1.0
0.5
0.0
Saline
LPS
Saline
LPS
Sedentary
Exercise
B
Cortex
Bax mRNA (RQ)
3
2
1
0
Saline
LPS
Saline
LPS
Sedentary
Exercise
C
Hippocampus
Bcl-2 mRNA (RQ)
1.5
1.0
0.5
0.0
Saline
LPS
Saline
LPS
Sedentary
Exercise
D
Cortex
Bcl-2 mRNA (RQ)
3
2
1
0
Saline
LPS
Saline
LPS
Sedentary
Exercise

5.3.7 Effects of LPS and exercise on protein expression of TrkB, Akt, MAPK ERK1/2, CREB and NF- κB in the hippocampus and cortex

We have demonstrated that neither LPS nor prior short-term exercise affected mRNA expression of neurotrophins and growth factors. Therefore, we decided to analyse further whether a single LPS challenge and short-term prior physical exercise would modulate the protein expression TrkB receptor and BDNF downstream activation of Akt, MAPK-ERK1/2 and CREB, in the hippocampus and cortex of mice. Accordingly, hippocampal and cortical samples were analysed by western blot. Similar to the PCR results observed in the mRNA expression of TrkB receptors, there was no difference between the groups in the total and phosphorylated protein expression of TrkB receptor in hippocampus (Total TrkB p=0.2528; pTrkB p=0.9875; pTrkB/TrkB p=0.7881, two-way ANOVA Bonferroni *post-hoc*, Figure 5.17 A-C) and cortex (Total TrkB p=0.4141; pTrkB p=0.8331; pTrkB/TrkB p=0.7152, two-way ANOVA Bonferroni *post-hoc*, Figure 5.18 A-C).

In addition, total and phosphorylated protein content of Akt in the hippocampus (Total Akt p=0.7410; pAkt p=0.9314; pAkt/Akt p=0.5931, two-way ANOVA, Bonferroni *post-hoc*, Figure 5.19 A-C) and cortex (Total Akt p=0.4479; pAkt p=0.3787; pAkt/Akt p=0.9352, two-way ANOVA, Bonferroni *post-hoc*, Figure 5.20 A-C) were not altered either by LPS or short-term prior exercise. Also, no changes were observed in mRNA expression of MAPK-ERK1/2 in the hippocampus (Total ERK1/2 p=0.3036; pERK1/2 p=0.8993; pERK/ERK p=0.3781, two-way ANOVA, Figure 5.21 A-C) and cortex (Total ERK1/2 p=0.7018; pERK1/2 p=0.7793; pERK/ERK p=0.9225, two-way ANOVA, Bonferroni *post-hoc*, Figure 5.22 A-C).

Moreover, similarly, to the results described above, neither LPS nor prior exercise affected total and phosphorylated protein levels of the cellular transcription factor CREB, in both regions, hippocampus (Total CREB p=0.4137; pCREB p=02852; pCREB/CREB p=0.8232, two-way ANOVA, Bonferroni *post-hoc*, Figure 5.23 A-C) and cortex (Total CREB p=0.9302; pCREB p=0.8135; pCREB/CREB p=0.7448, two-way ANOVA, Bonferroni *post-hoc*, Figure 5.24 A-C). Finally, as NF-κB is a pivotal mediator of the inflammatory response, we decided to investigate the effects of LPS and prior short-term exercise on protein expression of NF-κB in

hippocampus of mice. Although a trend for an increased ratio of phosphorylated/total NF-κB protein expression was observed in EX-LPS mice, no significant difference was observed between the groups in the expression of total and phosphorylated content of NF-κB in the hippocampus (Total NF-κB $p=0.4132$; pNF-κB $p=0.8997$; pNF-κB/NF-κB $p=0.1606$, two-way ANOVA, Bonferroni *post-hoc*, Figure 5.25 A-C)).

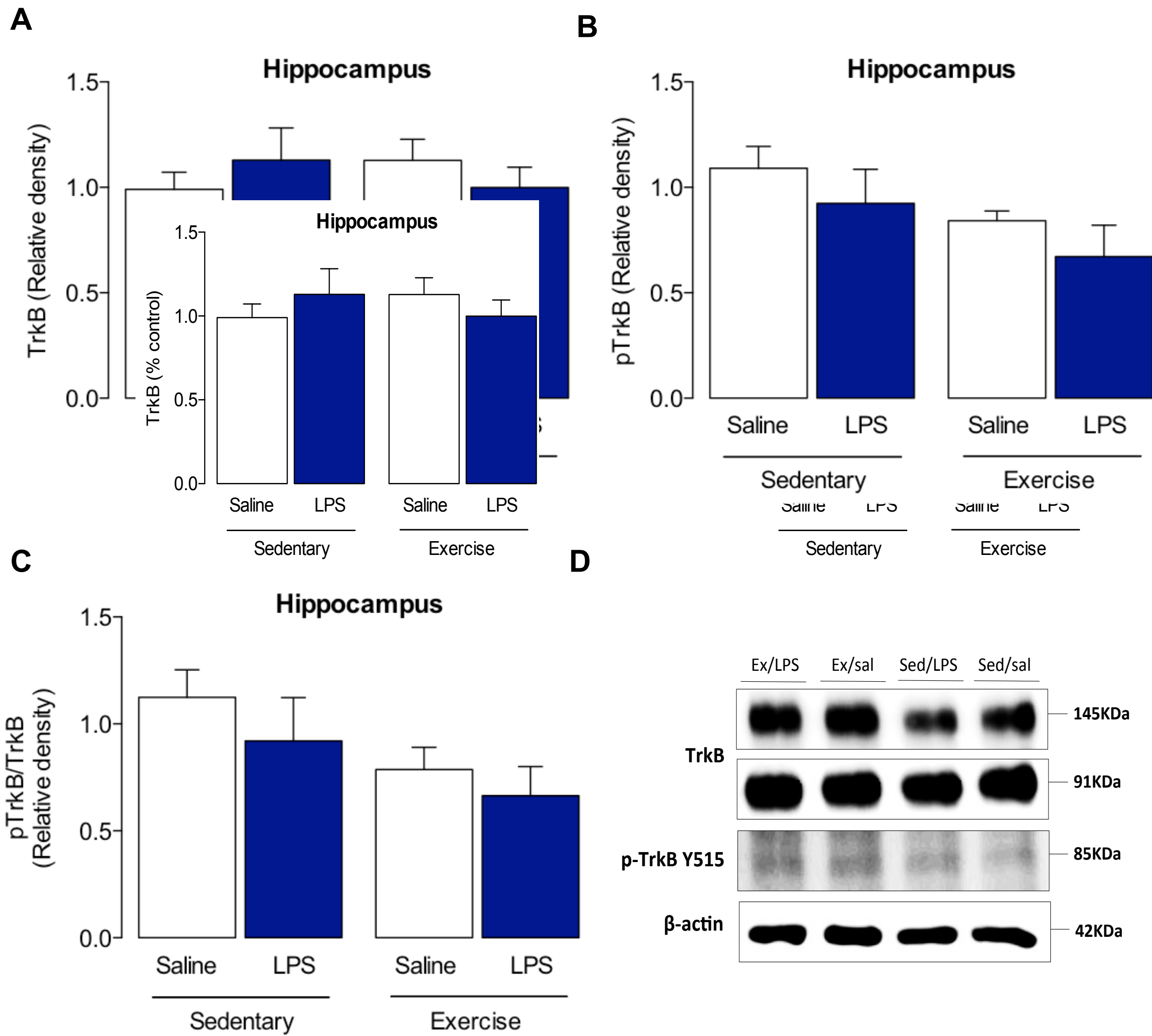


Figure 5.17 - Effect of LPS injection and prior exercise on TrkB receptor protein expression in hippocampus

Protein expression of total and phosphorylated TrkB receptor, in hippocampus, was measured using Western Blot. Neither LPS nor prior exercise affected the expression of TrkB receptor in hippocampus **(A)**. Phosphorylated TrkB receptor and the ratio of phosphorylated TrkB by total TrkB protein content were not affected either by LPS or prior exercise **(B, C)**. Representative blots of total TrkB, phosphorylated TrkB (Y515) and β-Actin in hippocampus **(D)**. β-Actin was used as loading control of protein content. Data are expressed as relative density and presented as mean ± SEM (n=5 to 6), two-way ANOVA with Bonferroni *Post hoc* analysis.

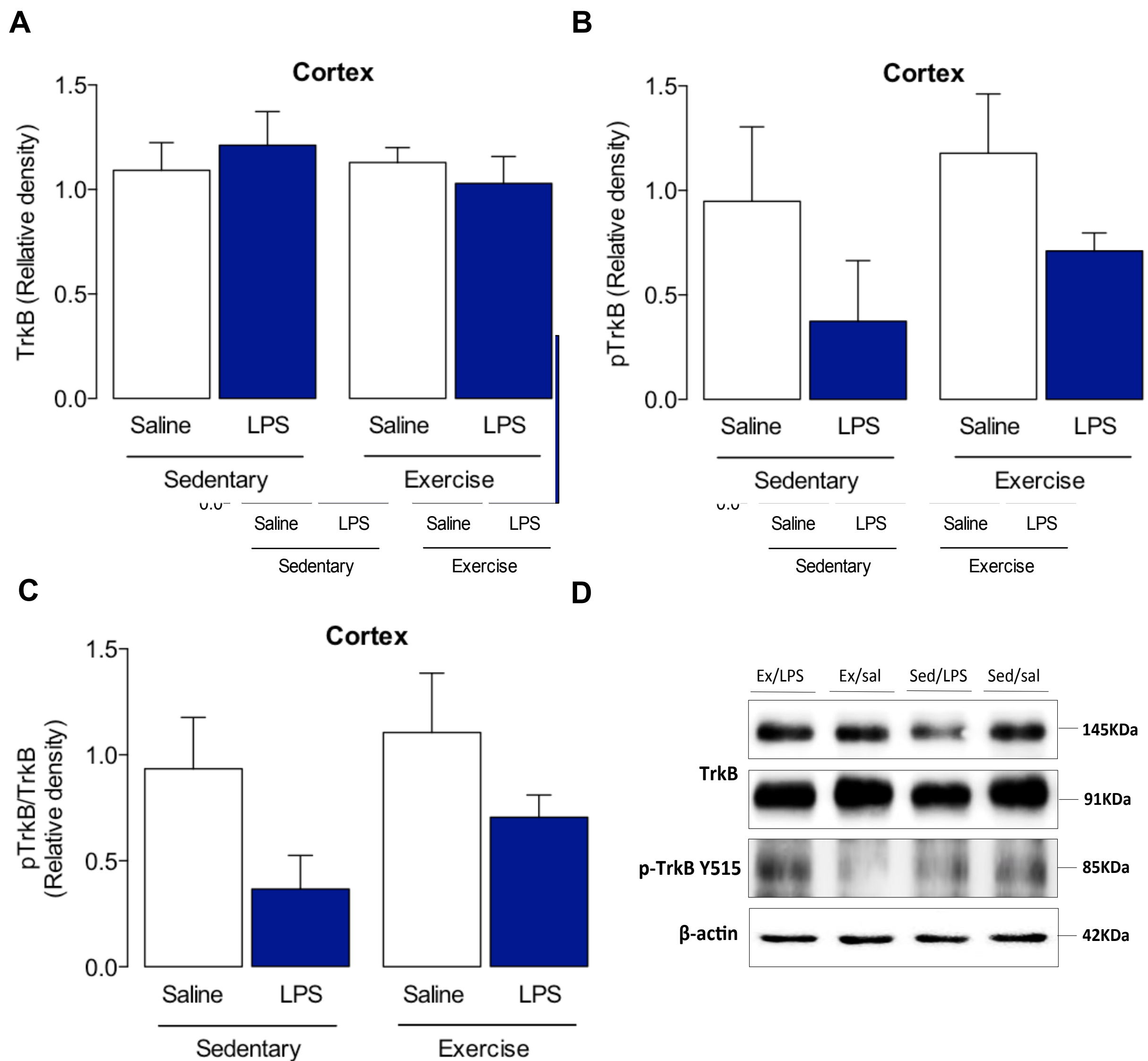


Figure 5.18 - Effect of LPS injection and prior exercise on TrkB receptor protein expression in cortex

Protein expression of total and phosphorylated TrkB receptor, in cortex, was measured using Western Blot. Neither LPS nor prior exercise affected the expression of TrkB receptor in cortex **(A)**. Phosphorylated TrkB receptor and the ratio of phosphorylated TrkB by total TrkB protein content were not affected either by LPS or prior exercise **(B, C)**. Representative blots of total TrkB, phosphorylated TrkB (Y515) and β-Actin in cortex **(D)**. β-Actin was used as loading control of protein content. Data are expressed as relative density and presented as mean ± SEM (n=5 to 6), two-way ANOVA with Bonferroni *Post hoc* analysis.

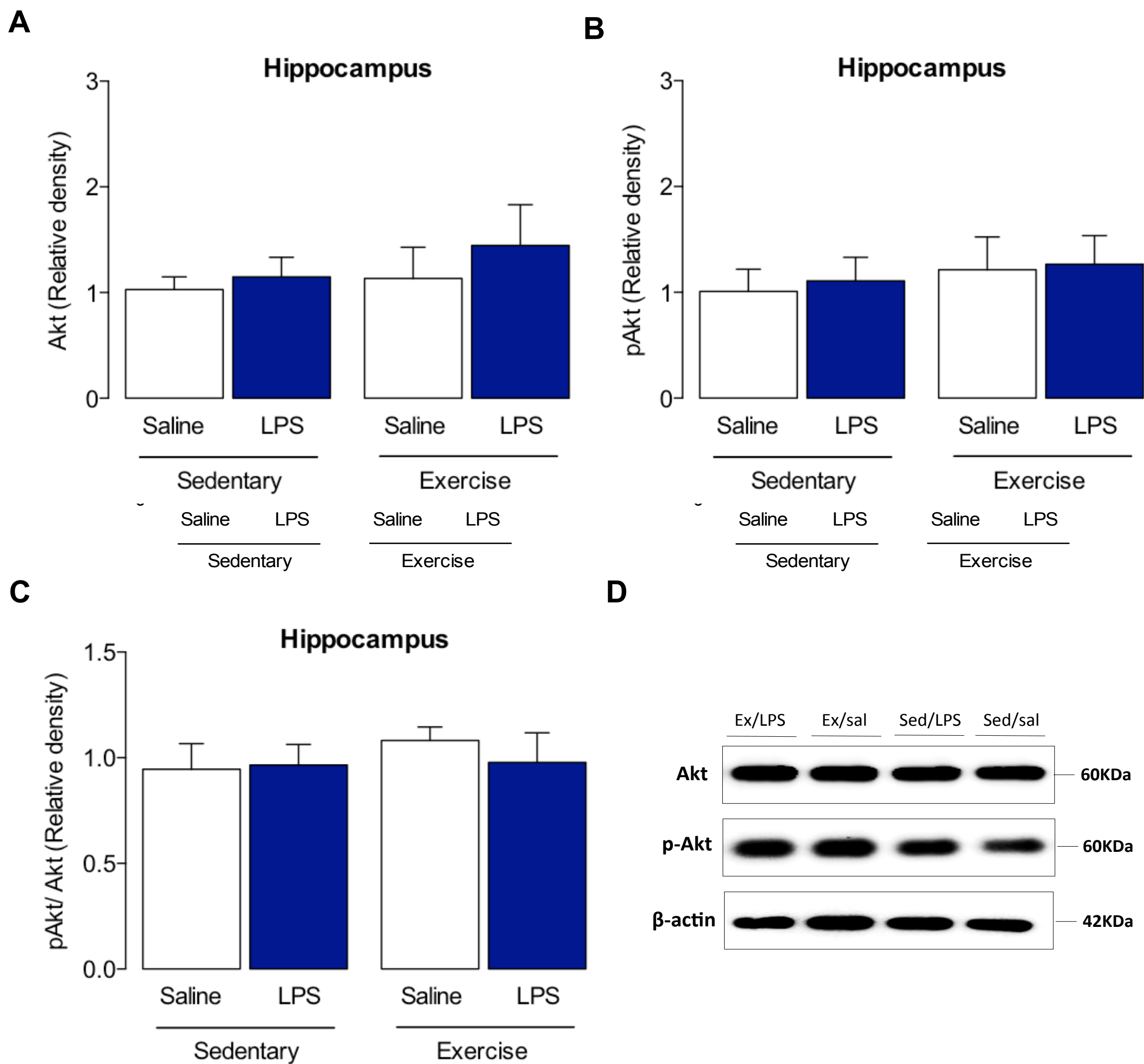


Figure 5.19 - Effect of LPS injection and prior exercise on Akt protein expression in hippocampus

Protein expression of total and phosphorylated Akt, in hippocampus, was measured using Western Blot. Neither LPS nor prior exercise affected the expression of Akt in hippocampus **(A)**. Phosphorylated Akt and the ratio of phosphorylated Akt by total Akt protein content were not affected either by LPS or prior exercise **(B, C)**. Representative blots of total Akt, phosphorylated Akt and β-Actin in hippocampus **(D)**. β-Actin was used as loading control of protein content. Data are expressed as relative density and presented as mean ± SEM (n=5 to 6), two-way ANOVA with Bonferroni *Post hoc* analysis.

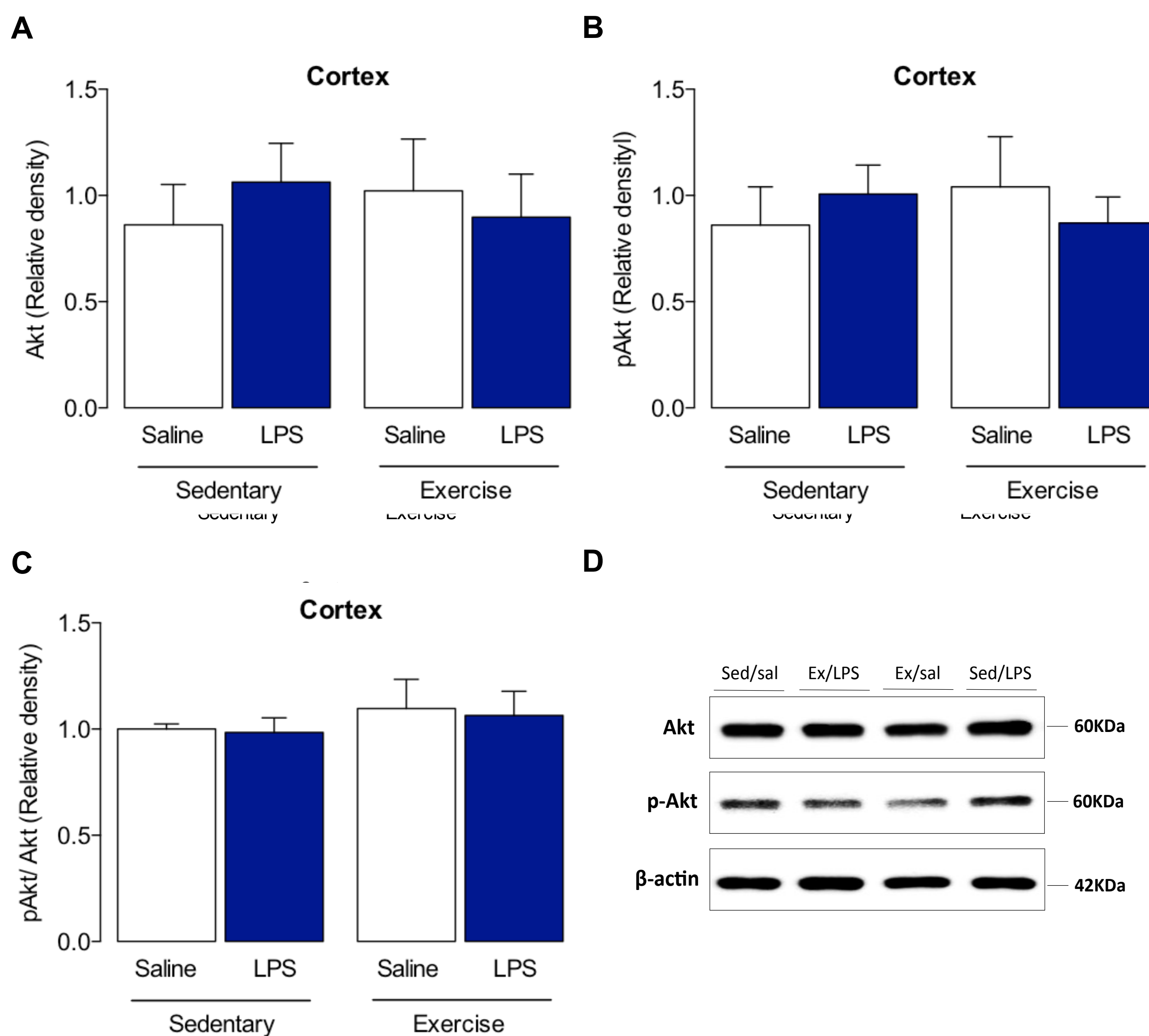


Figure 5.20 - Effect of LPS injection and prior exercise on Akt protein expression in cortex Protein expression of total and phosphorylated Akt, in cortex, was measured using Western Blot. Neither LPS nor prior exercise affected the expression of Akt in cortex **(A)**. Phosphorylated Akt and the ratio of phosphorylated Akt by total Akt protein content were not affected either by LPS or prior exercise **(B, C)**. Representative blots of total Akt, phosphorylated Akt and β-Actin in cortex **(D)**. β-Actin was used as loading control of protein content. Data are expressed as relative density and presented as mean ± SEM (n=5 to 6), two-way ANOVA with Bonferroni *Post hoc* analysis.

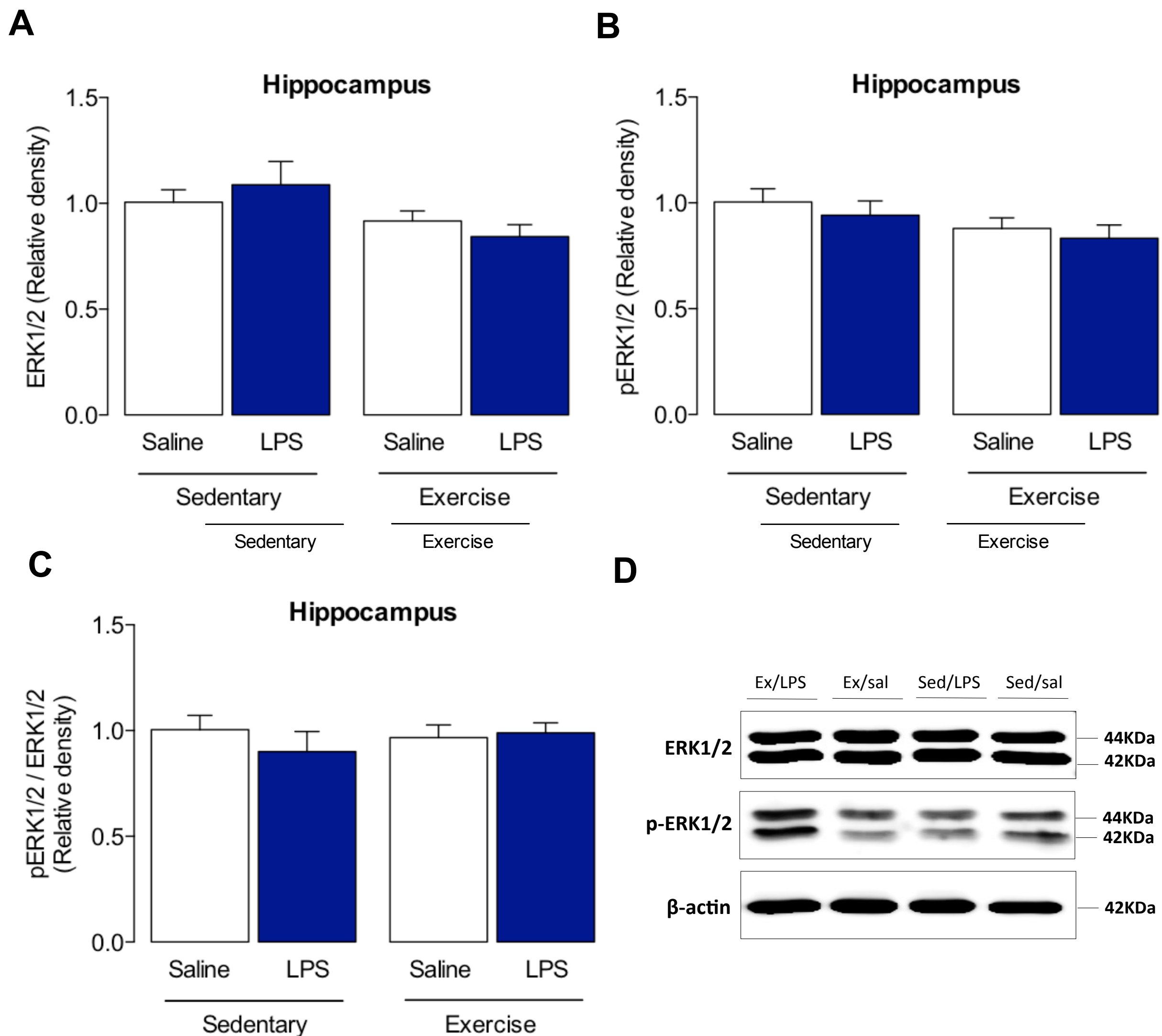


Figure 5.21 - Effect of LPS injection and prior exercise on ERK1/2 protein expression in hippocampus

Protein expression of total and phosphorylated ERK1/2, in hippocampus, was measured using Western Blot. Neither LPS nor prior exercise affected the expression of ERK1/2 in hippocampus **(A)**. Phosphorylated ERK1/2 and the ratio of phosphorylated ERK1/2 by total ERK1/2 protein content were not affected either by LPS or prior exercise **(B, C)**. Representative blots of total ERK1/2, phosphorylated ERK1/2 and β-Actin in hippocampus **(D)**. β-Actin was used as loading control of protein content. Data are expressed as relative density and presented as mean ± SEM (n=5 to 6), two-way ANOVA with Bonferroni *Post hoc* analysis.

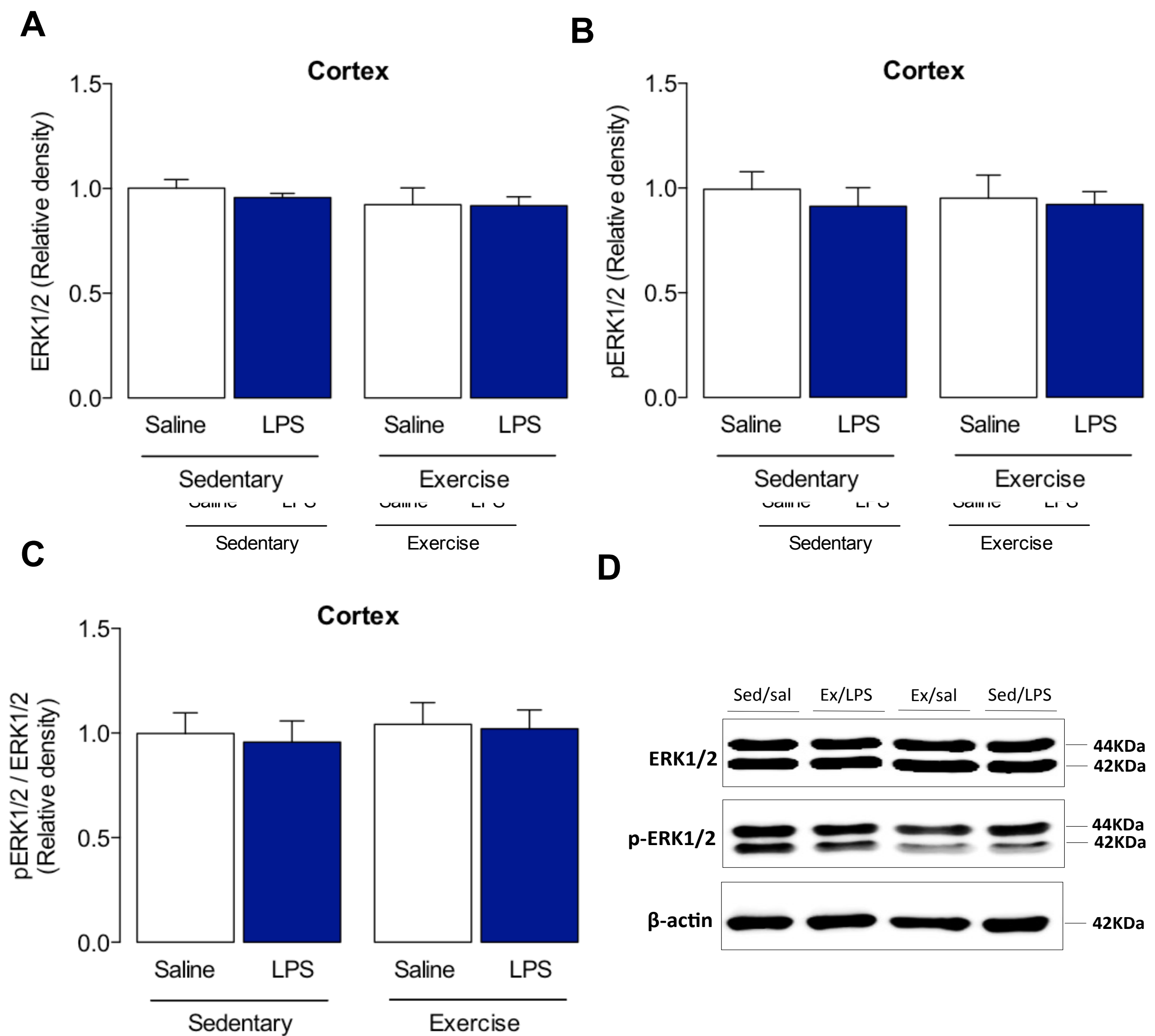


Figure 5.22 - Effect of LPS injection and prior exercise on ERK1/2 protein expression in cortex

Protein expression of total and phosphorylated ERK1/2, in cortex, was measured using Western Blot. Neither LPS nor prior exercise affected the expression of ERK1/2 in cortex **(A)**. Phosphorylated ERK1/2 and the ratio of phosphorylated ERK1/2 by total ERK1/2 protein content were not affected either by LPS or prior exercise **(B, C)**. Representative blots of total ERK1/2, phosphorylated ERK1/2 and β-Actin in cortex **(D)**. β-Actin was used as loading control of protein content. Data are expressed as relative density and presented as mean ± SEM (n=5 to 6), two-way ANOVA with Bonferroni *Post hoc* analysis.

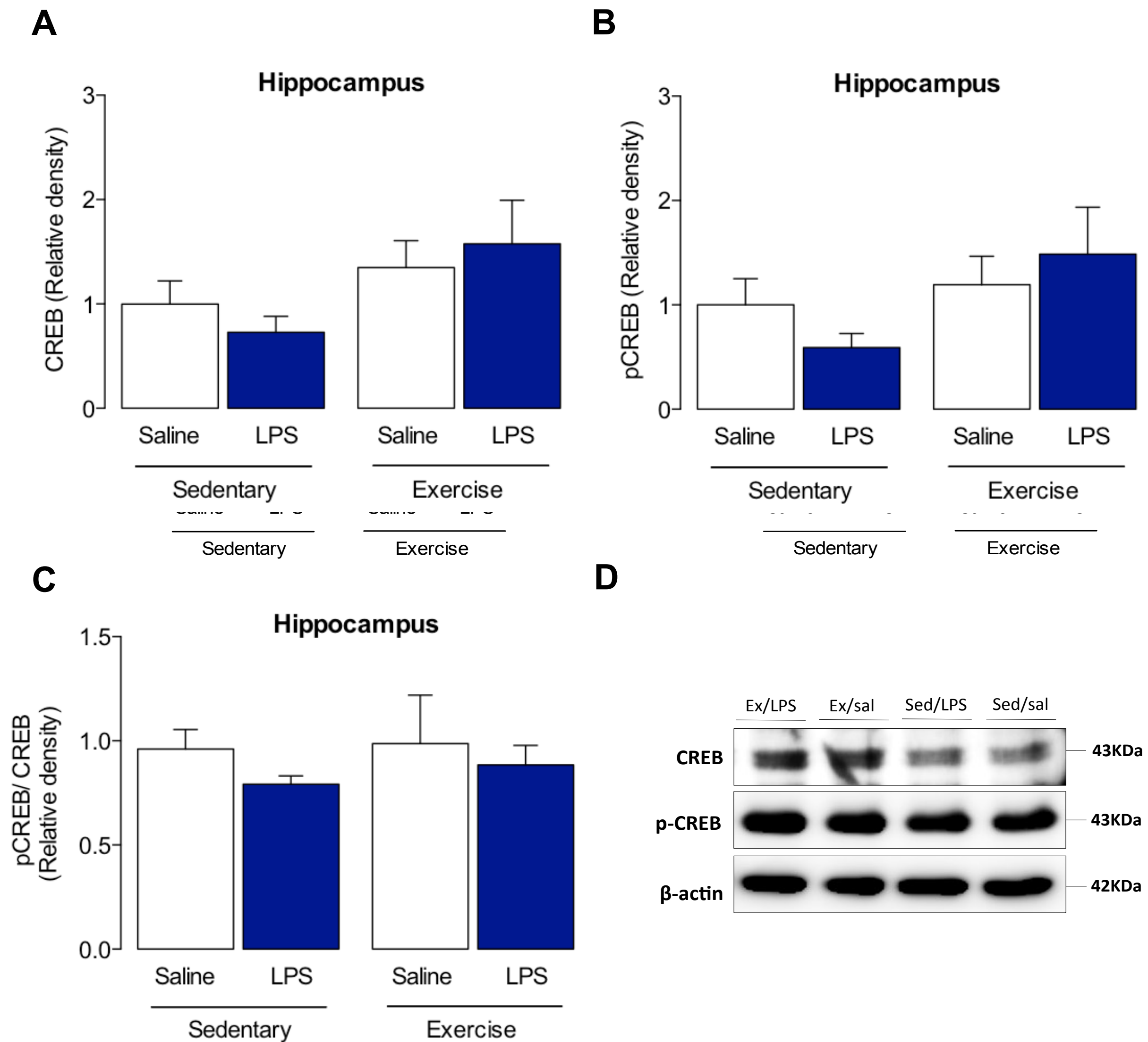


Figure 5.23 - Effect of LPS injection and prior exercise on CREB protein expression in hippocampus

Protein expression of total and phosphorylated CREB, in hippocampus, was measured using Western Blot. Neither LPS nor prior exercise affected the expression of CREB in hippocampus **(A)**. Phosphorylated CREB and the ratio of phosphorylated CREB by total CREB protein content were not affected either by LPS or prior exercise **(B, C)**. Representative blots of total CREB, phosphorylated CREB and β-Actin in hippocampus **(D)**. β-Actin was used as loading control of protein content. Data are expressed as relative density and presented as mean ± SEM (n=5 to 6), two-way ANOVA with Bonferroni *Post hoc* analysis.

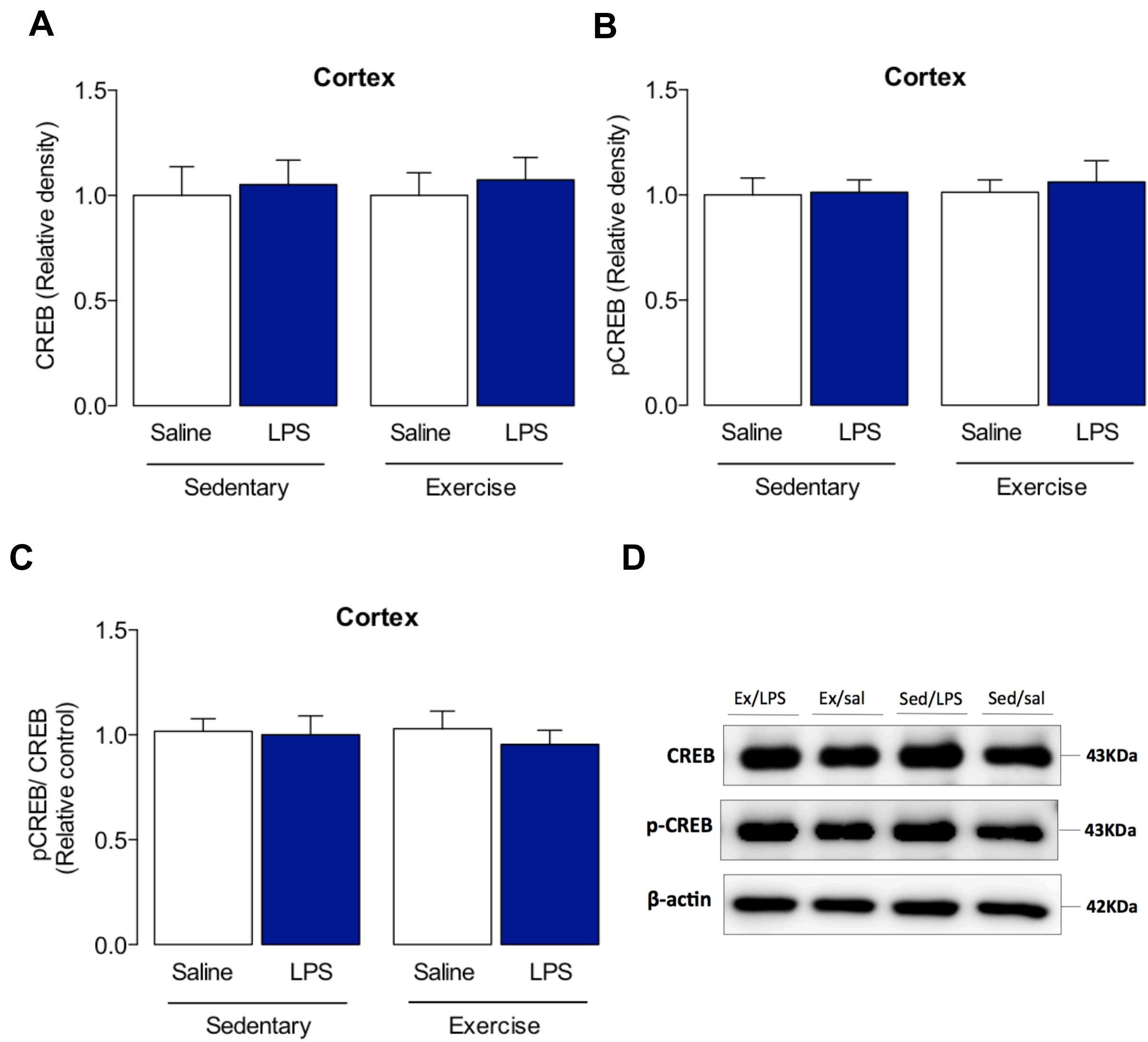


Figure 5.24 - Effect of LPS injection and prior exercise on CREB protein expression in cortex Protein expression of total and phosphorylated CREB, in cortex, was measured using Western Blot. Neither LPS nor prior exercise affected the expression of CREB in cortex **(A)**. Phosphorylated CREB and the ratio of phosphorylated CREB by total CREB protein content were not affected either by LPS or prior exercise **(B, C)**. Representative blots of total CREB, phosphorylated CREB and β-Actin in cortex **(D)**. β-Actin was used as loading control of protein content. Data are expressed as relative density and presented as mean ± SEM (n=5 to 6), two-way ANOVA with Bonferroni *Post hoc* analysis.

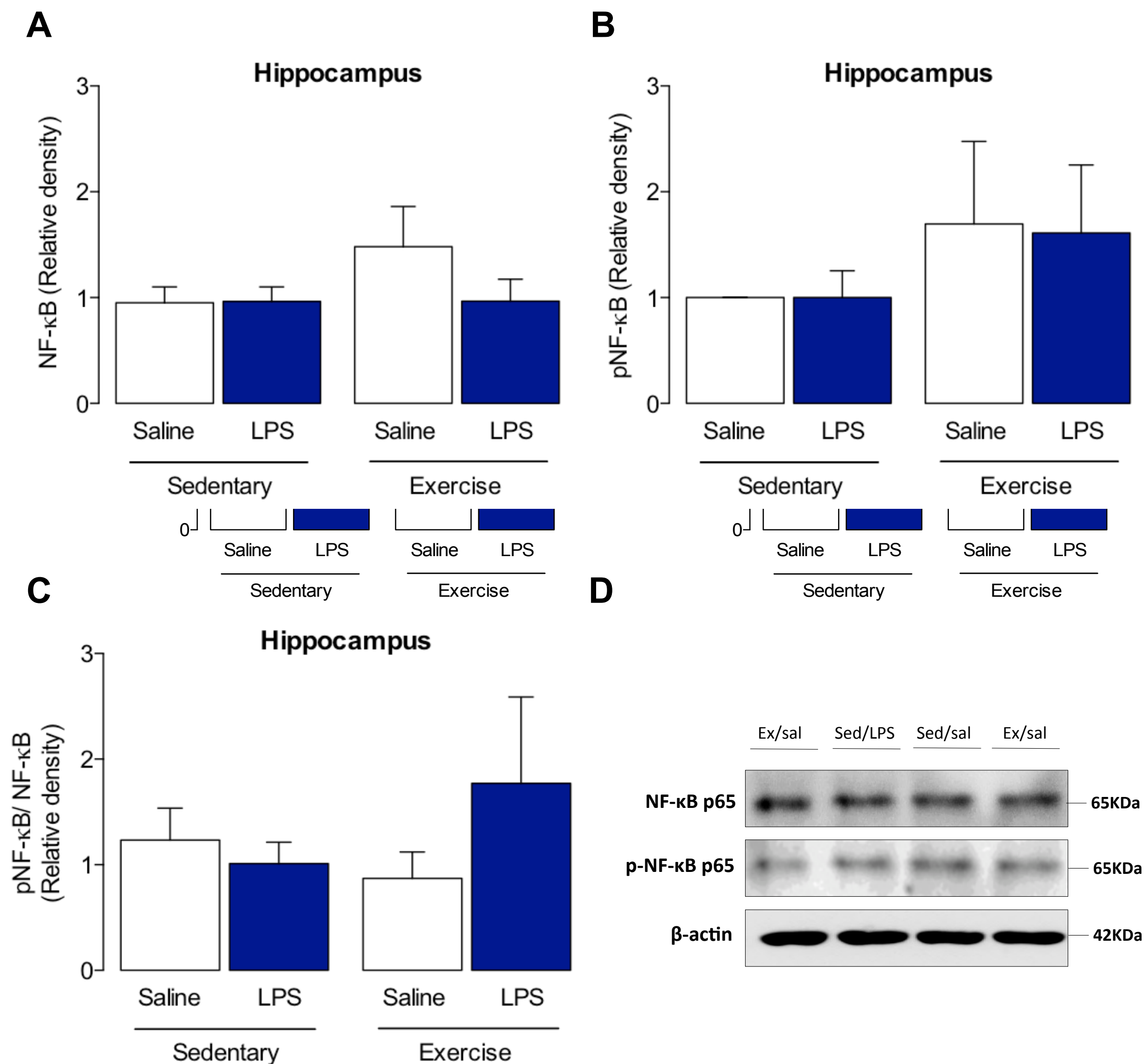


Figure 5.25 - Effect of LPS injection and prior exercise on NF-κB protein expression in hippocampus

Protein expression of total and phosphorylated NF-κB, in hippocampus, was measured using Western Blot. Neither LPS nor prior exercise affected the expression of NF-κB in hippocampus **(A)**. Phosphorylated NF-κB and the ratio of phosphorylated NF-κB by total NF-κB protein content were not affected either by LPS or prior exercise **(B, C)**. Representative blots of total NF-κB, phosphorylated NF-κB and β-Actin in hippocampus **(D)**. β-Actin was used as loading control of protein content. Data are expressed as relative density and presented as mean ± SEM (n=5 to 6), two-way ANOVA with Bonferroni *Post hoc* analysis.

5.3.8 Effects of LPS and exercise on the expression of M1/M2 markers of microglial polarisation in an enriched population of microglia

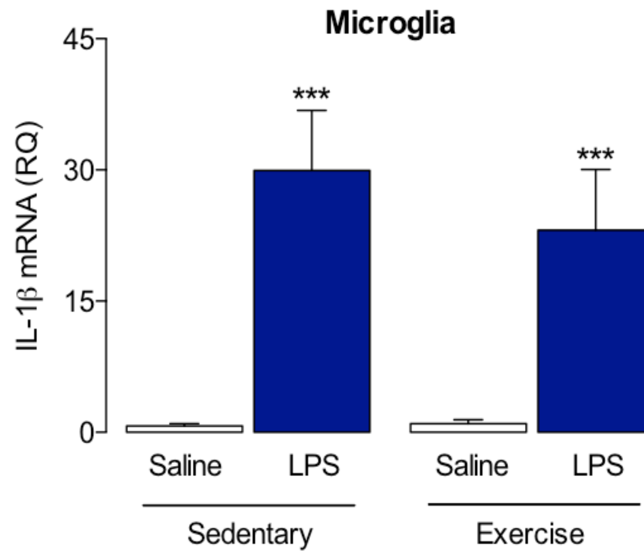
After analysis of mRNA expression of cytokines and markers of activation of astrocytes and microglia in tissue prepared from homogenised hippocampus, we investigated the potential modulatory effects of LPS and exercise on M1 and M2 phenotype in an enriched microglia population prepared from brain tissue of these mice. LPS injection significantly increased mRNA expression of the pro-inflammatory cytokines IL-1β ($p<0.001$, two-way ANOVA LPS vs. Saline, fold change SED-SAL: 0.72±0.28; SED-LPS: 29.95±6.89; EX-SAL: 1.01±0.45; EX-LPS: 23.10±6.96, Bonferroni *post-hoc*, Figure 5.26 A) and TNF-α ($p<0.001$, two-way ANOVA, LPS vs. Saline, fold change SED-SAL: 1.11±0.22; SED-LPS: 5.23±1.22; EX-SAL: 0.88±0.39; EX-LPS: 3.00±0.86, Bonferroni *post-hoc*, Figure 5.26 B), in microglia prepared from sedentary mice, mimicking the change observed in hippocampus (Figure 5.4 A-B and 5.5 A-B, respectively). Interestingly, expression of TNF-α was unaltered in microglia of exercise mice compared to sedentary LPS-injected mice ($p=0.1093$, two-way ANOVA, Figure 5.26 B).

Although it seems that LPS may tend to increase mRNA expression of iNOS ($p=0.4592$, two-way ANOVA LPS vs. Saline, fold change SED-SAL: 0.65±0.16; SED-LPS: 2.09±1.67; EX-SAL: 0.54±0.12; EX-LPS: 0.69±0.19, Bonferroni *post-hoc*, Figure 5.27 A) and IL-6 ($p=0.6468$, two-way ANOVA LPS vs. Saline, fold change SED-SAL: 1.03±0.36; SED-LPS: 2.39±1.12; EX-SAL: 0.44±0.25; EX-LPS: 1.20±0.48, Bonferroni post-hoc, Figure 5.27 B), statistical analysis showed that neither LPS nor exercise affected these markers.

Expression of M2 microglia phenotype markers Arg-1, Ym1 and Mrc1, were also assessed in the enriched microglial preparation. Statistical analysis demonstrated significant difference in LPS increased the expression of Arg-1 ($p<0.001$, two-way ANOVA, fold change SED-SAL: 0.66±0.22; SED-LPS: 1.71±0.36; EX-SAL: 0.37±0.08; EX-LPS: 2.32±0.28, Bonferroni *post-hoc*, Figure 5.28 A) and Ym-1 ($p<0.001$, two-way ANOVA, fold change SED-SAL: 1.58±0.80; SED-LPS: 8.09±2.27; EX-SAL: 4.96±3.03; EX-LPS: 17.14±4.94, Bonferroni *post-hoc*, Figure

5.28 B) only in the microglial cells prepared from EX mice However, no change in mRNA expression of Mrc1 was observed following LPS injection in either sedentary or exercise groups ($p=0.8905$, two-way ANOVA, fold change SED-SAL: 1.09 ± 0.17; SED-LPS: 0.89 ± 0.29; EX-SAL: 1.26 ± 0.27; EX-LPS: 1.13 ± 0.12, Bonferroni *post-hoc*, Figure 5.28 C).

A



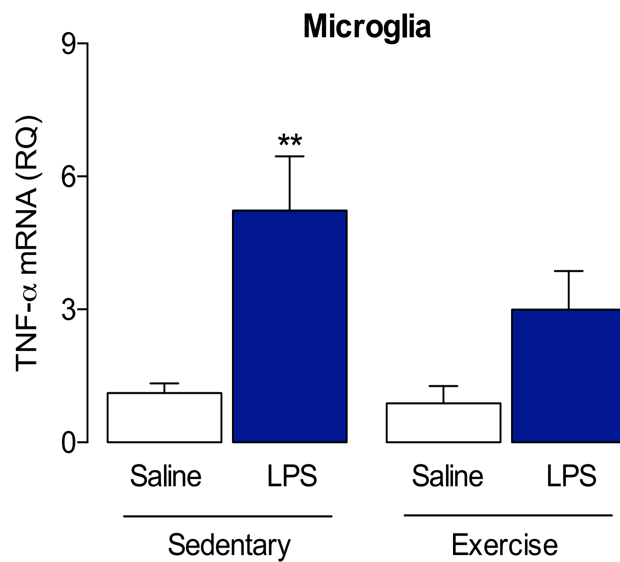
icroglia

***

Saline LPS

Exercise

B



croglia

Saline LPS

Exercise

ıre 5.26 - Effect **pression of IL-1β and**

-α in microglia

' hours after i.p. iı β and TNF-α in isolated

oglia was assess ısed in microglia in both

) and EX groups, creased the expression

\NF-α only in micr d as mean ± SEM (n=5

). ***p<0.001, Sal √A with Bonferroni *post*

analysis

Microglia

IL-6 mRNA (RQ)

6
4
2
0

Saline LPS Saline LPS

Sedentary Exercise

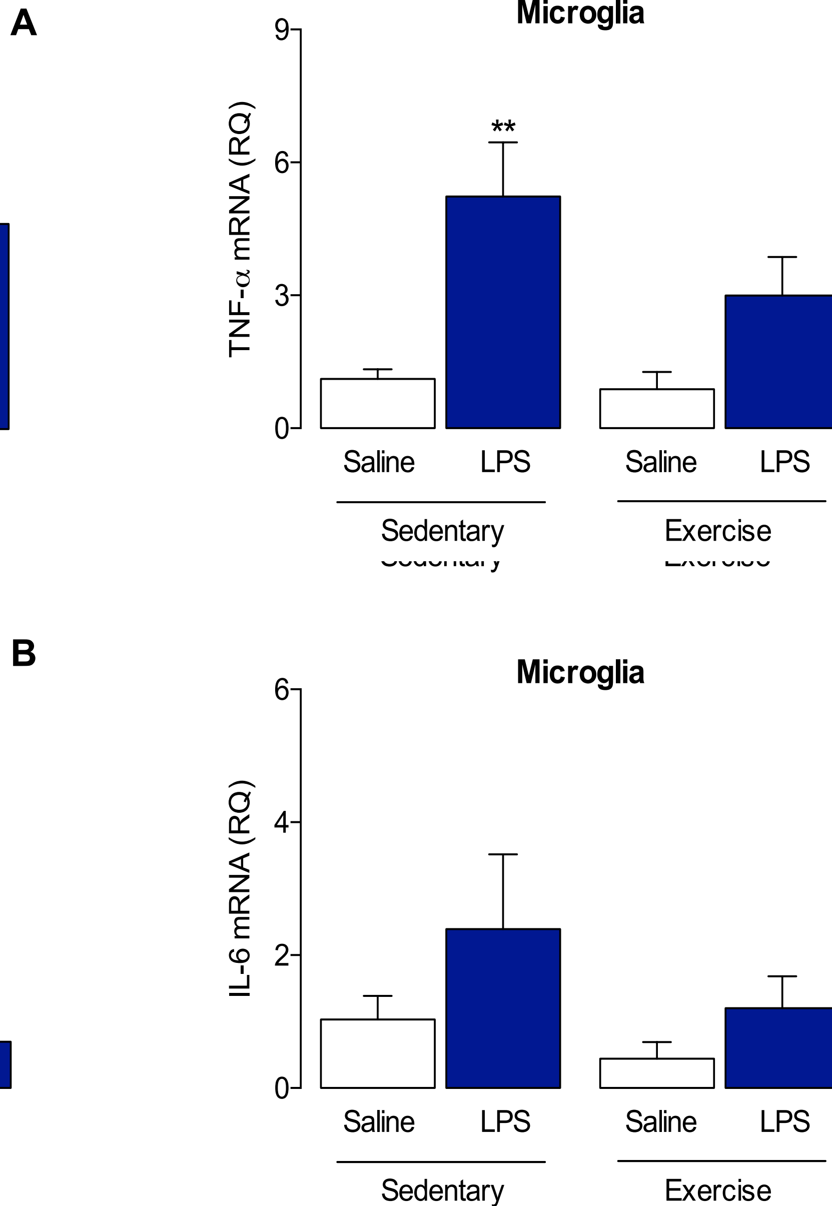
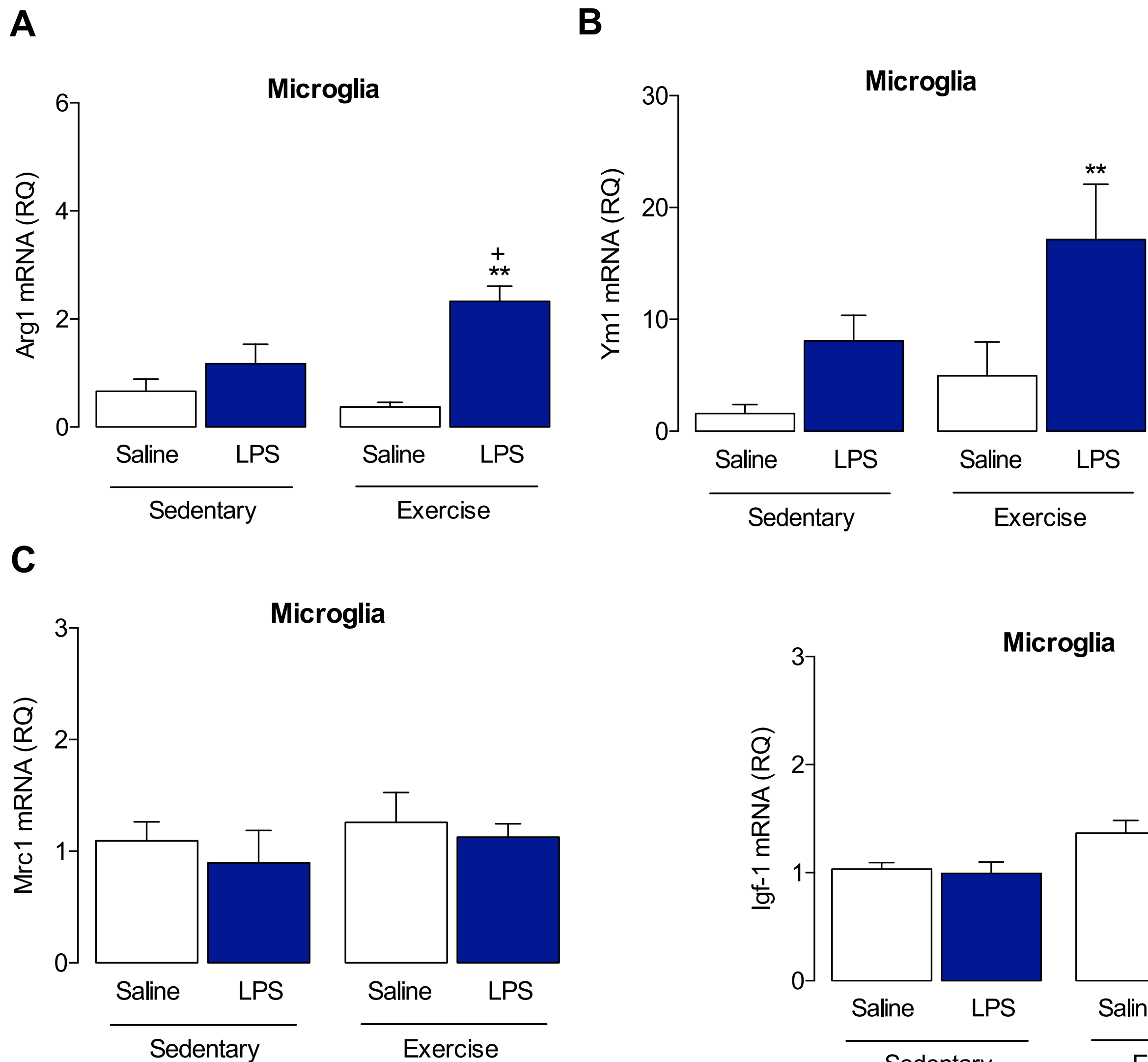


Figure 5.27 - Effect of LPS injection and prior exercise on mRNA expression of iNOS and IL-6 mRNA microglia

Four hours after i.p. injection of LPS, mRNA expression of M1 markers, iNOS and IL-6 in isolated microglia was assessed by RT-PCR. Neither LPS nor prior exercise affected the mRNA expression of iNOS and cytokine IL-6 in the enriched population of microglia **(A, B)**. Data are presented as mean ± SEM (n=5 to 6), two-way ANOVA with Bonferroni *post hoc* analysis

Arg1
2
0
Saline
LPS
Saline
LPS
Sedentary
Exercise



Microglia
Igf-1 mRNA (RQ)
3
2
1
0
Saline
LPS
Saline
LPS
Sedentary
Exercise

exercise on mRNA expression of Arg-1, Ym1

ɔression of M2 markers, Arg-1, Ym1 and Mrc1 in
Expression of Arg-1 and Ym1 was significantly
EX, but not SED, mice **(A, B)**. Neither LPS nor
ne enriched population of microglia **(C)**. Data are
Saline vs. LPS; $^{+}$p<0.05, SED-LPS vs. EX-LPS;
is

5.3.9 Effects of LPS and exercise on the expression of fractalkine receptor Cx3cr1, growth factor Igf-1 and cell-surface glycoprotein CD44 in an enriched population of microglia

Within the CNS, fractalkine receptor Cx3cr1 is restricted and abundantly expressed in microglia cells and it has been demonstrated to play a crucial role in the control of the biological activity of microglia cells. Since our data suggested that exercise may be priming microglial cells towards the M2 phenotype, we decided to address if LPS injection and prior short-term exercise would affect the expression of fractalkine receptor Cx3cr1 in the enriched microglia population. However, statistical analysis showed that mRNA expression of Cx3cr1 was unaffected by either LPS or prior exercise in microglia ($p=0.9578$, two-way ANOVA, fold change SED-SAL: 1.06 ± 0.15; SED-LPS: 0.80 ± 0.13; EX-SAL: 0.98 ± 0.22; EX-LPS: 0.75 ± 0.19, Bonferroni *post-hoc*, Figure 5.29 A). Since it has been demonstrated that microglial cells are an important source of Igf-1, which can also be up regulated by physical exercise, we decided to analyse the expression of Igf-1 in the microglia after LPS injection and prior exercise. No changes were observed in mRNA expression of Igf-1 in the enriched population of microglia in any group ($p=0.1854$, two-way ANOVA, fold change SED-SAL: 1.03 ± 0.06; SED-LPS: 0.99 ± 0.10; EX-SAL: 1.36 ± 0.12; EX-LPS: 0.99 ± 0.16, Bonferroni *post-hoc*, Figure 5.29 B).

Finally, it has been suggested that expression of CD44 may be up regulated in microglia exposed to inflammatory agents. Therefore, we investigated whether LPS injection and prior exercise would modulate mRNA expression of CD44 in microglia cells. Our results show that mRNA expression of CD44 was increased only in microglia prepared from SED- LPS mice ($p<0.01$, two-way ANOVA LPS vs. Saline, fold change SED-SAL: 1.02 ± 0.10; SED-LPS: 3.52 ± 0.56; EX-SAL: 1.16 ± 0.12; EX-LPS: 2.22 ± 0.57, Bonferroni *post-hoc*, Figure 5.29 C).

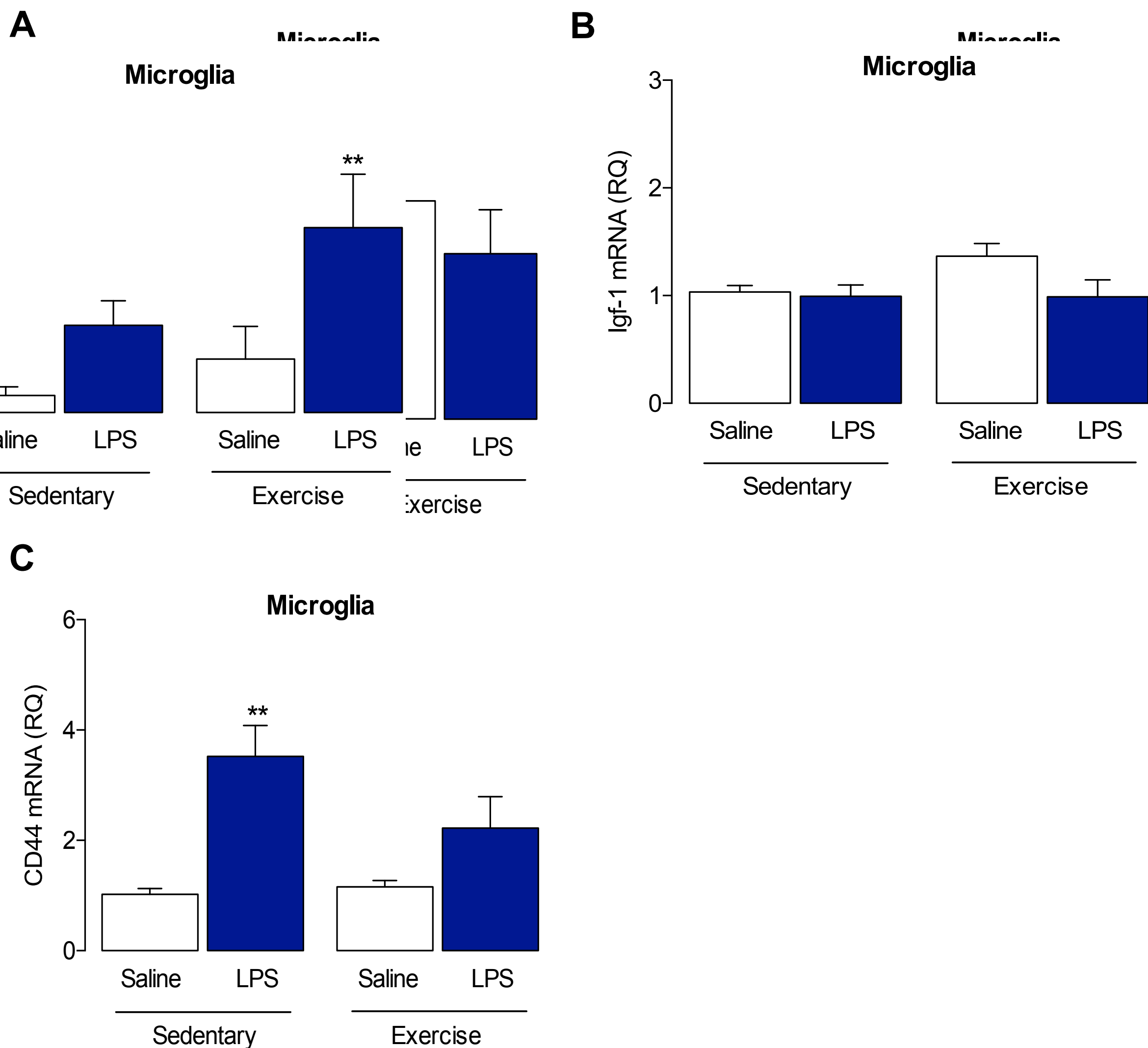


Figure 5.29 - Effect of LPS injection and prior exercise on mRNA expression of Cx3cr1, Igf-1 and CD44 in microglia

Four hours after i.p. injection of LPS, mRNA expression of Cx3cr1, Igf-1 and CD44 in isolated microglia cells was assessed by RT-PCR. Expression of Cx3cr1 and Igf-1 in microglia was not affected by LPS or prior exercise (**A, B**). LPS induced an increase in mRNA expression of CD44 only in microglia isolated from SED-LPS mice and exercise attenuated this increase **(C)**. Data are presented as mean ± SEM (n=5 to 6). **p<0.01, Saline vs. LPS; two-way ANOVA with Bonferroni *post hoc* analysis.

5.3.10 Effects of LPS and exercise on the expression of A1/A2 markers of astrocyte activation in an enriched astrocyte population

Recently, it has been suggested that astrocytes can assume an A1/A2 phenotype, similar to microglia (Roybon et al, 2013). The results above suggest that exercise can prime microglia towards an M2 state, therefore we investigated whether this effect could also be observed in isolated enriched population of astrocytes. An enriched population of astrocytes was prepared from brain tissue of sedentary and exercise mice treated with saline or LPS. Similar to the results observed in hippocampus, LPS significantly increased the expression of GFAP in astrocytes of SED mice ($p<0.001$, two-way ANOVA LPS vs. Saline, fold change SED-SAL: 0.87±0.07; SED-LPS: 1.49±0.06; EX-SAL: 0.77±0.11; EX-LPS: 1.14±0.13, Bonferroni *post-hoc*, Figure 5.30 A), and this effect was not observed in EX mice ($p=0.0255$, two-way ANOVA SED-LPS vs. EX-LPS, Figure 5.30 A). However, mRNA expression of TNF-α was significantly increased to identical levels in SED and EX mice injected with LPS ($p<0.001$, two-way ANOVA LPS vs. Saline, fold change SED-SAL: 1.49±0.43; SED-LPS: 8.69±1.13; EX-SAL: 1.90±0.94; EX-LPS: 9.24±1.56, Bonferroni *post-hoc*, Figure 5.30 B), with no effect of exercise ($p=0.6624$, two-way ANOVA, Figure 5.30 B).

Neither LPS nor EX affected mRNA expression of iNOS ($p=0.8231$, two-way ANOVA, fold change SED-SAL: 1.97±0.41; SED-LPS: 1.06±0.13; EX-SAL: 0.82±0.28; EX-LPS: 0.81±0.21, Bonferroni *post-hoc*, Figure 5.31 A) or IL-6 ($p=0.5421$, two-way ANOVA, fold change SED-SAL: 1.16±0.26; SED-LPS: 1.66±0.40; EX-SAL: 1.08±0.21; EX-LPS: 1.98±0.39, Bonferroni *post-hoc*, Figure 5.31 B) in an enriched population of astrocytes. A trend towards increased expression of IL-10 mRNA was observed in EX-LPS mice but this was not statistically significant ($p=0.3548$, two-way ANOVA, fold change SED-SAL: 2.26±1.48; SED-LPS: 1.57±0.54; EX-SAL: 2.84±1.70; EX-LPS: 6.08±3.46, Bonferroni *post-hoc*, Figure 5.32 A). mRNA expression of Arg-1 in astrocytes was increased to similar levels in both SED and EX groups ($p<0.001$, two-way ANOVA LPS vs. Saline, fold change SED-SAL: 0.89±0.30; SED-LPS: 9.35±2.00; EX-SAL: 0.83±0.19; EX-LPS: 7.04±2.08, Bonferroni *post-hoc*, Figure 5.32 B) with no effect

of exercise observed ($p=0.4035$, two-way ANOVA, Figure 5.32 B). Finally, neither LPS nor EX induced any change in mRNA expression of Mrc1 in astrocytes ($p=0.2178$, two-way ANOVA, fold change SED-SAL: 1.02±0.12; SED-LPS: 0.92±0.14; EX-SAL: 1.57±0.44; EX-LPS:0.78±0.06, Bonferroni *post-hoc*, Figure 5.32 C), similar to results the observed previously in hippocampus and isolated microglia.

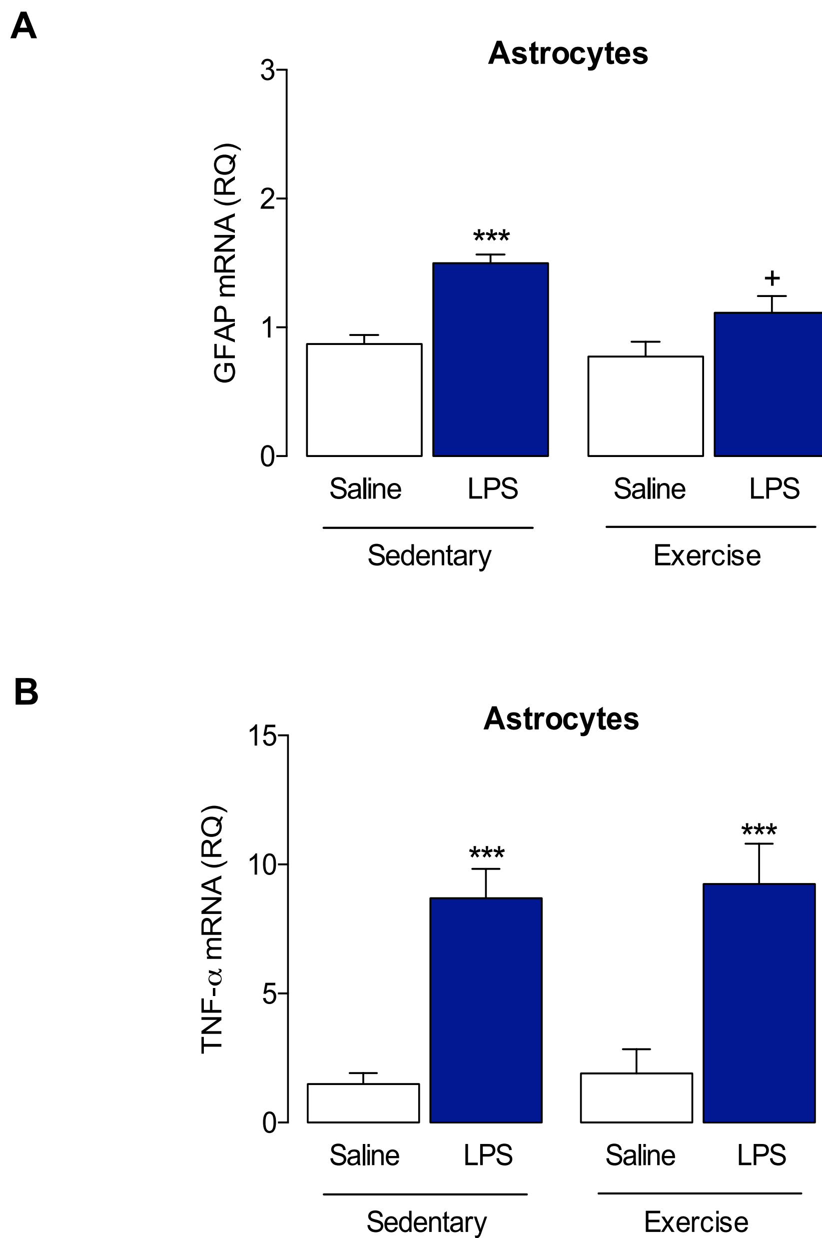


Figure 5.30 - Effect of LPS injection and prior exercise on mRNA expression of GFAP and
-α in astrocytes … r hours after i.p. i … α in isolated astrocytes … s, was assessed … antly increased by LPS … ED and EX grou … e **(A)**. LPS induced an … ease in mRNA e … LPS and EX-LPS mice, … no effect of exe … 6). ***p<0.001, Saline … LPS; +p<0.05, SE … *post hoc* analysis.

ytes
Saline
LPS
Exercise
Astrocytes
IL-6 mRNA (RQ)
3
2
1
0
Saline
LPS
Saline
LPS
Sedentary
Exercise

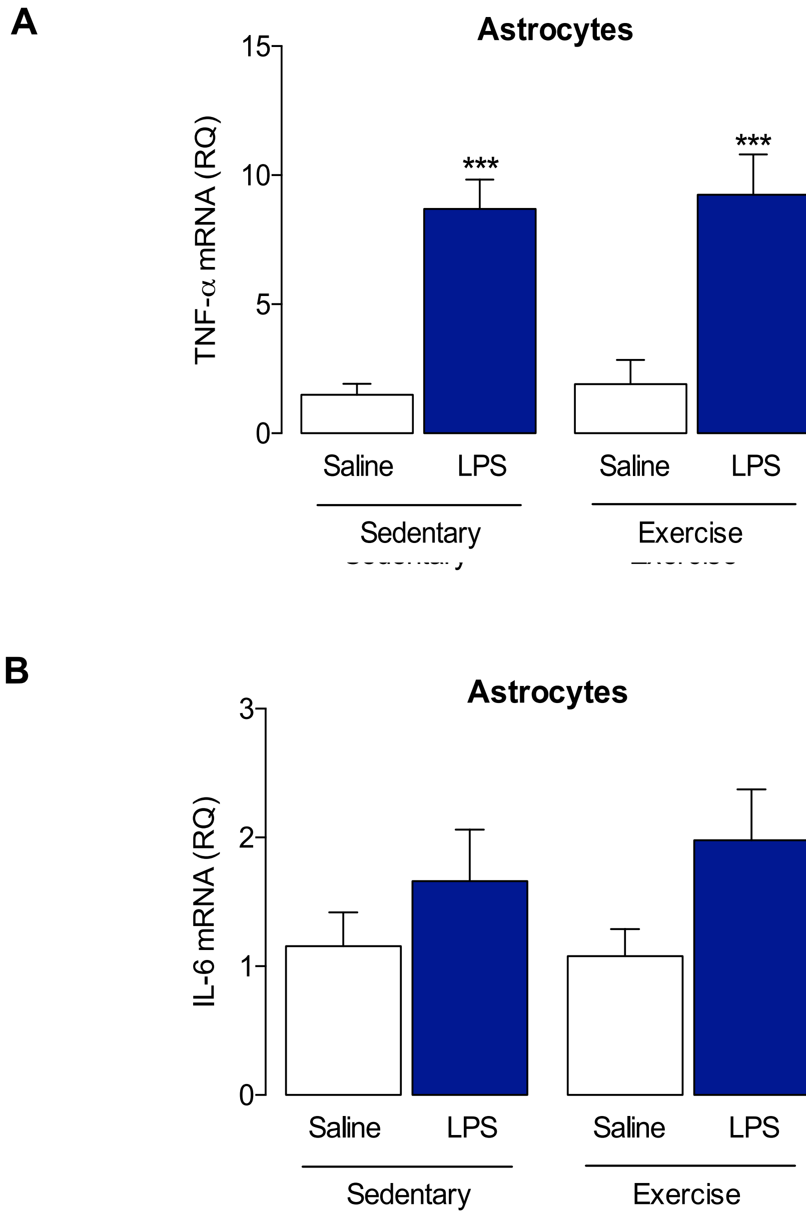


Figure 5.31 - Effect of LPS injection and prior exercise on mRNA expression of iNOS and IL-6 in astrocytes.

Four hours after i.p. injection of LPS, mRNA expression of iNOS and IL-6 in isolated astrocytes, was assessed by RT-PCR. Expression of iNOS and IL-6 in astrocytes was not affected by LPS or prior exercise **(A, B)**. Data are presented as mean ± SEM (n=5 to 6), two-way ANOVA with Bonferroni *post hoc* analysis.

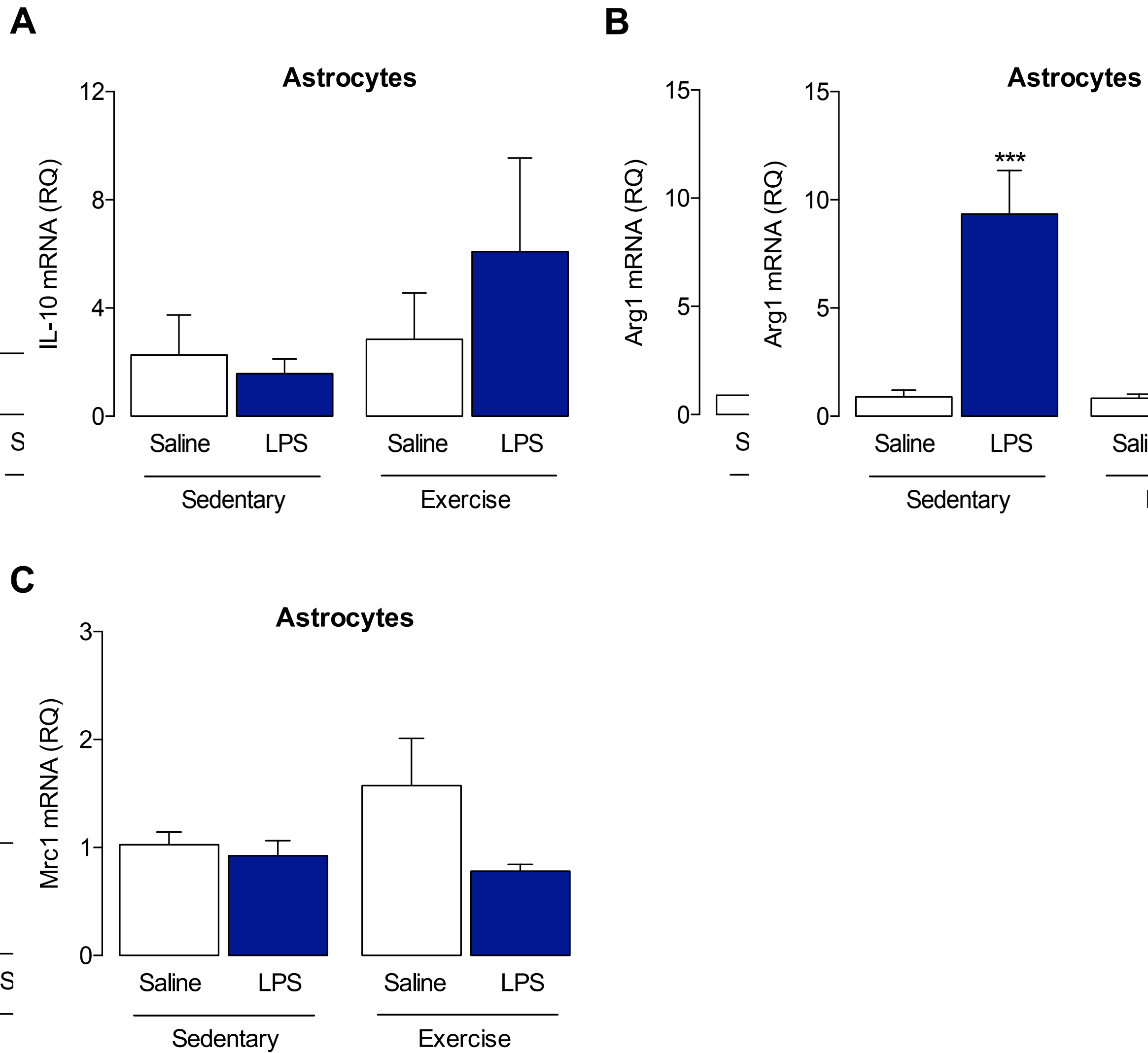


Figure 5.32 - Effect of LPS injection and prior exercise on mRNA expression of IL-10, Arg-1 and Mrc1 in astrocytes

Four hours after i.p. injection of LPS, mRNA expression of IL-10, Arg-1 and Mrc1 in isolated astrocytes, was assessed by RT-PCR. Expression of IL-10 in astrocytes was not affected by LPS or prior exercise **(A)**. LPS induced an increase in mRNA expression of Arg-1 in astrocytes isolated from both, SED and EX groups **(B)**. Neither LPS nor prior exercise affected mRNA expression of Mrc1 in astrocyte cells. Data are presented as mean ± SEM (n=5 to 6). ***p<0.001, Saline vs. LPS; two-way ANOVA with Bonferroni *post hoc* analysis.

5.3.11 Effects of LPS and exercise on mRNA expression of growth factors BDNF and GDNF and cell-surface glycoprotein CD44 in an enriched astrocyte population

Astrocytes have been demonstrated to play an important role in the secretion of trophic factors, including glial-cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF). Therefore, we decided to address whether prior exercise and LPS systemic injection would modulate mRNA expression of GDNF and BDNF in the enriched population of astrocytes. There was no significant difference in expression of BDNF ($p=0.4840$, two-way ANOVA, fold change SED-SAL: 1.03 ± 0.12; SED-LPS: 0.61 ± 0.13; EX-SAL: 1.20 ± 0.17; EX-LPS: 0.73 ± 0.32, Figure 5.33 A). or GDNF ($p=0.9680$, two-way ANOVA, fold change SED-SAL: 1.03 ± 0.10; SED-LPS: 0.92 ± 0.22; EX-SAL: 0.90 ± 0.08; EX-LPS: 0.80 ± 0.20, Bonferroni *post-hoc*, Figure 5.33 B) in astrocytes,

Similar to microglia, astrocytes are an important source of CD44 in the CNS, although its functions in astrocytes cells still remains poorly understood. For this reason, we analysed mRNA expression of CD44 in the enriched population of astrocytes obtained from all groups. mRNA expression of CD44 was upregulated in SED-LPS and EX-LPS mice, with no effect of exercise on CD44 mRNA expression ($p<0.001$, two-way ANOVA LPS vs. Saline, fold change SED-SAL: 1.01 ± 0.07; SED-LPS: 3.43 ± 0.23; EX-SAL: 1.09 ± 0.08; EX-LPS: 2.85 ± 0.79, Bonferroni *post-hoc*, Figure 5.33 C).

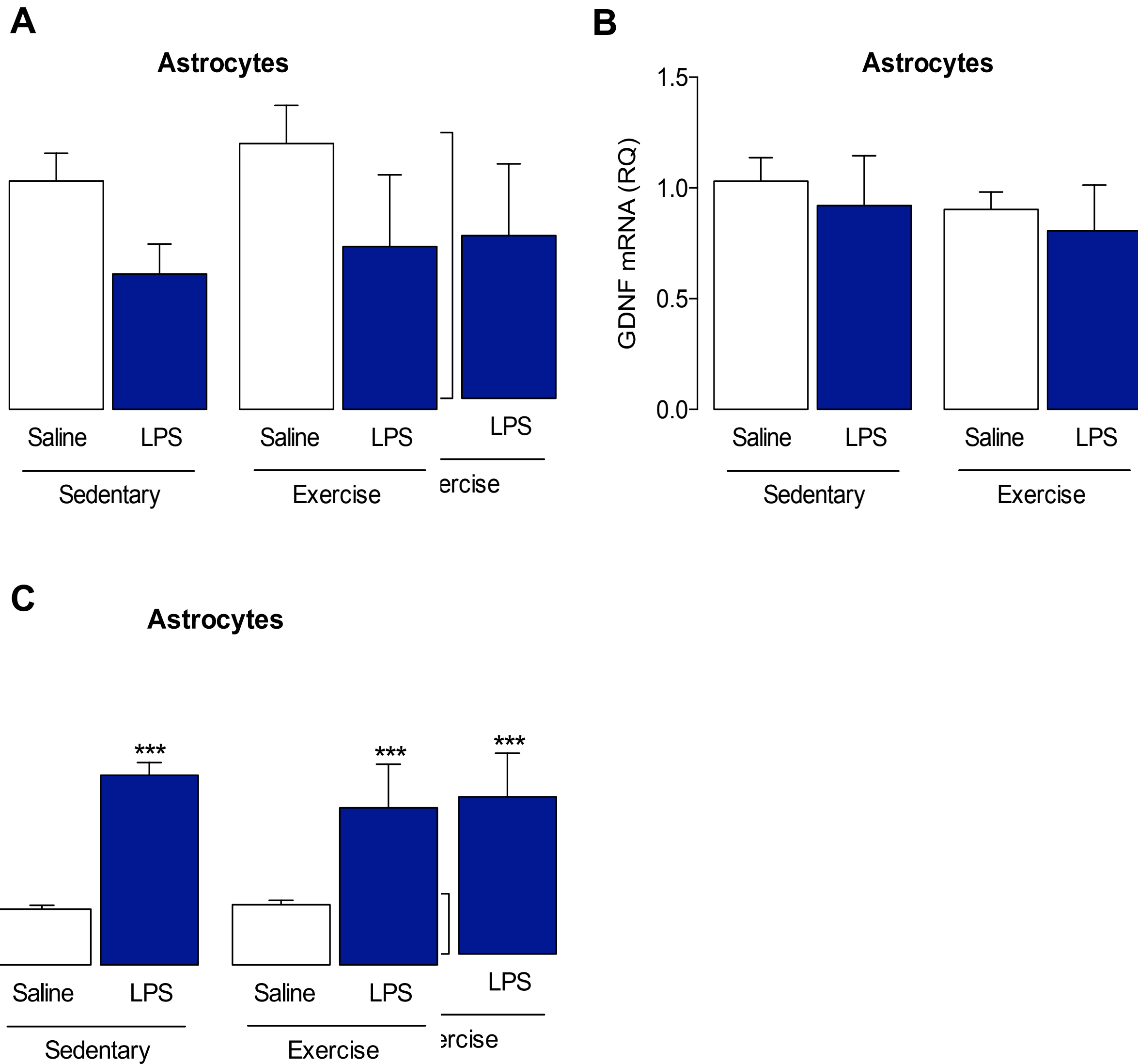


Figure 5.33 - Effect of LPS injection and prior exercise on mRNA expression of BDNF, GDNF and CD44 in astrocytes

Four hours after i.p. injection of LPS, mRNA expression of BDNF, GDNF and CD44 in isolated astrocytes was assessed by RT-PCR. Neither LPS nor prior exercise affected mRNA expression of BDNF and GDNF in astrocyte cells. **(A, B)**. LPS induced an increase in the expression of CD44 in astrocytes isolated from both, SED and EX groups **(C)**. Data are presented as mean ± SEM (n=5 to 6). ***p<0.001, Saline vs. LPS; two-way ANOVA with Bonferroni *post hoc* analysis.

5.4 Table of results

5.4.1 Cytokines

	Sed/Sal		Sed/LPS		Ex/Sal		Ex/LPS	
IL-1β	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↑		↔		ATTENUATED ↑	
cortex	↔		↑		↔		ATTENUATED ↑	
microglia	↔		↑		↔		↑	
TNF-α	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↑		↔		ATTENUATED ↑	
cortex	↔		↑		↔		ATTENUATED ↑	
microglia	↔		↑		↔		PREVENTED ↑	
astrocytes	↔		↑		↔		↑	
IL-6	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↑		↔		ATTENUATED ↑	
microglia	↔		↔		↔		↔	
astrocytes	↔		↔		↔		↔	
IL-10	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↑		↔		ATTENUATED ↑	
cortex	↔		↑		↔		ATTENUATED ↑	
astrocytes	↔		↔		↔		↔	
IL-4	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↑		↔		ATTENUATED ↑	
cortex	↔		↔		↔		↔	

Table 5.1 - Effect of exercise on cytokines 4 hours after LPS challenge

Illustrative table with the outcome effect of exercise on mRNA and protein expression of cytokines in the hippocampus, cortex, microglia and astrocytes 4 hours after LPS challenge. (↑) Increased in mRNA and/or protein expression. (↔) No changes in mRNA and/or protein expression.

5.4.2 Glial cell markers

	Sed/Sal		Sed/LPS		Ex/Sal		Ex/LPS	
GFAP	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↑		↔		ATTENUATED ↑	
cortex	↔		↑		↔		PREVENTED ↑	
astrocytes	↔		↑		↔		ATTENUATED ↑	
Iba-1	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔		↔		↔	
cortex	↔		↔		↔		↔	
microglia	↑		↑				PREVENTED ↑	
astrocytes	↑		↑				PREVENTED ↑	
CD11b	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↑		↔		PREVENTED ↑	
cortex	↔		↔		↔		↔	
NF-κB	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔		↔		↔	
iNOS	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↑		↔		ATTENUATED ↑	
microglia	↔		↔		↔		↔	
astrocytes	↔		↔		↔		↔	
Arg-1	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↑		↔		PREVENTED ↑	
microglia	↔		↔		↔		↑	
astrocytes	↔		↑		↔		↑	
Mrc1	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔		↔		↔	
microglia	↔		↔		↔		↔	
astrocytes	↔		↔		↔		↔	
Ym1	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
microglia	↔		↔		↔		↑	

Table 5.2 - Effect of exercise on glial cells markers 4 hours after LPS challenge

Illustrative table with the outcome effect of exercise on mRNA and protein expression of glial cells markers in the hippocampus, cortex, microglia and astrocytes 4 hours after LPS challenge. (↑) Increased in mRNA and/or protein expression. (↔) No changes in mRNA and/or protein expression.

5.4.3 Inflammatory and apoptotic markers

	Sed/Sal		Sed/LPS		Ex/Sal		Ex/LPS	
Cx3cl1	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔		↔		↔	
cortex	↔		↔		↔		↔	
Cx3cr1	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔		↔		↔	
cortex	↔		↔		↔		↔	
microglia	↔		↔		↔		↔	
CD44	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↑		↔		ATTENUATED ↑	
cortex	↔		↑		↔		ATTENUATED ↑	
microglia	↔		↑		↔		PREVENTED ↑	
astrocytes	↔		↑		↔		↑	
Bax	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔		↔		↔	
cortex	↔		↔		↔		↔	
Bcl-2	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔		↔		↔	
cortex	↔		↔		↔		↔	

Table 5.3 - Effect of exercise on inflammatory and apoptotic markers 4 hours after LPS challenge

Illustrative table with the outcome effect of exercise on mRNA and protein expression of inflammatory and apoptotic markers in the hippocampus, cortex, microglia and astrocytes 4 hours after LPS challenge. (↑) Increased in mRNA and/or protein expression. (↔) No changes in mRNA and/or protein expression.

5.4.4 Growth factors, neurotrophins and signalling proteins

	Sed/Sal		Sed/LPS		Ex/Sal		Ex/LPS	
BDNF	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔	↔	↔	↔	↔	↔	↔	↔
cortex	↔	↔	↔	↔	↔	↔	↔	↔
astrocytes	↔		↔		↔		↔	
TrkB	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔		↔		↔	
cortex	↔		↔		↔		↔	
p75	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔		↔		↔	
cortex	↔		↔		↔		↔	
NGF	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔		↔		↔	
cortex	↔		↔		↔		↔	
Igf-1	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔		↔		↔	
cortex	↔		↔		↔		↔	
cortex	↔		↔		↔		↔	
VEGF	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus	↔		↔		↔		↔	
cortex	↔		↔		↔		↔	
GDNF	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
astrocytes	↔		↔		↔		↔	
Akt	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus		↔		↔		↔		↔
cortex		↔		↔		↔		↔

	Sed/Sal		Sed/LPS		Ex/Sal		Ex/LPS	
CREB	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus		↔		↔		↔		↔
cortex		↔		↔		↔		↔
ERK1/2	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**	**mRNA**	**protein**
hippocampus		↔		↔		↔		↔
cortex		↔		↔		↔		↔

Table 5.4 - Effect of exercise on growth factors, neurotrophins and signalling proteins 4 hours after LPS challenge

Illustrative table with the outcome effect of exercise on mRNA and protein expression of growth factors, neurotrophins and signalling proteins in the hippocampus, cortex, microglia and astrocytes 4 hours after LPS challenge. (↑) Increased in mRNA and/or protein expression. (↔) No changes in mRNA and/or protein expression.

5.5 Discussion

We have demonstrated in the previous chapters that early-life exposure to exercise had positive effects in spatial and non-spatial learning and memory during the lifespan that persisted until old age and that this enhancement in cognitive function was also accompanied by decreased anxiety and depression-like behaviour. Moreover, these behavioural changes were associated with a built-up cognitive reserve, mediated in parts by a persistent anti-inflammatory modulation of early-life exposure to exercise. The generation of our previous findings drove us to investigate further the effect of exercise in modulating experimentally-induced acute neuroinflammation and its effects on cognitive function. Therefore, the aim of this chapter was to address the effects of prior short-term exercise on spatial memory impairment in a mouse model of neuroinflammation induced by LPS and to verify the involvement of inflammation, an important feature of ageing, in this effect. First, we investigated a possible protective effect of prior short-term exercise on spatial learning and memory impairment in the OD task, 4 hours after a single LPS challenge. Finally, we investigated the possible mechanisms involved in the effect of exercise protecting against spatial memory impairment, focusing on the anti-inflammatory effect of exercise. We assessed whether the effects of exercise on memory were mediated by exercise-induced changes in general inflammatory markers in the hippocampus and cortex of LPS-injected mice as well as by a direct effect of exercise in modulating the activation of glial cells in this model of neuroinflammation.

5.5.1 Behaviour

Systemic administration of LPS is well known to induce behavioural changes, collectively called “sickness behaviour”, characterised by neuroinflammation and cognitive impairment (Cunningham et al., 2005). In our study, although acute systemic administration of LPS induced changes in cytokine expression in the brain that will be discussed below, this did not interfere with general exploratory and locomotor behaviour of mice during the OD task, since total object exploration did not differ between groups. Accumulating evidence suggests that LPS interferes in the consolidation of memory processes. For example, acute administration of LPS

prior to training impairs contextual fear conditioning and in the performance of MWM (Pugh et al., 1998). while chronic LPS injection impairs spatial memory (Sparkman et al., 2005). Extending the findings from previous studies, we demonstrated short-term spatial memory impairment in the OD task, in adult mice that were injected with LPS 4 hours prior to training and testing. However, 10 days of prior treadmill running was able to prevent the impairment in spatial memory in the OD as demonstrated by an increased exploration of the displaced object to levels similar to the exercised saline-injected mice.

Supporting our findings, the majority of the studies in the literature demonstrated that exercise attenuates LPS-induced impairment in cognition in rodents. Five weeks of treadmill exercise have been shown to restore spatial learning and memory performance in the MWM in mice repeatedly injected with LPS; this was accompanied by significant attenuation of LPS-induced decrease in hippocampal neurogenesis (Wu et al., 2007). Most recently, Littlefield and colleagues (2015), demonstrated that 9 weeks of voluntary running attenuated an LPS-induced decrease in neurogenesis and modulated microglia towards a pro-neurogenic state, indicated by increased co-expression of BDNF and Iba-1 positive cells, in aged mice (Littlefield et al., 2015), suggesting that the ability of exercise to counteracts LPS-induced neurogenesis reduction and consequently cognitive impairment in mice may in part, results from its modulation of microglia activation.

5.5.2 Potential mechanisms underlying altered behaviour

Systematic inflammation is characterised by an increased concentration of circulating cytokines, which can impact the CNS, trigging a neuroinflammatory response and thereby affecting cognitive function (Perry, 2004). Our findings demonstrated that LPS induced an increase in mRNA expression of several pro-inflammatory cytokines, IL-1β, TNF-α and IL-6 in the hippocampus and cortex of adult mice, 4 hours after its administration, in agreement with previous studies in the literature that reported increased mRNA and protein levels of IL-1β and TNF-α in the mouse brain, 1 hour following LPS administration that persisted for a week (Qin et al., 2008). Also, systemic administration of LPS, IL-1β and TNF-α, in mice, has been show to induce an increase in hippocampal mRNA expression of IL-1β,

TNF-α and IL-6, 2 hours after the challenges (Skelly et al., 2013). We chose the 4 hour timpoint to ensure an adequate inflammatory response in the brain. We demonstrated that 9 consecutive days of prior treadmill running attenuated the increase in mRNA expression of pro-inflammatory cytokines IL-1β, TNF-α and IL-6 induced by LPS in both hippocampus and cortex, suggesting a potent effect of exercise in minimising the central inflammatory response to systemic LPS administration and corroborating previous studies that have demonstrated exercise to counteract the increase of pro-inflammatory cytokines induced by endotoxins (Tanaka et al., 2010). For instance, Barrientos and collaborators (2011) showed that 6 weeks of voluntary wheel running reduced hippocampal mRNA expression of IL-1β in aged mice challenged with a single systemic injection of *Escherichia coli* and this reduction was associated with rescuing of impairment in the contextual fear conditioning by exercise (Barrientos et al., 2011). This finding supports our results and leads us to suggest that the attenuation of exacerbated cytokine production within the brain may be an important protective mechanism induced by exercise against LPS-induced memory impairment, given the described role of IL-1β in reducing BDNF expression (Cortese et al., 2011), disrupting LTP (Lynch, 2010) and promoting hippocampal-dependent memory impairments (Gemma and Bickford, 2007)).

Nevertheless, LPS is not only reported to upregulate pro-inflammatory cytokines, but also to stimulate an early increase in the expression of anti-inflammatory cytokines, such as IL-10 (Martin et al., 2014), suggesting this may be a compensatory mechanism to counterbalance and counteract the inflammatory response. Interestingly, extending the present findings, our results demonstrate an LPS-induced increase in mRNA expression of IL-10 and IL-4 in the mouse brain. However, exercise attenuated the increase in IL-10 in the hippocampus and completely blocked the LPS-induced increase in mRNA expression of IL-10 in the cortex and of IL-4 in the hippocampus, 4 hours after systemic LPS administration. These results indicate that the effect of exercise in attenuating the increase in anti-inflammatory cytokines induced by LPS is, in part, due to the reduction in pro-inflammatory cytokines induced by exercise, suggesting exercise is promoting a counterbalance in cytokines production in the brain of LPS-injected mice.

In addition, it has been reported that intraperitoneal administration of LPS, not only increases the production of pro-inflammatory cytokines within the CNS, but also stimulates microglia and astrocyte activation (Godbout et al., 2005). Indeed, peripheral production of cytokines, induced by endotoxins, can modulate neuroinflammation in several ways, for example increased cytokine levels in areas such as choroid plexus, can activate the transport of cytokines across the BBB. Another mechanism underlying LPS-induced neuroinflammation is the direct activation of brain resident immune cells by LPS, through the activation of TLR4 (Chakravarty and Herkenham, 2005), stimulating these cells to increase the secretion of cytokines within the CNS. Similarly, normal aging, that is featured by chronic and low-grade inflammation and cognitive decline, also primes microglia towards the classic inflammatory phenotype, which is characterised by increased production of pro-inflammatory cytokines IL-1β, TNF-α and IL-6 (Sierra et al., 2007).

Under normal conditions, glial cells contribute to the regulation of several functions in the mature brain including controlling neurotransmitter turnover, neuronal metabolism support, synaptic modulation and neurogenesis and consequently, glia influence the process of learning and memory. However, these cells, mainly microglia cells, are extremely sensitive to small changes in the environment, such as an increase in pro-inflammatory cytokine expression and, once activated, they can exacerbate neuroinflammation, increasing production of cytokines and ROS, thereby contributing to the disruption of brain function (Blank and Prinz, 2013). In concordance with that, we have described above the effect of LPS in increasing cytokine expression in the hippocampus and cortex, and interestingly, we demonstrated LPS to also modulate markers of glial cell activation, such as GFAP, CD11B and iNOS in the whole tissue. Most interestingly, as exercise protected against this exaggerated LPS-induced increase in mRNA expression of cytokines, we observed an effect of exercise in counteracting the increase in mRNA expression of glial cell markers in hippocampus and cortex, suggesting that likely, this effect was in response to an exacerbated production of pro-inflammatory cytokines. Moreover, these findings suggest that the effect of exercise in counteracting cognitive impairment induced by LPS, observed in the OD task, may be partly associated with its attenuation of LPS-induced glial cell activation. Even

though GFAP mRNA expression was increased in the hippocampus and cortex 4 hours after systemic administration of LPS and exercise attenuated this increase, minimal effects of LPS and exercise were observed on the A1/A2- associated markers in the analysis of isolated astrocytes, suggesting a prominent response of microglial cells rather than astrocytes to LPS and to acute exercise modulation.

Although Iba1 expression has been shown to be upregulated in activated microglia and was found to be increased along with IL1β expression (Autieri et al., 2000), we failed to show changes in Iba-1 mRNA expression in the entire hippocampus and cortical tissue. However it does not necessarily indicate a lack of microglia activation induced by LPS in our study, because the microglia cytoskeletal rearrangements may not accurately represent an active inflammatory profile (Norden et al., 2016). Indeed, in a model of prion disease for example, LPS administration has been show to increase expression of cytokines in microglia independent of significant changes in microglial morphology (Cunningham et al., 2005). Indeed, although Iba-1 mRNA expression was not altered in the whole tissue, our analysis in isolated microglia showed an LPS-induced increase in the mRNA expression of classical markers of the M1 phenotype of microglia activation, such as IL-1β and TNF-α and interestingly, exercise protected against the increase in one of these markers, the pro-inflammatory cytokine TNF-α.

Age-primed microglia have been reported to produce a prolonged M1 response following immune challenge (Dilger and Jonhson, 2008), and it is likely that exercise changes the responsiveness of these cells to an LPS-induced effect. In this line, previous studies have shown increased expression of both M1- and M2-associated markers in aged mice, in response to LPS challenge (Henry et al., 2009). Indeed, when the whole hippocampus was analysed we demonstrated an increased mRNA expression of Arg-1 in LPS-injected mice, but our results from the analysis of isolated microglia failed to mimic this response. However, our results demonstrated a significant effect of exercise in increasing classical M2 phenotype markers, such as Arg-1 and Ym1 in response to LPS, suggesting that even though exercise minimally altered microglial cell response to LPS towards M1-associated state, exercise generated a significant positive modulation of M2 phenotype markers, which could, in part, be mediating the protection of exercise

against LPS-induced impairments in spatial memory we observe. Supporting this findings, it has been demonstrated that microglia, activated by IL-4 towards a M2 state, can stimulate NPCs differentiation, promoting neurogenesis and oligodendrogenesis. Although the exact mechanism of microglia in supporting neurogenesis in the adult brain remains unclear, it has been associated with the action of microglia in maintaining the surrounding environment, in part by its important phagocytic role (Butovsky et al., 2006). Fewer M2 cells are associated with lower levels of neuroprotective factors like Igf-1 or BDNF (Kohman and Rhodes, 2013), which can be produced by microglia cells, suggesting the lack of an appropriate M2 response might be an important mechanism underlying neurodegeneration and weighting the importance of M1/M2 balance in ageing and diseases characterized by chronic neuroinflammation. Furthermore, perhaps, an adequate M2 phenotype modulation could induce an appropriate neurotrophin expression in the brain microenvironment that contributes to the promotion of neurogenesis and cognitive benefits.

In this chapter, we have not investigated the effects of exercise to protect against inflammation-induced disruption in neurogenesis, but we analysed expression of growth factors and neurotrophins, such as BNDF, and the downstream signalling activated by BDNF-TrkB binding, which are associated with synaptic plasticity and neurogenesis, and although exercise is well described to induce upregulation of BDNF and consequently activate BDNF downstream signalling. we failed to show such regulation by exercise of this protein and the pathways it stimulates. Also, in our study, 4 hours after LPS administration, no changes were induced in mRNA expression of BDNF, mimicking previous reports (Shaw et al., 2001). However, opposite to the findings presented here, previous studies in our laboratory observed an increase in BDNF concentration immediately after the cessation of exercise, associated with enhancement in cognitive functions (Griffin et al., 2009, O'Callaghan et al., 2007). On the other hand, Wu and colleagues (2007), demonstrated BDNF levels to return to baseline levels after 4 hours of exercise and it was found to remain at baseline levels until 24 hours after exercise cessation. Here, since exercise was stopped 24 hours after tissue collection, it is likely that the changes in BNDF mRNA expression would be transient.

Similarly, no difference was observed in the mRNA and protein expression of TrkB receptor in the hippocampus and cortex. Give BDNF has been widely shown to play a central role mediating the exercise-induced enhancement on cognitive functions, changes in TrkB expression would be expected to be accompanied by changes in BDNF expression. As we failed to demonstrate exercise-induced increase in the expression of BDNF, this could explain the lack of increase in TrkB receptor expression. In this line of view, Widenfalk and collaborators (1999) also showed that exercise-induced changes in BDNF and TrkB expression occur in a running length-dependent manner (Widenfalk et al., 1999), suggesting that BDNF-TrkB modulation may depend on the duration of exercise session.

Moreover, evidence from literature investigating the molecular mechanisms involved in exercise-induced improvement in brain function indicates the activation of BDNF signalling pathways, such as MAPK/ERK and Akt. However, similar to our BDNF and TrkB results, we failed to show such an effect of exercise in upregulating these pathways and this could be explained by the lack of increase in BDNF and TrkB expression by exercise. Moreover, the duration of exercise intervention could also interfere with the BDNF pathway activation, as it has been found that acute exercise more robustly activates CaMK signalling, while the MAPK/ERK pathway is likely to be more important in chronic long-term exercise (Molteni et al., 2002). These considerations of the time course of protein induction and the exercise intervention used in this study may explain our lack of exercise-induced increase in these pathways. In addition, the positive effects of exercise in hippocampal function may involve the activation of other growth factors besides BDNF, such as Igf-1, NGF and VEGF (Carro et al., 2001, Ding et al., 2006). The exercise protocol used in this study did not affect the mRNA expression of these growth factor and this lack of results may be associated with the time-course of induction of these factors, and the intensity of the exercise protocol. These results suggest that the effect of exercise in mediating protection against LPS-induced impairment in spatial memory observed here is not associated with the exercise-modulation of BDNF and other growth factor signalling and it is likely modulated by the anti-inflammatory effect of prior exercise.

5.5.3 Summary

In summary, our results demonstrated prior short-term exercise protected against LPS-induced spatial memory impairment in the OD task, 4 hours after systemic LPS administration. The mechanisms mediating the effects of exercise are likely to involve the anti-inflammatory modulatory action of exercise, as we demonstrated exercise attenuated central expression of inflammatory cytokines, decreasing expression of general inflammatory markers and priming microglia towards a M2 alternative state phenotype rather than an evident effect of exercise mediated by increase in BNDF signalling.

The effects of cytokines and glial cell activation have been associated with disrupted BDNF signalling and impairment in the process of neurogenesis, thus we would expect an anti-inflammatory modulation of exercise to protect against inflammation-induced cognitive impartment through this mechanism. However, we did not observed changes in BDNF-stimulated signalling pathways, perhaps because of the length of our intervention or the time of analysis, and we did not analyse neurogenesis in this study, we could suggest that further tissue analysis may help elucidate the underlying changes mediating the behavioural changes observed. Moreover, we could suggest that a significant modulation of exercise in counteracting LPS-induced increase in the expression of pro-inflammatory cytokines, such as IL-1β, may be the major manifestation of the anti-inflammatory effect of exercise and subsequent protection of cognitive function, as several studies have reported IL-1β plays a pivotal role in cognitive disruption induced by age or injury. Finally, the results of this study strongly support our findings from previous chapters, and indicate the anti-inflammatory effect observed in this study could persist even months after exercise cessation.

Chapter 6

General Discussion

Chapter 6: General Discussion

6.1 Discussion

This study demonstrates, for the first time, the importance of early-life engagement in physical activity to more successful ageing in a mouse model of age. Moreover, this study extended current findings in the literature regarding the effects of exercise on learning and memory and the involvement of inflammation and neurogenesis in mediating these effects. To our knowledge, for the first time, we demonstrated that early-life exposure to physical exercise had a rapid (within 2 months) enhancing effect on learning and memory that persisted throughout the lifespan and protected against age-induced impairment in spatial and other forms of memory impairment, even months after exercise ceased.

There are numerous studies reporting the effects of different exercise modalities on cognition that link this effect to improvements in synaptic plasticity and neurogenesis in young and aged animals (van Praag, 1999). In support of this hypothesis, Chen and colleagues have reported that exercise enhances hippocampal neurogenesis and rescues learning and memory impairments in hippocampal-lesioned adult rats (Chen et al., 2006). In addition, previous studies from our laboratory demonstrated short-term forced exercise to enhance spatial and non-spatial memory in rats accompanied by an increase in BDNF concentration in the brain. Also, Marlatt and collaborators (2005), indicated that voluntary running throughout middle-age improves spatial-memory in the MWM and is associated with increased hippocampal BDNF and hippocampal neurogenesis (Marlatt et al., 2012), demonstrating a pro-neurogenic and pro-cognitive effect of exercise. Therefore, our findings not only confirm and corroborate these studies of the benefits of exercise in enhancing learning and memory, but they also support the emerging evidence of the impact of enriched experiences during youth in building cognitive reserve, which could protect against the adverse effects of both the normal ageing process and neurodegeneration (Stern, 2012).

Stern and colleagues have studied the existence of such reserve, and whether it is responsible for promoting brain resilience against deleterious effects of insults and ageing (Stern, 2012). This reserve theory proposes that lifestyle factors such as social and physical activity may contribute to an individual's capability to maintain cognitive abilities even in the face of pathologies or ageing, by increasing brain resources and consequently making the brain more flexible and capable of recruiting necessary resources (Stern, 2006). Evidence supporting the existence of this reserve includes the findings from several laboratories that enriched environment exposure throughout the lifespan protects against age-induced cognitive decline associated with a decrease in inflammatory markers in the brain, protection against impairment in neurogenesis and preservation of integrity of specific brain regions (Cao et al., 2017, Birch and Kelly, 2018). Another study in rats demonstrated that exposure to voluntary running wheel, in early-life, protected against cognitive deficits in contextual fear conditioning and induced neurogenesis and enhanced activity of the newborn neurons, even 4 months after exercise cessation (Shevtsova et al., 2017). These studies support our findings and also indicate a possible involvement of a building of cognitive reserve as a consequence of early-life exposure to new experiences, mediating the persistent cognitive enhancement observed in the first study. They also suggest the possible involvement of structural brain changes induced by life experiences, including preserved neurogenesis and maintenance of brain volume, in the process of building this reserve.

Moreover, in the past years, Kempermann (2008) also proposed the existence of a neurogenic reserve, which is partially based on the idea that new neurons that are not fully integrated into the circuitry could remodel their connections in response to experiences, suggesting that life experiences not only influence the number of newborn neurons, but could also mould the functionality of these new cells. In this context, exercise has been shown to affect not only NSCs proliferation, but also differentiation and survival of newborn neurons in the DG of middle age mice (Wu, 2008). In addition, in a more recent study, exercise was also found to reorganise the morphology, physiology and early network of new neurons, contributing not only to the survival of newborn neurons, but also to their integration and functionality (Sah et al., 2016).

Our study strongly demonstrated the persistence of permanent molecular and structural brain modifications induced by exercise, even after months in the absence of exercise, including increased synaptogenesis and neurogenesis as well as increases in whole brain and hippocampal volume. Our findings support the hypothesis that structural changes induced by life experiences are involved in building a cognitive reserve and suggest that this reserve mediates the persistent effect of exercise on cognition, even months after the end of exercise. Disruption of synaptic plasticity and neurogenesis have been widely reported during the ageing process. Also, age-induced decrease in the volume of specific brain regions, including the hippocampus, have been reported in individuals with MCI and in normal ageing (Raz et al., 2004). However, in this study we did not observe any age effect in the expression of the synaptogenesis-related proteins analysed, hippocampal and total brain volumes or neurogenesis. This lack of observed age-related molecular and structural brain alterations, could be partially influenced by the constant handling and by the exposure of sedentary mice to the stationary treadmill and to the learning and memory tasks carried out during the entire period, which could contribute to protecting against these alterations due to the effect of a minimal enriched environment exposure throughout the lifespan, without affecting the manifestation of cognitive impairment due to age. Supporting this hypothesis, a recent study from our laboratory demonstrated that lifelong exposure to an enriched environment, in the absence of exercise, protected against age-related structural brain modifications (Birch and Kelly, 2018).

Although no changes in synaptogenesis and neurogenesis and no reduction in brain region volumes were observed in age, our results demonstrated that exercise induced significant modifications in these parameters, even 10 months after exercise cessation, suggesting these molecular and structural modifications induced by the early-life exposure to physical exercise are likely to be involved in the process of building up cognitive reserve, which may be mediating the learning and memory improvements observed in our first study. Moreover, together with the beneficial effects of early-life exercise in cognitive function observed in our study, we demonstrated a reduction in anxiety and depression-like behaviours in exercised mice at old age. In parts these findings can be associated with the persistent effect of exercise in enhancing neurogenesis, as studies have

demonstrated that neurogenesis also plays an important role in control of emotional processes, such as anxiety and depression, and impaired hippocampal neurogenesis has been linked to increased anxiety and depression-like behaviour as wells as being a feature of several psychiatric disorders (Revest et al., 2009).

There are many ways in which exercise may mediate such beneficial modulation in synaptic plasticity, neurogenesis and consequently in cognition. Exercise may affect brain functions by increasing blood flow and brain perfusion, increase the delivery of oxygen and nutrients to the brain and change the CNS microenvironment, which may be important in supporting neurogenesis (So et al., 2017). Moreover, exercise could increase neurogenesis through the modulation of neurotransmission and upregulation of several growth factor and neurotrophins within the brain. Among these factors, BDNF is the most widely-reported candidate in mediating the pro-neurogenic and pro-cognitive effects of exercise (Cotman and Berchtold, 2002). Another way in which exercise can mediate pro-cognitive and pro-neurogenic effects is by modulating inflammation and glial cell activation. Evidence supports the importance of a "positive" microenvironment for the process of neurogenesis, from proliferation to survival and network incorporation (Kohman and Rhodes, 2013). The maintenance of this positive microenvironment is complex, and several factors are likely to be involved in this, including vasculature changes, adequate balance of growth factors and neurotrophins and glial cell support (Morrens et al., 2012, Lee et al., 2002). In this context, the immune system has been shown to contribute (positively and/or negatively) to this microenvironment through the activation of resident brain immune cells (microglia and astrocytes) and by infiltration of peripheral inflammation molecules into the CNS (Yirmiya and Goshen, 2011).

Ageing is accompanied by chronic and low-grade inflammation, involving the presence of increased levels of pro-inflammatory cytokines and glial cell activation. Exercise has been widely described to exert important anti-inflammatory modulation. This modulation is primarily initiated in the periphery and the possible mechanisms described to mediate this, include exercise-induced increase in production and release of cytokines from contracting skeletal muscle (such molecules called myokines, of which IL-6 is the best known), and reduction in the

expression of TLRs on monocytes and macrophages, contributing to a subsequent inhibition in production of pro-inflammatory cytokines. However, anti-inflammatory effects of exercise are not restricted to the periphery, and as the brain is no longer considered to be an "immune privileged site", it is known that peripheral immune cells communicate with the brain, and consequently, it is proposed that exercise can modulate neuroinflammation and glia cells activation in the CNS (Spielman et al., 2016). Interestingly, the anti-inflammatory modulation of exercise was one of the most prominent effects of exercise observed in this study. In this study, we observed age-induced increase in brain expression of pro-inflammatory cytokines and early-life exposure to physical exercise attenuated the increase in the expression of several pro-inflammatory cytokines, including IL-1β and TNF-α as well as increasing significantly the expression of cortical and hippocampal levels of anti-inflammatory cytokine IL-10, even months after exercise cessation. For instance, elevated brain expression of IL-1β is reported to impair neurogenesis by affecting cell proliferation and differentiation, indicating that IL-1β can act directly on NPCs (Green and Nolan, 2012) Moreover, TNF-α has been shown to reduce cell proliferation in the DG and promote apoptosis of hippocampal NPCs (Hofer et al., 2011, Seguin et al., 2009), suggesting that the modulation of exercise attenuating pro-inflammatory cytokines may be restoring the balance in the brain microenvironment and promoting positively the process of neurogenesis, even months after exercise cessation.

Ageing is also associated with mild and persistent inflammation, and glia cells in the aged brain have been shown to be primed towards the classic inflammatory phenotype, as microglia in aged animals express high levels of MHC II, CD86 and pro-inflammatory cytokines (Godbout et al., 2005). The age-primed activation of microglia and astrocytes towards a pro-inflammatory phenotype, may contribute towards a disruption of the brain microenvironment, contributing to impairments in neurogenesis. In part, the age-related decrease in hippocampal neurogenesis has been associated with microglia activation and increased expression of pro-inflammatory cytokines, such as IL-1β, in the brain (Kuzumaki et al., 2010) and thus, exercise may contribute positively by counteracting neuroinflammation and restoring neurogenesis. Surprisingly, our study demonstrated that early-life exercise attenuated the age-priming effect on microglia cells towards a classical

activation, but had minimal effects in markers of “alternative” microglia activation. However, we observed a more robust and persistent effect of early-life exposure to physical exercise in attenuating the classically reactive state of isolated astrocytes, since astrocyte reactivity was found to be decreased in specific-hippocampus subregions, in exercised mice. The direct modulation of glia by exercise has been demonstrated previously in microglia cells, supporting our findings. A study showed that voluntary running in aged mice reduced microglia proliferation and decreased the number of M1 state activated microglia (Kohman et al., 2012). Most recently, another study suggested that voluntary running had minimal effect on M2 markers, such as Arg-1, IL-1ra, TGF-β, and CD206, in the hippocampus of adult and aged mice following IL-4/IL-13 administration (Littlefield and Kohman, 2017). However, to the best of our knowledge, the direct activation of astrocytes by exercise has not been reported before now.

In this context, as priming of classic activation of glia cells by the ageing process could be detrimental to neurogenesis and cognition, the effects of exercise to modulate an alternative glial cell activation and attenuation of the shift towards a pro-inflammatory state, may support these processes. Evidence suggests that anti-inflammatory cytokines may support neurogenesis. For instance, treatment of NPCs with IL-4 stimulated microglia increased the proportion of newborn neurons (Butovsky et al., 2006) and microglia cells stimulated with IL-10 have been reported to enhance NPCs proliferation, with no effect on differentiation (Kyota et al., 2011). This studies are compatible with our findings and lead us to suggest that the effect of exercise in attenuating classical activation of glial cells could be mediating the persistent effects of exercise in increasing hippocampal synaptogenesis and neurogenesis resulting in the persistent cognitive enhancement observed in the first study, even months after exercise cessation. Interestingly, the modulatory effect of exercise on inflammation induced by LPS was similar to the modulation of exercise in the inflammatory profile, 10 months after exercise cessation. In this study, exercise-mediated protection against cognitive impairment was associated with drastic attenuation of the LPS-induced increase in brain expression of inflammatory cytokines and modulation of microglia and astrocyte activation stimulated by LPS administration.

Therefore, regarding changes in the inflammatory profile induced by exercise, this study can suggest that exercise has a strong anti-inflammatory role. Interestingly, this modulation was found to occur immediately after a short-term exercise intervention and to persist for months after a long term early-life exposure to physical exercise, differing only in the cell type affected by exercise in the two studies. While the short-term exercise robustly affected microglia activation, the persistent effect of early-life exercise more effectively modulated astrocyte activation. Taken together, the results presented here, leads us to speculate that the persistent effects of exercise in modifying the inflammatory profile, even months in the absence of exercise, was due to an very strong effect of exercise in the brain inflammatory profile during the period of exercise that persisted even long after exercise cessation.

In conclusion, for the first time, we demonstrated that exercise has an important role in enhancing cognition and emotional behaviour as well as protecting against cognitive impairments. We suggest that this improvement was mediated in large part by the anti-inflammatory effects of exercise, which positively modulated the brain environment and consequently protected against the disruption of brain mechanism structure and function induced by neuroinflammation and age, as reported in this study. Accordingly, this anti-inflammatory effect of exercise was shown to be directly involved in the preservation and enhancement of the mechanisms of hippocampal neurogenesis and synaptogenesis induced by exercise in our study and consequently the protection of the integrity of important brain regions. Finally, all these changes could be contributing to building a cognitive reserve that renders the brain more resilient, thereby mediating the protection against cognitive decline during the age process, observed here.

6.2 Limitations and future directions

This study has identified a long-term persistent effect of early-life exposure to physical exercise, protecting the cognitive decline induced by age and suggesting the involvement of exercise-induced positive changes in brain inflammatory profile driving persistent molecular and structural brain alterations, such as synaptogenesis, neurogenesis and brain volume changes, thereby contributing to

a greater cognitive reserve and consequently reflecting in the cognitive and emotional behavioural improvements observed in this study. However, even though such changes have been shown in our study, further research is warranted to more clearly define the mechanism by which exercise is building-up the cognitive reserve and mediating its effects on cognition. In addition, some unexpected observations raised some questions within this field of research that merit further study.
The results demonstrated a persistent modulation by exercise of peripheral and central inflammatory responses, however, in the periphery we have demonstrated a discrete persistent modulation of exercise in pro-inflammatory cytokine expression in the liver. The gut-brain axis, which consists of bidirectional interaction between the central and the enteric nervous system linking emotional and cognitive brain areas with peripheral intestinal microbiome, have been shown to influence neuro-endocrine systems associated with stress response, anxiety and memory function (Carabotti et al., 2015). As mentioned in the material and methods section, gut samples were collected and have been sent to the Microbiome Institute in University College Cork and RNA sequence analysis is being carried out to analyse possible changes in the microbiome that may modulate the neuroinflammatory responses observed and a possible effect of exercise in modulating any age-induced changes. Moreover, regarding peripheral inflammation, serum samples and bone marrow were also collected from the mice in the first study, and further analysis of serum samples as well as generation of bone marrow-derived macrophages could contribute to further understanding the mechanisms mediating the persistent anti-inflammatory of exercise.

Moreover, as we have demonstrated a persistent anti-inflammatory effect of exercise not only in peripheral but also in central tissues, particularly glial cells activation, further analyses of glial cell markers co-localised with markers of glial cell activation by immunohistochemistry, may help us to identify more clearly the specific effects of exercise in modulating microglia and astrocytes. Furthermore, microglia cells have been reported to be primed towards a M1 state due to age, and this primed state has been associated with changes in the brain metabolic state, characterised by a shift in microglia metabolism towards the glycolytic state (Orihuela et al., 2016). In this context, given the positive modulation of exercise in

controlling the activation state of this glial cells that persisted months after exercise cessation, a detailed analysis of possible effects of exercise in modulating changes in metabolic shift in microglia from the aged brain may expand our knowledge of the effects of exercise on these cells. Also, given that this metabolic shift in microglia metabolism is associated with reduced microglia phagocytic capacity, a process important in shaping neurogenesis (Sierra et al., 2010), analysis of microglia phagocytic capacity is an important point to be considered.

To elucidate further the effects of exercise on cell proliferation, differentiation and survival, immunohistochemistry analysis could be extended to investigate the expression of Ki67 and DCX markers, to analyse a possible persistent effect of exercise also in the proliferation of NSCs. Moreover, analysis of neurogenesis in the tissue of mice from the second group (the model of neuroinflammation), may link the beneficial effect of exercise attenuating inflammation to changes in neurogenesis that are reflected in the cognitive protection observed. Also, further analysis of MRI images and investigation of the more possible structural brain changes induced-by exercise, could contribute to support the presence of more permanent changes mediated by exercise, reinforcing the involvement of cognitive reserve in mediating the effects observed in this study. Moreover, as it has been described that within the SGZ of the dentate gyrus, only a few newborn cells are incorporated into the circuitry, and the majority of them are presumed to die before they achieve maturity (Kempermann et al., 2004), a further investigation regarding apoptosis in the DG, might reveal a fuller picture of the effects of exercise on cell turnover in the brain.

Furthermore, an important effect of exercise mediating brain modifications is through the increase of blood flow to the CNS and improvement in brain perfusion, contributing also, to the process of angiogenesis (Leosco et al., 2007). Thus, analysis of possible effects of exercise on angiogenesis may contribute to further elucidate the beneficial effects of exercise we observe here. Finally, as the most best-described mechanism by which exercise mediates cognitive enhancement is through its effects in increasing BDNF expression and activating the BDNF-TrkB downstream signalling, and by increasing other growth factors and neurotrophins, further analysis of the tissue, using different and more sensitive methods, like

immunohistochemistry, may provide further information on whether these key signalling pathways do in fact mediated any of the cognitive changes we report here.

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