DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

A_{2A} receptor blockade in the control of microglia impact upon neurons during early development

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Doutora Catarina Alexandra dos Reis Vale Gomes (Centro de Neurociências e Biologia Celular) e a orientação institucional do Professor Doutor Ângelo José Ribeiro Tomé (Universidade de Coimbra).

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2013

The experimental work described in this thesis was performed at the Center for Neuroscience and Cell Biology (CNC), University of Coimbra, under the supervision of Doctor Catarina Alexandra dos Reis Vale Gomes.

O trabalho experimental descrito nesta tese foi realizado no Centro de Neurociências e Biologia Celular, Universidade de Coimbra, sob a orientação da Doutora Catarina Alexandra dos Reis Vale Gomes.

Entrega sempre a tua beleza
sem cálculo, sem palavras.
Calas-te. E ela diz por ti: eu sou.
E com mil sentidos chega,
chega finalmente a cada um.

Rainer Maria Rilke,
in "O Livro das Imagens"

AGRADECIMENTOS

Embora a capa desta tese só vá levar o meu nome, há um grupo de pessoas sem as quais este trabalho não teria sido possível. Em primeiro lugar, quero agradecer à minha orientadora, Catarina Gomes, pela sua inexorável criatividade, abertura, apoio, dedicação e orientação. Ensinou-me a paciência e o cuidado. O esforço e o empenho. A audácia de enfrentar a vida que nos é dada com os recursos que temos. A Catarina é realmente um exemplo a seguir e tive muito gosto e orgulho em tê-la como orientadora.

Agradeço ao Professor Rodrigo Cunha pela oportunidade concedida de realizar esta tese no grupo de investigação *Purines at CNC*, por toda a partilha científica e confiança. Agradeço, também ao Professor Ângelo Tomé e Professor Ramiro de Almeida por todo o conhecimento transmitido, apoio e disponibilidade.

À Maria Joana por toda a ajuda dispensada durante o meu trabalho, pela paciência e pelo apoio, pela energia e sorriso contagiante e contagioso! Era impossível estar perto dela e não ter vontade de compartilhar aquela energia.

Agradeço também a todos aqueles que comigo partilharam o laboratório LEF do Centro de Neurociências e Biologia Celular de Coimbra, passado e presente, pela companhia, alegria e companheirismo. De forma especial aos 'mestrandos cá do sítio': Anna Pliássova, Tiago Silva e Rui Beleza! Agradeço também a todos os elementos do *Medical Mycology – Yeast Research Group* (MMYRG), nomeadamente à Professora Teresa Gonçalves pela amabilidade na cedência de espaço!

Gostaria de agradecer a todos os meus familiares e amigos, que durante o mestrado me foram 'apaparicando' das mais variadas formas. Deste modo, por forma a agradecer a fidelidade e o apreço demonstrado por todos aqueles, que de uma forma ou de outra, foram escrevendo a PAIXÃO pela vida ao meu lado, o meu sincero BEM-HAJA!

Por último, mas não menos importante, agradeço aos meus pais, aos meus avós, à minha madrinha Ana e ao meu tio Luís por estarem sempre lá para mim. Um grande beijo à Mariana e sonhos arco-íris, afilhada!

Bem-hajam,

Gonçalo Pires Cristóvão

In addition to the work directly related with this thesis, I also participated in other projects. The work developed resulted in a manuscript *in preparation* for submission and was presented in different national and international scientific meetings (listed below). This parallel work allowed me to acquire experience in different methodological/technical approaches:

- phagocytic assays (confocal microscopy of internalized apoptotic cells);
- quantification of proteins (Western blot and enzyme-linked immunosorbent assay, ELISA);
- metabolism (nuclear magnetic resonance, NMR);
- enzymatic activity;
- primary cultures of microglia; cell lines.

George J, Cristóvão G, Queiroz F, Rodrigues L, Gonçalves T, Meyer-Fernandes R, Cunha RA, Gomes CA. **ATP-derived adenosine boosts microglial proliferation in inflammatory-like conditions** (manuscript *in preparation*).

George J, Cristóvão G, Queiroz F, Gomes CV, Cunha RA. **Lipopolysaccharide and glutamate determine different levels of extracellular ATP and dictate opposite microglial proliferation levels**. XLIII Annual Meeting of the Portuguese Society for Pharmacology, Porto, Portugal, Feb 06-08, 2013 (oral communication).

Cristóvão G, Pinto MJ, Ryu H, Jeon NL, Gomes CV, Almeida RD, Cunha RA. **Neuron-microglia interactions in the developing CNS: focus on the role of microglia before synapse formation.** XIII Meeting of the Portuguese Society for Neuroscience, Luso, Portugal, May 30-Jun 01, 2013 (oral communication and poster).

Cristóvão G, Viegas M, Vieira O, Cunha RA, Gomes CV. **Microglia phagocytic ability is preserved irregardless changes in ATP levels and depends upon adenosine A_{2A}R tonic activation.** XIII Meeting of the Portuguese Society for Neuroscience, Luso, Portugal, May 30-Jun 01, 2013 (oral communication and poster).

Lemos C, Cristóvão G, Jarak I, Cunha RA, Gomes CV, Carvalho RA. **Adenosine A_{2A} receptors control the metabolic changes associated with microglia activation as revealed by NMR isotopomeric analysis.** 43rd Annual Meeting of the Society for Neuroscience, San Diego, California, Nov 9-13, 2013 (poster accepted).

ABSTRACT

Yolk-sac derived primitive macrophages, precursors of microglial cells, begin migrating into and colonizing the brain between embryonic days 8 and 9.5 (E8 and E9.5), well before synaptogenesis. Due to their unique phagocytic function in the brain, microglia has been proposed as the sculptor scavenger of the developing brain: communication between neurons and microglial cells at the synaptic level results in the elimination of unwanted synapses – a process termed *synaptic pruning*. During brain development but before synaptogenesis, neurons establishing neural circuits require trophic support to synapse onto the respective target cell. Recently, it was reported that microglial elimination or inactivation compromises neuronal survival in developing brain, raising a new hypothesis for microglial role during early development, besides the well-known phagocytic ability: a supportive role.

Other studies have shown that microglial cells are required for the development of mature synapses during phases of development subsequent to synapse formation (e.g. regulation of the number of functional synapses) or to regulate adult neurogenesis. Several factors synthesized by target neurons or surrounding glia may exert a supportive role for the growing axon; however, to date, it was not clearly identified the mechanism by which microglia could interfere with pre-synaptic differentiation, neither the exogenous or endogenous substances able to control these microglial responses.

In the present work we investigated the potential contribution of microglial cells to: (i) the accumulation of synaptic vesicles with presynaptic material along the axon – synapsin puncta –; (ii) the length of the axonal network; and (iii) the growth cone morphology, in the absence and in the presence of microglial modulators: lipopolysaccharide (LPS) and adenosine A_{2A} receptor ($A_{2A}R$) ligands.

Adenosine is a neuromodulator which activates different receptor subtypes; $A_{2A}R$ activation controls important microglial functions, namely if the cells are conditioned by the presence of the classical activator, LPS, a bacterial antigen. Importantly, the experiments were conducted in

co-cultures of developing neurons and microglia established in appropriate devices to allow selective pharmacological blockade of microglial $A_{2A}R$ previous to the activation of neuronal $A_{2A}R$.

The present results: (i) show that microglial activation by the bacterial antigen LPS increases the density of synapsin puncta in developing neurons and that $A_{2A}R$ blockade does not affect synapsin density in both activated and non-activated microglia; (ii) suggest that neuronal and microglial $A_{2A}R$ may exert differential roles in the regulation of the length of the axonal network; (iii) show that microglia tends to increase the growth cone area.

Altogether, these data suggest that, besides their role in *synaptic pruning*, microglia may be involved in events leading to axonal maturation, likely providing spatial information for synapse positioning and controlling axonal network. These novel observations reinforce the concept that any change of microglial cells during the early phases of brain development may result in synaptic abnormalities associated with neurodevelopmental disorders.

Keywords: adenosine A_{2A} receptor, brain development, microglia-neuron interaction.

RESUMO

Os precursores da microglia (macrófagos primitivos) migram para e colonizam o cérebro entre os dias embrionários 8 e 9.5 (E8 e E9.5), antes da formação de sinapses. Atendendo à sua capacidade fagocítica, tem sido descrito como preponderante o papel da microglia na definição de contactos sinápticos: a comunicação entre os neurónios e a microglia ao nível sináptico resulta na eliminação de sinapses – um processo denominado de *synaptic prunning*. No desenvolvimento do sistema nervoso, mas antes da sinaptogénese, o estabelecimento de circuitos neuronais requer suporte trófico ao axónio em desenvolvimento até à respectiva célula-alvo. Recentemente foi descrito que a eliminação da microglia ou a sua inactivação comprometem a sobrevivência neuronal, gerando uma nova hipótese para o papel da microglia durante o neurodesenvolvimento, para além da já conhecida capacidade fagocítica: um papel de suporte.

Outros estudos têm demostrado que a microglia é necessária para o desenvolvimento de sinapses maduras em fases precoces do desenvolvimento (por exemplo, na regulação do número de sinapses funcionais) ou na regulação da neurogénese adulta. Vários factores libertados pelos neurónios-alvo ou pelas células gliais podem exercer um papel de suporte para o crescimento axonal; contudo, não foram identificados os mecanismos pelos quais a microglia poderá interferir com a diferenciação pré-sináptica, em particular quando sujeita a substâncias exógenas ou endógenas capazes de controlar as suas repostas.

No presente trabalho investigámos a contribuição da microglia: (i) na acumulação de vesículas com material pré-sináptico ao longo do axónio – *synapsin puncta*; (ii) no comprimento da rede axonal; e (iii) no cone de crescimento, na ausência e na presença de moduladores microgliais, o lipopolissacárido (LPS) e um antagonista selectivo dos receptores A_{2A} de adenosina ($A_{2A}R$).

A adenosina é um neuromodulador que activa diferentes subtipos de receptores; a activação dos $A_{2A}R$ controla importantes funções microgliais, nomeadamente quando condicionadas pela presença do LPS. As experiências foram realizadas numa co-cultura de neurónios imaturos em

desenvolvimento e de células da microglia em dispositivos que permitem a modulação farmacológica selectiva dos $A_{2A}R$ da microglia, antes da activação dos $A_{2A}R$ neuronais.

Os resultados do presente trabalho: (i) indicam que na presença do LPS, a microglia induz um aumento do número de *synapsin puncta* em neurónios. Este número não é afectado pela presença de um antagonista selectivo dos $A_{2A}R$, na ausência e/ou na presença de LPS; (ii) sugerem que os receptores $A_{2A}R$ neuronais e microgliais podem exercer diferentes funções na regulação do comprimento da rede axonal; e (iii) revelam que a microglia tende a aumentar a área do cone de crescimento.

Os presentes resultados sugerem que, para além do seu papel de *synaptic prunning*, a microglia pode estar envolvida nos processos de maturação axonal, e condicionar o posicionamento espacial da sinapse, uma hipótese que requer investigação futura. O presente trabalho corrobora a tese de que qualquer alteração ao nível da microglia pode resultar em perturbações do neurodesenvolvimento decorrentes de alterações sinápticas.

Palavras-chave: receptor A_{2A} de adenosina, neurodesenvolvimento, interacção microglia-neurónio.

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LIST OF ABBREVIATIONS

A_1R – adenosine A_1 receptor subtype

A_{2A}R – adenosine A_{2A} receptor subtype

A_{2B}R – adenosine A_{2B} receptor subtype

A_3R – adenosine A_3 receptor subtype

AC – adenylate cyclase

ADA – adenosine deaminase

AM – amoeboid microglial cells

AMP – adenosine 5'-monophosphate

AMY – amygdala

ANOVA – analysis of variance

ATP – adenosine 5'-triphosphate

BDNF – brain-derived neurotrophic factor

BM – bone marrow

BSA – bovine serum albumin

bv – blood vessels

cAMP – cyclic adenosine 5'-monophosphate

CCL2 – CC-chemokine ligand 2

CD – cluster of differentiation

C-domain – central domain

CNS – central nervous system

CRAMP – cathelicidin-related antimicrobial peptide

CSF1 – colony-stimulating factor 1

CSF1R – conoly-stimulating factor 1 receptor

CX_3CL1 – CX3C-chemokine ligand 1 or fractalkine

CX_3CR1 – fractalkine receptor

DAPI – 4',6-diamidino-2-phenylindole

DG – dentate gyrus

DIV – days in vitro

E –embryonic age

EDTA – ethylenediamine tetraacetic acid

ELISA – enzyme-linked immunosorbent assay

F-actin – filamentous-actin

FBS – fetal bovine serum

FM – fetal macrophage

GPCR – G protein-coupled receptor

Iba 1 – ionized calcium-binding adapter molecule 1

IL – interleukin

IR – immunoreactivity

LPS – lipopolysaccharide

M – monocyte

MCP – microfluidic culture platform

MHC II– major histocompatibility complex II

mQH_2O – mili-Q water

N – nucleus

NIH – national institute of health

NMR – nuclear magnetic resonance

NO – nitric oxide

P – postnatal day

PAF – paraformaldehyde

PAMP – pathogen-associated molecular pattern

PBS – phosphate buffered saline

PDL – poly-D-lysine

PDMS – poly-dimethylsiloxane

P-domain – peripheral domain

PKA – cAMP-dependent protein kinase

PRM – primitive ramified microglial cells

PRR – pattern-recognition receptor

PU.1 – transcription factor Spi-1

RGMa – repulsive guidance molecule a

RM – ramified microglial cells

ROI – regions of interest

ROS – reactive oxygen species

RPMI – Roswell Park Memorial Institute

RT – room temperature

SCH58261 – 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4 triazolol[1,5c]pyrimidine

SEM – standard error of mean

SV – synaptic vesicle

TBS – tris buffered saline

TLR – toll-like receptor

TNF – tumour necrosis factor

TSP – thrombospondin

T-zone – transition zone

CHAPTER 1

– INTRODUCTION –

1. INTRODUCTION

1.1. OVERVIEW OF BRAIN DEVELOPMENT

Neurons, as well as other cells of the nervous system are derived from the dorsal ectoderm of the embryo. During development, the embryonic ectoderm appears and forms the neural plate along the dorsal side of the embryo. The most anterior part of the neural tube expands rapidly due to cell proliferation, and gives rise to the brain (Kiernan and Rajakumar, 1998).

Neural development includes the migration of immature neurons from different parts of the developing brain, the outgrowth of axons and dendrites from neurons, the guidance of the motile growth cone towards postsynaptic partners and the generation of synapses between these axons and their postsynaptic partners (Kiernan and Rajakumar, 1998).

1.1.1. NEURONAL MATURATION

During development of the central nervous system (CNS), neurons evolve through several stages, characterized by evident morphological and functional changes (Figure 1.1.) (Dotti *et al.*, 1988; Craig and Banker, 1994). The ability of neuronal cells to polarize is essential for the organization of the nervous system, but the ultimate objective is the formation of a synaptic contact. For this, developing axons undergo elongation and possess at their tips an actin-rich growth cone, which is a highly motile structure (Craig and Banker, 1994; Yoshimura *et al.*, 2006).

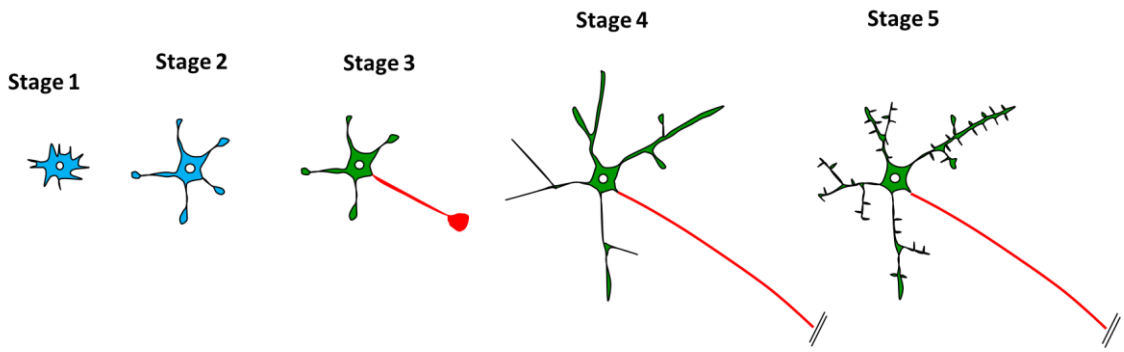


Figure 1.1. – Processes of neuronal polarization in cultured hippocampal neurons. Stage 1, immature neurons display intense lamellipodial and filopodial protrusion activity (0 days *in vitro,* DIV0); **stage 2**, multiple immature neurite extension (DIV1-2); **stage 3**, neuronal symmetry breaks and a single neurite growth rapidly to become the

axon (red) (DIV2-4); **stage 4**, rapid axon and dendrite outgrowth, branching (DIV4-15); **stage 5**, cultured neurons form synaptic contacts and establish a neuronal network (DIV15-25) (adapted from Yoshimura *et al.*, 2006; Polleux and Snider, 2010).

In a first stage, hippocampal neurons extend lamellipodia, which contain cross-linked networks of actin filaments, and filopodia, tensile structures composed of bundled filamentous-actin (F-actin). In stage 1, neurites appear to arise preferentially at lamellipodial patches (Figure 1.1., stage 1) (Yoshimura *et al.*, 2006; Arimura and Kaibuchi, 2007; Polleux and Snider, 2010).

After several hours, neurons extend and form a number of immature neurites (outgrowth of the minor processes) (Figure 1.1., stage 2) (Yoshimura *et al.*, 2006; Arimura and Kaibuchi, 2007; Polleux and Snider, 2010). The neurites are morphologically similar, and undergo repeated, random growth and retraction. However, several hours after the appearance of minor processes, one of these processes begins to extend at a higher rate, becoming longer than the other neurites. This process is the axon, and the cell has become polarized (Figure 1.1., stage 3) (Yoshimura *et al.*, 2006; Arimura and Kaibuchi, 2007; Polleux and Snider, 2010).

The other minor processes continue to undergo brief spurts of growth and retraction, maintaining their net length, for up to a week, when they become mature dendrites. Dendritic growth begins only after DIV 4, 2-3 days after axonal outgrowth. So, the dendritic growth is slower than axonal growth and several dendrites grow at the same time (Figure 1.1., stage 4) (Yoshimura *et al.*, 2006; Arimura and Kaibuchi, 2007; Polleux and Snider, 2010).

During the stage 4, dendrites become thicker and begin to establish dendritic components and to construct premature dendritic spines. When the axon and dendrites are mature, neurons form synaptic contacts that enable the trans-cellular transmission of information (Yoshimura *et al.*, 2006; Arimura and Kaibuchi, 2007; Polleux and Snider, 2010). This is the moment where the growth cone undergoes a profound transition from a highly motile structure to a functional presynaptic terminal (Figure 1.1., stage 5). Importantly, synaptic activity determines whether synapses will be stabilized or eliminated (Waites *et al.*, 2005).

1.1.2. GROWTH CONES GUIDANCE OF THE MATURATION AND ELONGATION OF DEVELOPING AXONS

Growth cones were discovered by Santiago Ramon y Cajal in 1890, but the first living growth cones extended by neurons in tissue culture were observed in 1907.

How does the axon detect and respond to axon guidance cues? In the developing nervous system, growth cone is a dilated terminal of axonal and dendritic processes equipped with surface receptors for extracellular signals, allowing axons to respond to diverse cues. As the growth cone moves outward, the axon elongates due, in part, to the polymerization of tubulin into microtubules, which give the axon its rigidity. In this structure three different compartments can be seen (Figure 1.2.): (1) the peripheral domain (P-domain), which is the most distal part of the growth cone. The P-domain is highlighted by lamellipodia and filopodia, that probe the extracellular environment; (2) the transition zone (T-zone), situated in the interface between the actin-rich P-domain and the central domain (C-domain); and (3) the C-domain, which is enriched in cellular organelles and has a dense microtubule array that extends from the axonal shaft to support the growth cone movement (Huber *et al.*, 2003; Vitriol and Zheng, 2012).

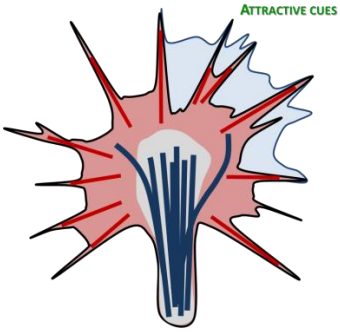


Figure 1.2. – Schematic representation of a growth cone. The three domains of the growth cone: growth cone's P-domain contains actin-rich lamellipodia (light red shaded) and filopodia (dark red lines); newly formed lamellipodia on the side undergoing a positive turning response is shown in blue; microtubules in the growth cone are largely restricted to the C-domain (dark blue lines) (adapted from Vitriol and Zheng, 2012).

During neuronal development, neurons project axons over long distances in order to reach their final targets; the neuronal growth cones, which are highly motile structures at the tip of a growing axon, follow specific pathways: they sense and respond to spatially and temporally

distributed guidance cues (environmental cues), that guide them to their appropriate direction (Yu and Bargmann, 2001; Dickson, 2002; O'Donnell *et al.*, 2009). There are various types of evolutionary conserved guidance molecules and receptors, both attractants and repellents (Tessier-Lavigne and Goodman, 1996; Huber *et al.*, 2003; Garbe and Bashaw, 2004; O'Donnell *et al.*, 2009; Kolodkin and Tessier-Lavigne, 2011). The activation of guidance receptors elicits intracellular signalling events, which control cytoskeleton activity to steer the growth cone (e.g. reorganization of actin filaments or stabilization of microtubules) (Huber *et al.*, 2003; Garbe and Bashaw, 2004; O'Donnell *et al.*, 2009).

Many molecules had been identified as modulators of growth cone dynamics. Important in the context of the present work, the main intracellular pathway operated by $A_{2A}R$, cyclic adenosine 5'-monophosphate (cAMP)-dependent protein kinase (PKA), is one of these players (Han *et al.*, 2007).

1.1.3. DIFFERENTIATION OF PRESYNAPTIC TERMINALS

Electrical and chemical signals are transmitted between neurons at specialized sites of contact – synapses, whose formation in the brain is important for learning, memory, perception, and cognition.

Presynaptic terminal differentiation is the period between axon formation and the establishment of the synapse. This period involves the coordinated action of several inter-dependent events: clustering and maturation of synaptic vesicles (SVs); presynaptic growth; precise alignment between postsynaptic density and the presynaptic active zone; cytoskeletal restructuring and assembly of vesicle recycling machinery; establishment of the active zone.

Two types of presynaptic terminals can be distinguished: at the ends of the axon ('*boutons terminaux*') or along the axon shaft ('*boutons en passant*') (Jin and Garner, 2008).

We could distinguish three different stages in presynaptic specialization: (1) biogenesis and transport, (2) trapping and stabilization, and (3) maturation and growth of synaptic components

(McAllister, 2007; Jin and Garner, 2008). Normally, synaptic components are constitutively generated in the cell soma and transported along the length of the axons by vesicular intermediates derived from the trans-Golgi network (Ahmari *et al.*, 2000), but the generation of mature SVs occurs after transport. Synapsins, SV-specific proteins, are associated with the cytoplasmic surface of small SVs (De Camilli *et al.*, 1983; Huttner *et al.*, 1983; Ziv and Garner, 2004).

Synaptogenesis is a process involving the formation of a neurotransmitter release site in the presynaptic neuron with a receptive field at the postsynaptic neuron. Synaptic proteins are crucial in the process: shortly after neurons differentiate and extend axonal and dendritic processes, many of the genes encoding synaptic proteins are turned on, resulting in the formation, accumulation and directional trafficking of vesicles (Garner *et al.*, 2002; Waites *et al.*, 2005). There are several secreted factors, receptors, and signalling molecules that make neurons receptive to form synapses. “Priming molecules” are target-derived factors that accelerate neuronal maturation or directly induce synapse formation. These factors that seems to make neurons competent to undergo synaptogenesis (Waites *et al.*, 2005), are synthesized by surrounding glia and/or target neurons, and have many activities (e.g. guide axonal projections to their correct targets, stimulate local arborization, promote neuronal differentiation and maturation and facilitate the initiation of synapse formation) (Gallo, 2011). Synaptic activity determines whether these synapses will be stabilized or eliminated, both during development and in the mature brain.

1.2. OVERVIEW OF MICROGLIA

Glial cells are an integral part of CNS networks and consist of two main populations: macroglia (astrocytes and oligodendrocytes) and microglia.

Microglial cells are distributed throughout the CNS, brain and spinal cord, and these cells are present in large numbers in all major divisions of the brain, although not uniformly distributed (Lawson *et al.*, 1990; Lyck *et al.*, 2009). Microglial cells are key players in the innate defensive system of the CNS, and their involvement in pathological conditions have been extensively investigated (Hanisch and Kettenmann, 2007; Kettenmann *et al.*, 2011; 2013).

Microglial cells are able to release factors that have been implicated in neuronal functions and behaviour (Hanisch, 2002). In non-pathologic conditions, microglial cells exhibit extensively ramified processes that perform a continuous surveillance of the surrounding CNS. Neurons have been suggested to maintain this phenotype, suppressing the activation of microglial cells through cell-cell contacts (Ransohoff and Cardona, 2010) and by the diffusion of neuronal mediators, namely CX_3C-chemokine ligand 1 (CX_3CL1) which binds and activates the cell-surface fractalkine receptor (CX_3CR1) on microglia, controlling cell activity (Rivest, 2003; Cardona *et al.*, 2006; Saijo and Glass, 2011).

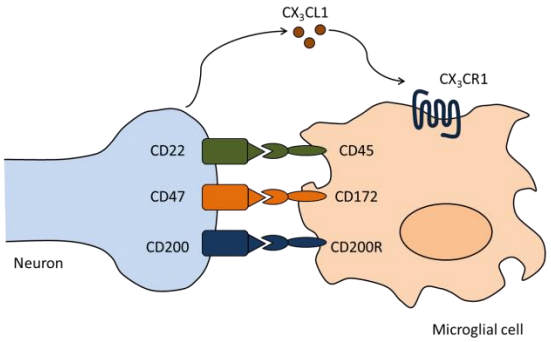


Figure 1.3. – Microglial phenotype in non-pathological conditions microglial phenotype. Phenotype of the microglial cells is maintained in part through neuron-derived signals, by the establishment of cell-cell contacts (e.g. cluster of differentiation 47, CD47, CD200 and CD22) and/or by the diffusion of neuronal mediators, namely CX_3CL1 (adapted from Saijo and Glass, 2011).

Microglial cells express pattern recognition receptors (PRRs), e. g. toll-like receptors (TLRs) that sense pathogen-associated molecular patterns (PAMPs) found on bacteria, viruses and fungi.

Besides PAMPs, other receptors are expressed by microglia, including P2 purinoreceptors and receptors for neurotransmitters (e.g. glutamate) that are important to detect changes in neuronal physiology (Inoue, 2008; Kettenmann *et al.*, 2011). Disturbances in homeostasis (for example an infectious or a traumatic stimuli) are detected by microglia, that exhibit a shift in morphology and increase the production of inflammatory mediators, such as cytokines, chemokines, reactive oxygen species (ROS), nitric oxide (NO) and neurotrophic factors (Bessis *et al.*, 2007). Such circunstances also trigger phagocytic activity of microglia, which is relevant to cope with damage (by elimination of infectious agents, dead and/or apoptotic neurons, debris …) (Schlegelmilch *et al.*, 2011).

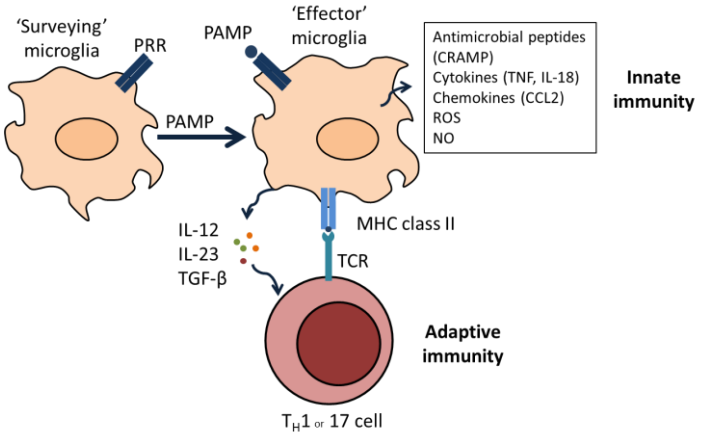


Figure 1.4. – Classically activated microglia participate in both innate and adaptive immune responses. PRRs are expressed by microglial cells that recognize various PAMPs found on bacteria, virus and fungi. Normally, PRR-mediated signalling induces the production of antimicrobial peptides (such as cathelicidin-related antimicrobial peptide (CRAMP)), cytokines (such as tumour necrosis factor (TNF), and interleukin-1 beta (IL-1β)), chemokines (such as CC-chemokine ligand 2 (CCL2), ROS and NO (adapted from Saijo and Glass, 2011).

The role of microglia in physiologic conditions started to be explored only in 2005 with two references reporting the ability of microglia to be active also in the healthy brain (Nimmerjahn *et al.*, 2005; Cardona *et al.*, 2006). In these conditions, microglial cells monitor neuronal activity and rectify changes, namely phagocytising (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005; Wake *et al.*, 2009; Tremblay *et al.*, 2010; Paolicelli *et al.*, 2011; Schafer *et al.*, 2012). However, their role in synapses formation during the CNS development is not completely established (Tremblay *et al.*, 2011). The fact that, during development, there is a massive invasion of microglia and an

increment of their density in the brain (Dalmau *et al.*, 1997, 1998), suggests a critical role of microglia in synaptogenesis and in the establishment of the neuronal network.

1.2.1. ORIGIN OF MICROGLIA

In 1913, Ramon y Cajal identified the so-called "third element" (non-astrocyte glial cells) of the CNS to distinguish from the "first element" (neuron) and "second element" (astrocyte). In 1919, Pio del Rio-Hortega, a student of Cajal, distinguished microglia and oligodendrocytes as separate components of the "third element" (del Rio-Hortega, 1919, 1921), after the invasion of the developing brain by mesodermal pial elements.

Del Rio-Hortega also suggested that microglia arise during embryonic development, indicating that they might represent a distinct developmental lineage of macrophages, even though they express a diversity of macrophage-associated markers, namely: CD11b, colony-stimulating factor 1 receptor (CSF1R), CX_3CR1, CD68 and ionized calcium-binding adapter molecule 1 (Iba-1) (Ginhoux *et al.*, 2010; Saijo and Glass, 2011; Greter and Merad, 2013). The lineage relationship between microglia and macrophages is clear; microglia are most closely related to bone marrow (BM)-derived and more distantly related to other haematopoietic and non-haematopoietic cell types (Figure 1.5.) (Saijo and Glass, 2011).

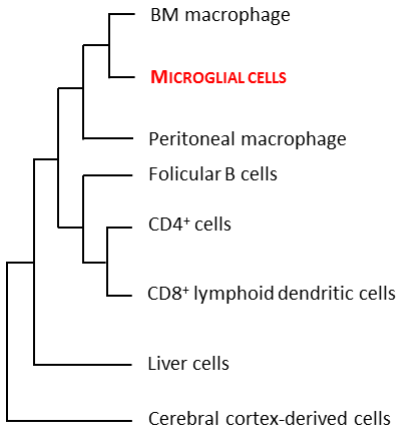


Figure 1.5. – Molecular relationships between different primary mouse haematopoietic lineage cells, brain cells, liver cells and microglia isolated from C57BL/6 wild-type mice. Microglia are most related to BM macrophages. Gene expression as determined by genoma-wide microarray analysis (adapted from Saijo and Glass, 2011).

After the first reports by del Rio-Hortega, microglial research has been dominated by intense discussion about their origin. A few years ago there were two hypothesis about the origin of microglia: (1) microglia differentiated within the CNS from yolk sac primitive macrophages formed during primitive haematopoiesis, an event that occurs in early embryonic ages and constitutes the source of microglia that colonizes the adult healthy brain; and (2) microglia from myeloid precursors that leave the blood stream and colonize the nervous parenchyma in inflammatory conditions. BM-derived monocytes do not contribute to the mature microglial pool in the absence of inflammation (Figure 1.6.) (Aguzzi *et al.*, 2013).

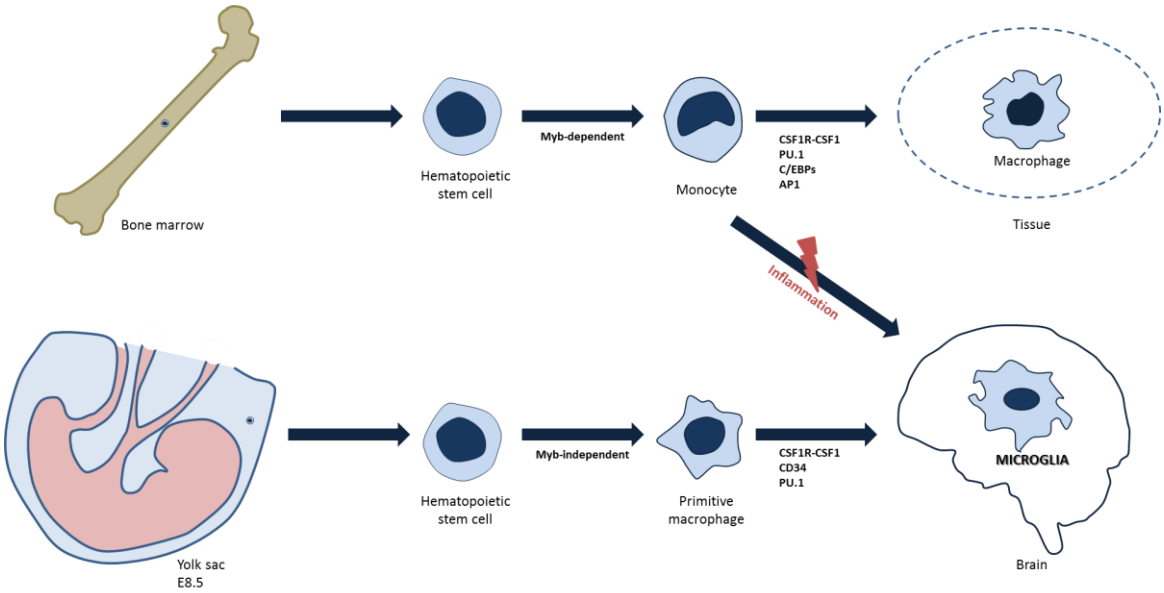


Figure 1.6. – Developmental relationship between microglia and macrophages. Microglial cells are derived from primitive macrophages in the fetal yolk sac formed during haematopoiesis independently of Myb, a requisite transcription factor for stem cell development in the BM. Microglial cells proliferation and differentiation is dependent on a set of transcription factors (for example, transcription factor Spi-1 (PU.1)) and growth factor receptors (for example, CSF1R and CD34) that overlaps with the set required for the development of tissue macrophages that arise from definitive haematopoiesis in the BM and the fetal liver. Macrophages are derived from haematopoietic stem cells in the BM. CNS macrophages found in the meninges choroid plexus, and perivascular space. During inflammation, microglia derive from precursor blood monocytes, but in the BM is dependent on a transcription factor Myb (adapted from Davalos *et al.*, 2005; Saijo and Glass, 2011; Schulz *et al.*, 2012; Aguzzi *et al.*, 2013; Greter and Merad, 2013).

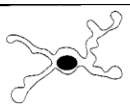
1.2.2. BRAIN COLONIZATION BY MICROGLIA

1.2.2.1. PRENATAL PERIOD – E8-E21 –

Microglial cells penetrate into the mouse brain before day 8 (Ginhoux *et al.*, 2010). There are three routes by which microglial cells can invade the immature CNS: (1) the parenchymal vascular network, (2) the surrounding meninges, and (3) the cerebral ventricles (Cuadros and Navascués, 1998; Monier *et al.*, 2006).

During neural development microglial cells increase in density and undergo progressive ramification (increase in the number and complexity of branches) (Dalmau *et al.*, 1997, 1998, 2003). During the prenatal period it is possible to categorize cells according to their morphology: amoeboid cells (AM), the first to be observed during embryonic development and considered to be the precursors of the mature ramified microglial cells (RM), 'surveying' microglia in adulthood. AM can be distinguished in three different subtypes: (1) type 1, present at E14 and at considerably larger numbers at E17. AM type 1 were observed with a preferential location next to the pial surface; (2) type 2, present at days E19 and E21. AM type 2 mainly occurred with a primary relation to blood vessels; and (3) type 3 with fine filopodia and/or pseudopodia (Table I.i).

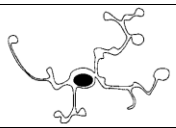
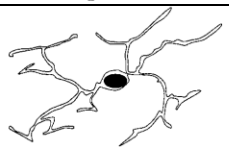
Table I.i – Classification of the morphologic types of microglial cell precursors in the prenatal rat hippocampus. (adapted from Dalmau *et al.*, 1997).

Type of cell	Shape	Cell processes	Measuring	Time course of appearance	Cell morphology
AM type 1	Roundish and sometimes lobular	None	15-40µm	From day E14	
AM type 2	Round	None, occasional filopodia	15-20µm	From day E19	
AM type 3	Pleomorphic	Filipodia and/or pseudopodia	20-50µm	From day E14	
PRM	Elongated	Scantly developed processes showing a beaded shape	50-110µm	From day E19	

1.2.2.2. POSTNATAL PERIOD – P0-P18 –

In the postnatal period microglial cells mature into highly ramified cells (Dalmau *et al.*, 1998). Primitive ramified microglial cells (PRM), first recognized at E19 (Dalmau *et al.*, 1997, 1998; Paolicelli *et al.*, 2011), and RM were distinguished in this period. PRM are found on E18 as stated above and are the most abundant cell type up to postnatal day 9 (P9). RM are rarely found before P9, but are the only type found in the brain from P18 (Table I.ii).

Table I.ii – Classification of the morphologic types of microglial cell precursors in the postnatal rat hippocampus. (adapted from Dalmau *et al.*, 1998).

Type of cell	Shape	Cell processes	Measuring	Time course of appearance	Cell morphology
AM type 2	Round	None, occasional filopodia	15-20µm	P0-P9, scarcely at P12	
AM type 3	Pleomorphic	Filopodia and/or pseudopodia	20-50µm	P0-P9, some at P15	
PRM	Oval to slightly elongated	Scantly developed processes showing a beaded shape	50-75/85µm	P0-P12, some at P15 and rarely at P18	
Resting / 'surveying' microglia	Oval to roundish	Fully developed processes	85-100µm	Some at P12, P15-P18	
Reactive-like / 'effector' microglia	Large, plump, round to oval	Retracted, coarse processes	40/50-80µm	Mainly from P9 to P18	

Microglial cells are present from E8 to P18 and are distributed through different brain areas (for example, cerebral cortex, subcortical white matter, and hippocampus), possibly with different morphologic features, different time of appearance and specific patterns of distribution.

The organization of microglial cells in the white and gray matter suggests that these cells may play active roles in developmental processes such as axon guidance, neurite growth, synaptogenesis, synaptic pruning and neurodevelopmental apoptosis (Kingham *et al.*, 1999; Marín-Teva *et al.*, 2004; Ullian *et al.*, 2004; Tremblay *et al.*, 2011).

The population of microglial cells increases from the prenatal to the postnatal period in the white and gray matter. However, a significant difference in the number of microglia cells occurs between these areas (Figure 1.7.): in gray matter, the density of microglial cell increases from P9 to P18 (except the dentate gyrus (DG), which shows a significant reduction); in white matter, there is a series of cyclic changes: increases from E21 to P0 and from P6 to P9, and decreases from E18 to E21, P0 to P6 and finally decreases from P9 to P18 (Lawson *et al.*, 1990; Dalmau *et al.*, 2003).

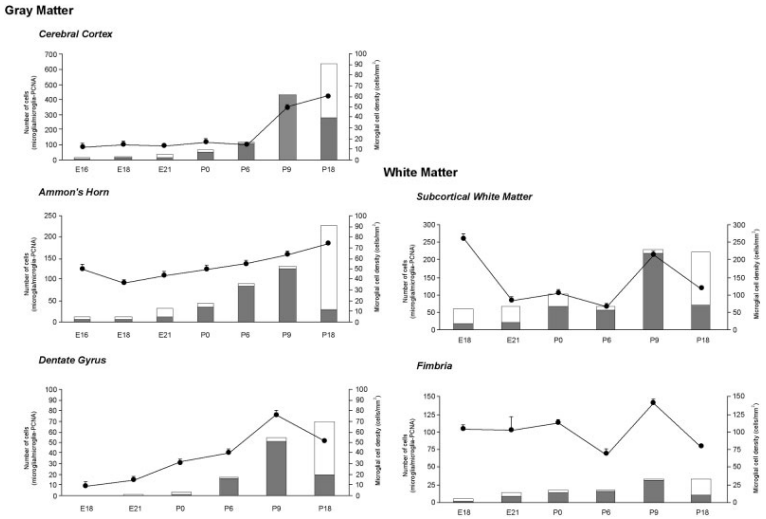


Figure 1.7. – Quantification of microglial cells in the developing rat brain. Histograms show the number of microglial cells in the ages selected (inferior black part: fraction of microglia that were actively proliferating.) Line graphs show the time course of microglial density (Dalmau *et al.*, 2003).

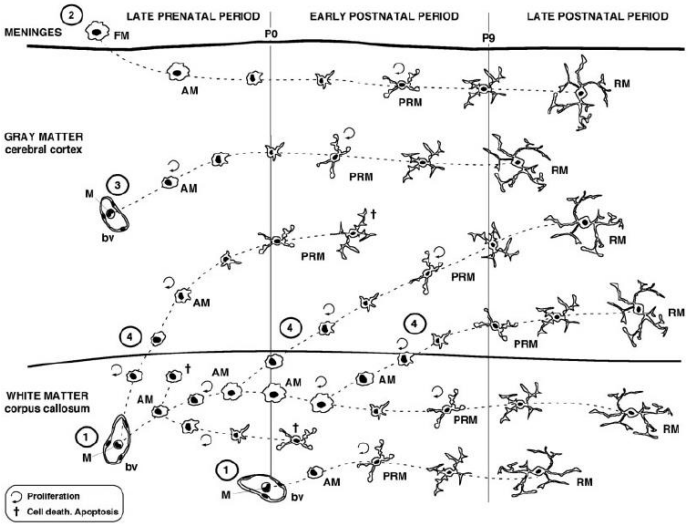


Figure 1.8. – Schematic representative of microglial population growth in the developing brain. AM, amoeboid microglia; PRM, primitive ramified microglia; RM, mature ramified microglia; M, monocyte; FM, fetal macrophage; bv, blood vessels (Dalmau *et al.*, 2003).

1.3. MICROGLIAL FUNCTIONS IN NEURAL DEVELOPMENT

The role of microglia in embryonic brain development is still largely uncharacterized, although increasingly investigated. Many studies indicate that microglial cells have a critical role in brain development/maturation, for example in the formation and maintenance of blood vessels (angiogenesis) (Beers *et al.*, 2006; Kubota *et al.*, 2009; Ginhoux *et al.*, 2010; Rymo *et al.*, 2011; Eyo and Dailey, 2013), in the induction of neuronal apoptosis (developmental apoptosis) (Marín-Teva *et al.*, 2004; Wakselman *et al.*, 2008; Rigato *et al.*, 2011; Swinnen *et al.*, 2013), in the phagocytic clearance of dead cells and neurites (phagocytic clearance) (Takahashi *et al.*, 2005; Fraser and Tenner, 2008; Ziegenfuss *et al.*, 2008; Sierra *et al.*, 2010, 2013; Linnartz *et al.*, 2012; Svahn *et al.*, 2013), and in brain masculinization and behaviour (Lenz *et al.*, 2012, 2013; Schwarz *et al.*, 2012). More recently, microglia role in synapse remodelling and plasticity began to be explored (Paolicelli *et al.*, 2011).

1.3.1. ROLE OF MICROGLIAL CELLS IN SYNAPSES

– DYNAMIC INTERACTIONS BETWEEN MICROGLIA AND SYNAPTIC ELEMENTS IN THE HEALTHY BRAIN –

Microglial cells have been closely associated with developing axon bundles (Cuadros *et al.*, 1993; Dalmau *et al.*, 1998; Herbomel *et al.*, 2001). Microglia monitor the functional state of synapses and respond to changes in synaptic activity during development. For this concept/hypothesis contributed recent studies showing interactions between microglia and synapses in the visual cortex of 3-5 week old mice and changes in microglial behavior were detected subsequently to changes in neuronal workload (Figure 1.9.) (Tremblay *et al.*, 2010).

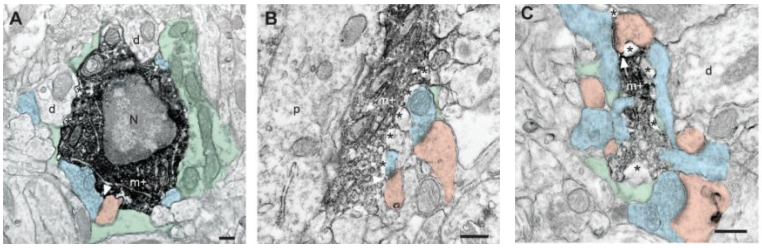


Figure 1.9. – Interactions between microglia and the synapses during normal sensory experience. (A–C) EM images showing Iba1-immunostained microglial (m+) cell bodies (A), as well as large (B) and small (C) processes, surrounded by extended extracellular space (asterisks) and contacting axon terminals (blue), dendritic spines (pink), perisynaptic astrocytes (green), and synaptic clefts (arrowheads). d, dendrite; N, nucleus; p, perikaryon. Scale bars, 250 nm (adapted from Tremblay *et al.*, 2010).

Another study where microglia mediated neuron and synapse elimination during development (2-3 postnatal week mice) was reported by Paolicelli *et al.* (2011). In basal conditions microglial cells are in a 'surveying state' in which their highly motile processes extend and retract to sense the neuronal environment. The dendritic spine density was increased in hippocampal neurons in mice lacking the CX_3CR1.

Post-mortem histological studies in humans describe a prominent accumulation of microglia at 10-12 weeks of gestation at the cortical plate-subplate junction, a position where the first synapses are detected, and later, at 19-30 weeks of gestation, at axonal crossroads in white matter (Verney *et al.*, 2010). This localized accumulation of microglia at points of axonal and synaptic development suggests that microglia may be actively involved in the refinement of neuronal connections, including axonal guidance and synaptogenesis (Paolicelli and Cross, 2011).

1.4. THE ADENOSINERGIC SYSTEM

Adenosine, a purine nucleoside, has important effects on biological processes (Dunwiddie and Masino, 2001; Ribeiro *et al.*, 2003). It is a fundamental neuromodulator and homeostatic regulator in the brain (Fredholm *et al.*, 2001).

Adenosine exerts its actions through the activation of four G-protein coupled receptors (GPCRs) A_1, A_{2A}, A_{2B}, and A_3 (Fredholm *et al.*, 2001; Latini and Pedata, 2001), that regulate different functions in the peripheral (Ohta and Sitkovski, 2001; Ohta *et al.*, 2007) and in the CNS, namely neurotransmission (Burnstock, 2013) and the effects of neurotrophic factors, which action is mediated by A_{2A}Rs (Diógenes *et al.*, 2004, 2007; Pousinha *et al.*, 2006; Gomes *et al.*, 2006, 2009, 2013; Fontinha *et al.*, 2008). In sum, adenosine is known as key molecule acting through combined presynaptic, postsynaptic and non-synaptic actions (Sebastião and Ribeiro, 2000).

Adenosine is released from most cells, including neurons but also glial cells. A_{2A}Rs are important regulators of different microglial functions, such as release of inflammatory mediators (Dai *et al.*, 2010; Saura *et al.*, 2005), proliferation (Gebicke-Haerter *et al.*, 1996; Gomes *et al.*, 2013), reactivity to LPS *ex vivo* (Rebola *et al.*, 2011), retraction and extension of processes (Orr *et al.*, 2009), secretion of brain-derived neurotrophic factor (BDNF) and BDNF-induced proliferation in inflammatory conditions (Gomes *et al.*, 2013).

To date, the role of adenosine in developing neurons, in particular during pre-synaptic differentiation, is limited to the involvement of A_{2B}R activation to mediate axon attraction (Corset *et al.*, 2000). However, the role of A_{2B}R has been controversial, and some evidences indicate that this receptor plays no role in signaling mechanism (Stein *et al.*, 2001; Bouchard *et al.*, 2004). Adenosine is also reported to induce neurite elongation in human neuroblastoma cells (Abbracchio *et al.*, 1989). Important in the context of the present thesis, it is also known that one important signalling pathway related with development is PKA, one of the main pathways activated by A_{2A}Rs (Figure 1.10.). For example, PKA activation is important in the

control of guidance receptors trafficking to the plasma membrane and/or regulated endocytosis of guidance receptors (O'Donnell *et al.*, 2009). These receptors are located in the axons or dendritic growth cones, and their activation could change the growth cone actin/microtubule cytoskeleton (O´Donnell *et al.*, 2009).

In light of all these observations, $A_{2A}R$ activation by adenosine emerges as a good candidate to control microglia impact upon neuron development.

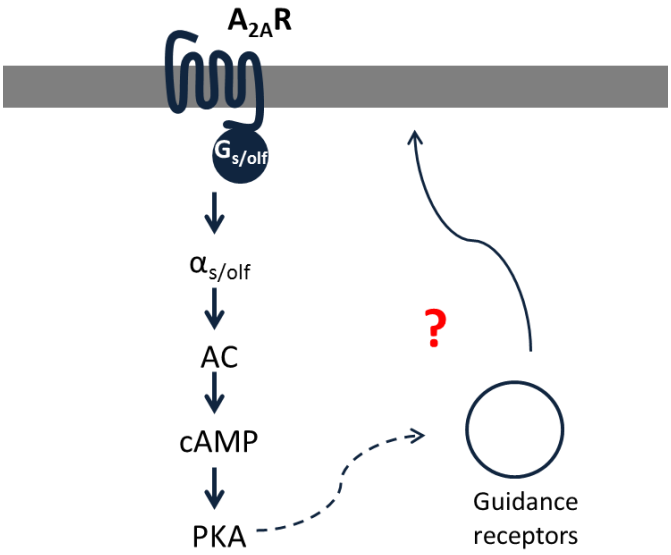


Figure 1.10. – The main signal transduction pathway used by $A_{2A}R$ – cAMP-PKA cascade. $A_{2A}Rs$ couple preferably to members of G_s or G_{olf} family of G protein, and their activation stimulates adenylate cyclase (AC), and increase the levels of cAMP and PKA activation. For example, PKA activation positively regulate the mobilization of an intracellular, vesicular pool of receptors (adapted from Sebastião and Ribeiro, 2000; O'Donnell *et al.*, 2009).

CHAPTER 2

– AIMS –

2. AIMS

Many studies approach the crosstalk between microglial cells and neurons in the adulthood, especially in pathological conditions; however, it is unclear if microglial cells have a role in developing brain besides the well-established phagocytic elimination of synapses or if developing neurons interfere with microglia reactivity.

The main goals of the present thesis are: (i) to clarify if the presence of developing axons/immature neurons affects microglia reactivity (as assessed by immunoreactivity for CD11b); (ii) to explore if microglial cells impact on developing neurons, namely on the density of synaptic proteins (as assessed by immunoreactivity for synapsin, synapsin puncta), axonal length, and growth cone area; and (iii) to clarify if challenging microglia with a classical activator, LPS, determines changes in axons under the control of microglial $A_{2A}R$.

In order to accomplish our goal, in particular to address microglia interactions with synapses, 'far' from the influence of neuronal cell body and the selective pharmacological modulation of microglia previous to the modulation of neurons, we used microfluidic chambers and took advantage of their fluidic properties (developing hippocampal neurons were cultured in one side, allowing axonal growth onto the other side, where a microglia cell line is previously cultured and pharmacologically treated).

CHAPTER 3

– MATERIALS AND METHODS –

3. MATERIALS AND METHODS

3.1. REAGENTS

Table III.i – Reagents.

REAGENT	SUPPLIER
Bovine Serum Albumin (BSA)	Sigma-Aldrich (Portugal)
di-Sodium Hydrogen Phosphate 7-hydrate PA-ACS ($Na_2HPO_4 \cdot 7H_2O$)	Panreac (Spain)
Ethylenediamine tetraacetic acid (EDTA)	Sigma-Aldrich (Portugal)
Fetal Bovine Serum (FBS)	Invitrogen (Spain)
Glucose ($C_6H_{12}O_6$)	Sigma-Aldrich (Portugal)
Paraformaldehyde (PAF)	Sigma-Aldrich (Portugal)
penicillin-streptomycin	Sigma-Aldrich (Portugal)
Poly-dimethylsiloxane (PDMS)	Sigma-Aldrich (Portugal)
poly-D-lysine (PDL)	Sigma-Aldrich (Portugal)
Potassium chloride (KCl)	Sigma-Aldrich (Portugal)
Potassium phosphate monobasic (KH_2PO_4)	Sigma-Aldrich (Portugal)
ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI)	Invitrogen (Spain)
Roswell Park Memorial Institute (RPMI)-1640 medium (R1383)	Sigma-Aldrich (Portugal)
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich (Portugal)
Sodium chloride (NaCl)	Sigma-Aldrich (Portugal)
Sodium hydroxide (NaOH)	Merck (Germany)
Sucrose ($C_{12}H_{22}O_{11}$)	Sigma-Aldrich (Portugal)
Triton X-100	Sigma-Aldrich (Portugal)
Trizma base [$NH_2C(CH_2OH)_3$]	Sigma-Aldrich (Portugal)
Trypan blue	Sigma-Aldrich (Portugal)
Trypsin, from porcine pancreas	Sigma-Aldrich (Portugal)
Tween 20 ($C_{58}H_{114}O_{26}$)	Sigma-Aldrich (Portugal)

3.2. DRUGS

Table III.ii – Drugs.

DRUG	CONCENTRATION	FUNCTION	SUPPLIER
Lipopolysaccharide (LPS) from *Escherichia coli*, serotype 055:B5	100 ng/mL	Classical activator of microglial cells	Sigma-Aldrich (Portugal)
7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo--[4,3-e]-1,2,4 triazolol[1,5c]pyrimidine (SCH58261) ($C_{18}H_{15}N_7O$)	50 nM	Adenosine A_{2A} receptor antagonist	Tocris (United Kingdom)

3.3. ANTIBODIES

Table III.iii – Primary and secondary antibodies and conditions used in immunocytochemistry. All antibodies were diluted in BSA 3% in PBS.

ANTIBODY	SUPPLIER	HOST	TYPE	DILUTION	FUNCTION
Anti-CD11b	Serotec (MCA711)	rat	monoclonal	1:100	microglia marker
Anti-Tau	Abcam (ab75714)	chicken	polyclonal	1:1000	axonal marker
Anti-βIII Tubulin [2G10]	Abcam (ab78078)	mouse	monoclonal	1:1000	axonal marker
Anti-synapsin I	Milipore (Cat. #AB1543)	rabbit	polyclonal	1:2000	synaptic vesicles marker
Anti-rat AlexaFluor 488	Invitrogen (A21208)	donkey	IgG (H+L)	1:1000	---
Anti-chicken AlexaFluor 568	Invitrogen (A21103)	goat	IgG (H+L)	1:1000	---
Anti-mouse AlexaFluor 594	Invitrogen (A21203)	donkey	IgG (H+L)	1:1000	---
Anti-rabbit AlexaFluor 647	Invitrogen (A21245)	goat	IgG (H+L)	1:1000	---
Alexa Fluor 633-conjugated phalloidin	Invitrogen (A22284)	---	---	5U/mL	stain for F-actin

3.4. MICROFLUIDIC CULTURE PLATFORM FOR CO-CULTURE OF MICROGLIAL CELLS AND RAT EMBRYONIC HIPPOCAMPAL NEURONS

Microfluidic culture platforms (MCP) were used to co-culture microglial cells and neurons, allowing the selective physical contact between axons and microglial cells (cell bodies do not contact microglia), one of the main goals of this work. This contact is allowed after microglia selective treatment with drugs, ensuring that these drugs do not affect neurons before microglia, exerting a “priming” effect on microglia previous to the reaction to axonal arrival. The ideal tool to address this goal was microfluidic chambers, small multicompartment devices with physical and fluidic isolation between two compartments.

The chambers are composed of a molded PDMS, a commonly suitable material used in biomedical microsystems to grow cells. In this work, the multi-compartment microfluidic co-culture platform is composed of one soma compartment for neurons and one axon/microglia compartment for axons and microglial cells. Each compartment measures 1.5 mm wide, 7 mm long and 100 µm height. The soma and axon/microglia compartments are connected by arrays of axon-guiding microchannels that function as physical barriers to confine neuron's somas in the soma compartment, while allowing axons to grow into axon/microglia compartment. Microglial cells loaded into axon/microglia compartment can interact only with axons but not with neuronal soma or dendrites, thus enabling localized axon-microglia interaction studies. The microchannels also enable fluidic isolation between soma and axon/microglia compartment, which measure 450 µm long, 10 µm wide and 3 µm height.

These devices allow the control of the microenvironment of cells, and in the last years have been emerged as potent utensils in neuroscience (Park *et al.*, 2006; Taylor and Jeon, 2010). MCP has been applied in several studies of neuronal processes (e.g. neurite formation, outgrowth and regeneration (Taylor *et al.*, 2003; Taylor *et al.*, 2005; Vahidi *et al.*, 2008) and co-culture of neurons with other cells of the CNS, such as astrocytes and oligodendrocytes (Yang *et al.*, 2009)).

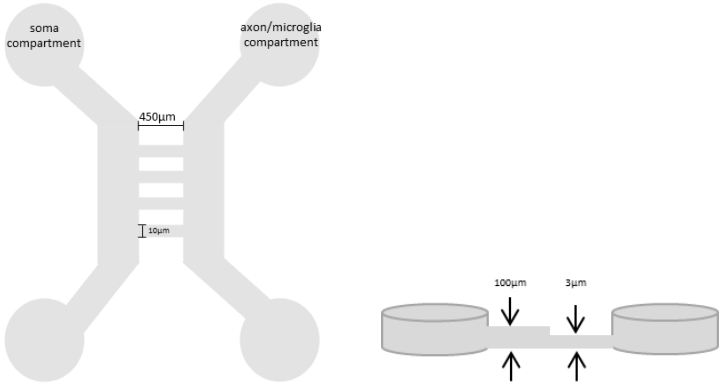


Figure 3.1. – Schematic representation of a microfluidic device. Microfluidic chambers have two compartments, the somal and the axonal/microglial, separated by a set of microgrooves. These devices were used to develop the co-culture of microglial cells and neurons, in which only axons enter a separate compartment and establish contact with microglial cells. MCP, because of its small multicompartment devices having a physical and fluidic isolation between

the compartments, allows a selective exposure of plated microglia (in one compartment), without affecting neurons (in the other compartment before axonal growth) (adapted from Taylor *et al.*, 2005).

3.4.1. PREPARATION OF MICROFLUIDIC CHAMBERS

The microfluidic device for neuron cell culture consists of a PDMS mold chamber placed against a glass coverslip. The PDMS mold was kindly fabricated and offered by Noo Li Jeon (School of Mechanical and Aerospace Engineering, Seoul National University, Seoul, Korea) and Hyun Ryu (Multiscale Mechanical Design, Seoul National University, Seoul, Korea). All procedures in the preparation of microfluidic chambers were performed by the group of Doctor Ramiro de Almeida (Pinto, 2010).

3.5. CELL CULTURE

3.5.1. CULTURE OF RAT EMBRYONIC HIPPOCAMPAL NEURONS

Primary cultures of hippocampal neurons were obtained from the hippocampus of Wistar rat embryos (18 days), handled according to the Portuguese law on Animal Care and European Union guidelines. The preparation of rat embryonic hippocampal neurons was performed by the group of Doctor Ramiro de Almeida.

3.5.2. CULTURE OF MICROGLIAL CELLS

A murine microglial cell line, N9 (kind gift from Professor Claudia Verderio, National Research Council, Neuroscience Institute, Cellular and Molecular Pharmacology, Milan, Italy) was grown in RPMI medium supplemented with 30 mM glucose, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were kept at 37°C under a humidified atmosphere with 95% O_2 and 5% CO_2. In microfluidic devices, 10µl of a 2×10^4 cells/mL cell suspension were added (the number of viable cells was evaluated counting trypan blue-excluding cellular elements) in the axonal/microglial side three days after plating hippocampal neurons (timepoint at which there

are still no axons growing through the channels). The co-culture was maintained for four more days, when pharmacological manipulations and/or immunocytochemistry analysis were performed.

3.6. MICROGLIAL CELLS PHARMACOLOGICAL TREATMENT

At DIV4, microglial cells were treated with LPS (100 ng/mL), a concentration previously reported to induce changes in $A_{2A}R$ density in microglial cells (Gomes *et al.*, 2013) and/or with the selective $A_{2A}R$ antagonist SCH58261 (50 nM) (it was previously reported that this concentration is selective for $A_{2A}R$) (Zocchi *et al.*, 1996) (Table III.ii). When LPS was tested in the presence of SCH58261, the antagonist was added twenty minutes before LPS. All treatments remained in the medium for six hours; and then washed out.

At DIV7, neurons were considered suitable to analyse the established endpoints after contacting microglial cells in axonal/microglial compartment.

3.7. IMMUNOCYTOCHEMISTRY

DIV7 co-cultures were pre-fixed in PAF 1% in phosphate buffered saline (PBS) (NaCl 137 mM, KCl 2.1 mM, KH_2PO_4 1.8 mM and $Na_2HPO_4.2H_2O$ 10 mM, at pH 7.4) for five minutes at room temperature (RT) in order to minimize the aggressive effect that PAF may have on fragile axons. After that, cells were fixed with PAF 4% (in PBS) for ten minutes at RT, and washed three times with tris-buffered saline (TBS). As fixation with PAF does not allow access of the antibody to the antigen, a permeabilization step was performed using a non-ionic detergent, Triton X-100 0.25% in TBS for five minutes at RT. After permeabilization, cells were washed once with TBS and non-specific binding blocked with BSA 3% in PBS for thirty minutes at RT. BSA interacts with other proteins, as well as with the antibodies used, increasing the competition of antibodies in binding

to their targets, with a consequent result of increasing selectivity to the antigen, decreasing the nonspecific binding. After washing the blocking solution, cells were incubated overnight at 4°C with primary antibodies (Table III.iii) with BSA 3% in PBS, as already mentioned. 20 µL of primary antibodies were added to the somal side and 15 µL to the axonal/microglial side, in order to guarantee the fluidic isolation. After incubation with the primary antibodies, cells were washed three times with TBS to remove excess primary antibodies (at least five minutes each wash), and then incubated with the secondary antibody (Table III.iii) in BSA 3% in PBS for one hour at RT. Cells were washed twice with Triton X-100 0.1% in TBS (five minutes each wash), and finally five minutes wash in TBS).

Antibodies mix (both primary and secondary) was centrifuged for twenty minutes at 16000 g at 4°C before application. In order to exclude non-specific labelling of the secondary antibodies, labelling was tested in the absence of primary antibodies. All immunocytochemistry steps were performed with the microfluidic devices assembled; after removing the last washing medium, microfluidic chambers were disassembled by slowly removing the mold from the coverslip. The glass coverslip was rinsed with mili-Q water (mQH_2O) and mounted in prolong mounting media with DAPI, a fluorescent stain that binds strongly to deoxyribonucleic acid (DNA) and is used in fluorescence microscopy to identify the nuclei. Preparations were dried overnight at 4°C and sealed with nailpolish before acquisition of images in the fluorescence microscope.

3.8. IMAGE ACQUISITION

For data acquisition and quantification, ten to twelve fluorescent images were blindly acquired using a Zeiss Axiovert 200 fluorescence microscope (Zeiss, Germany), and the images were acquired by AxioCam HRm camera and ZEN software (Carl Zeiss Imaging Systems), with an EC-Plan-Neofluar 40x oil objective (numerical aperture 1.3). Exposure times, as well as acquisition

settings, were conserved in individual experiments. The pixel size in the object space was 0.16 µm. In microfluidic chambers, images were randomly taken from the axonal/microglial side.

3.9. IMAGE QUANTIFICATION

All quantifications described below were performed using ImageJ software (National Institutes of Health, NIH). After acquisition, images were converted into 8-bit tiff images which were used (and manipulated as indicated when considered appropriate) for all the quantification procedures.

3.9.1. MICROGLIA REACTIVITY

CD11b immunoreactivity (IR) was analysed as an indicator of microglia reactivity. Identical background and threshold values were applied to all pictures to eliminate background. These images were used to quantify the total intensity of CD11b. The ratio between total CD11b intensity per CD11b area, as well as per cell number, was calculated to minimize eventual influence of changes in cell morphology or number upon total CD11b intensity.

3.9.2. SYNAPSIN PUNCTA PER AXONAL LENGTH

Axons were randomly chosen from exported 8-bit images and their lengths determined. Axons with similar appearance were selected; fragmented, bead-bearing axons or terminal regions were rejected. The selection was carried out in tau images, without observation of synapsin labelling. To quantify the number of synapsin puncta that correspond to each axonal length, synapsin channel images were thresholded (background and threshold values were applied to all pictures) and analysis of particles was performed. In each image the number of synapsin/µm was determined. In each experiment, ten to twelve images were analysed per condition.

3.9.3. LENGTH OF THE AXONAL NETWORK

In order to analyze changes in the outgrowth of axons within the axon/microglia compartment in the presence or absence of microglia, we quantified the total length of the axonal network from the microgrooves' end to the opposite side of the compartment. To combine in a single image the entire width of the axon/microglia compartment, 6 to 13 tile images (equally interspaced along the length of the compartment and without overlapping with each other) were acquired per chamber (Figure 3.2.). Each tile is composed of 10 individual images that were combined via stitching. These images were converted into 8-bit images and the threshold was applied to eliminate background. The total length of axons was measured by obtaining a representative skeleton of the axonal network using the ImageJ plugin Skeletonize, which was later analysed by the ImageJ plugin Analyze skeleton. This latter plugin will identify several axonal segments and their length will be measured. The total length of the axonal network corresponds to the sum of all individual segments' length.

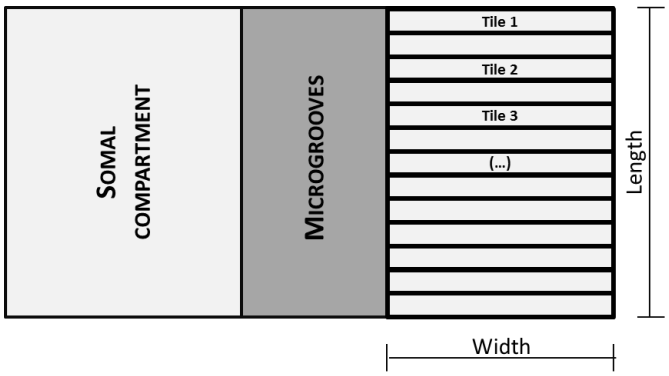


Figure 3.2. – Acquisition of images for measurement of the length of the axonal network. To combine in a single image the entire width of the axon/microglia compartment, 6 to 13 tile images (equally interspaced along the length of the compartment and without overlapping with each other) were acquired per chamber.

3.9.4. GROWTH CONE AREA

Polymerized actin is stained with Alexa Fluor 633-conjugated phalloidin. Growth cones present in phalloidin images were selected as regions of interest (ROI) and the threshold adjusted to eliminate background. The area of phalloidin in each ROI was quantified.

Next, we measured the distance between each growth cone and the most proximal microglial cell. Growth cones were grouped in two distinct populations: the ones that are within a 50 µm range from microglia and the remaining ones.

3.10. STATISTICAL ANALYSIS

Values were presented as means ± standard error of mean (SEM) of independent cultures. Statistical analysis was determined with Graph Pad Prism 6.0 software (GraphPad Software, San Diego, California). Student's *t* test, one-way or two-way analysis of variance (ANOVA) for independent means (followed by a Bonferroni post-hoc test for multiple comparisons), were used to define statistical differences, which were considered significant at $p < 0.05$.

CHAPTER 4

– RESULTS AND DISCUSSION –

4. RESULTS AND DISCUSSION

4.1. IMPACT OF AXONS ON MICROGLIAL REACTIVITY

4.1.1. THE PRESENCE OF AXONS DID NOT AFFECT MICROGLIA REACTIVITY

CD11b, a constitutive marker of microglia (and macrophages) was used as a marker of microglial reactivity, and CD11b IR (immunoreactivity) was evaluated as an indicator of this reactivity. In the present study, we used two types of normalization for measured CD11b IR, area and cell number.

Microglial cells cultured in microfluidic chambers exhibited different morphologies, even in the absence of neurons, as revealed by immunofluorescence staining with an antibody anti-CD11b. For this reason, it was decided to analyse IR and not morphology as an indicator of reactivity. As shown in Figure 4.1., the presence of axons did not affect CD11b IR in microglial cells per area (microglia: 61.24 ± 9.75; microglia plus axons: 72.70 ± 9.62, n=6, $p > 0.05$) (Figure 4.1.) or per cell number (microglia: 29292 ± 7857; microglia plus axons: 26261 ± 4877, n=6, $p > 0.05$) (Figure 4.1.).

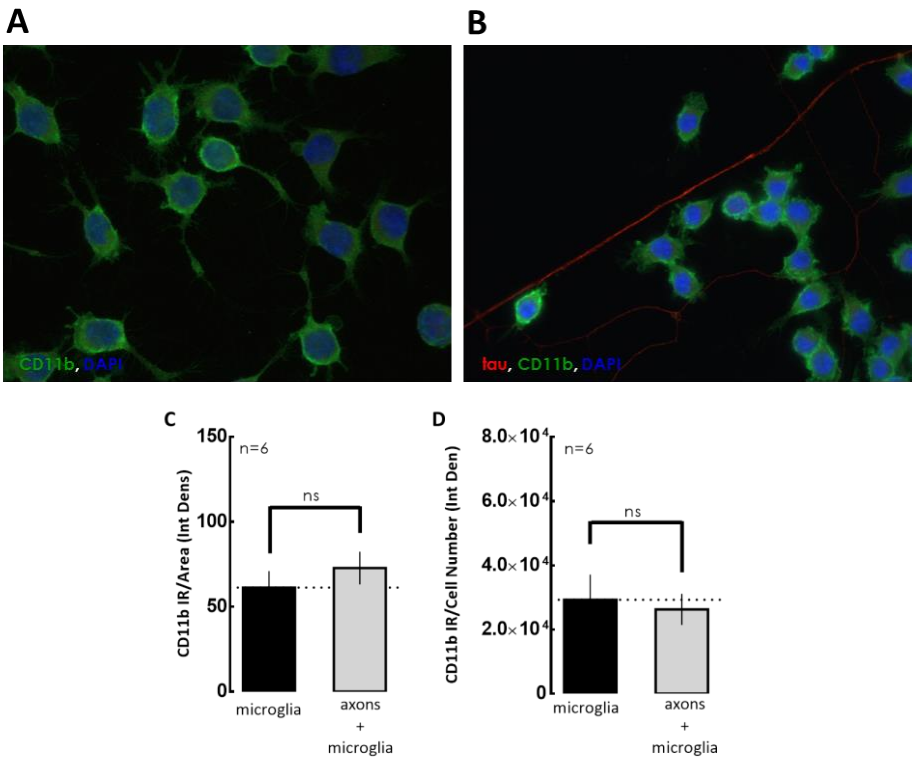


Figure 4.1. – The presence of axons did not cause alterations in CD11b IR of microglial cells. (A-B) Representative images of microglial cells in the absence of axons (A) and in the presence of axons (B). Microglial cells in the

axonal/microglial compartment at DIV7 stained for CD11b (microglia marker, green), DAPI (nuclei, blue) and tau (axonal marker, red). (C) CD11b IR quantification normalized per area. (D) CD11b IR quantification normalized per cell number. Results are expressed as mean ± SEM of *n* (as indicated above) independent experiments ($p > 0.05$, compared with isolated microglia, using paired Student's *t* test).

*

These data suggest that, in the experimental conditions used, the presence of axons did not change microglial reactivity. However, we do not exclude the possibility that changes in other markers may occur, for example Iba-1 (a marker for resting and activated microglia) or CD68 (a marker for activated microglia, mainly in the phagocytic state), because these three markers are differentially regulated (Louboutin *et al.*, 2010).

4.2. MICROGLIAL CELLS IMPACT UPON AXONS

It is still unknown if microglial cells affect developing neurons, and whether this effect is mediated by a local interaction between microglial cells and the growing axons (without directly affecting the soma). Previous studies show that, upon axotomy, microglia secrete thrombospondin (TSP) (Chamak *et al.*, 1995; Moller *et al.*, 1996), which belongs to the family of extracellular matrix proteins able to induce synaptogenesis (Christopherson *et al.*, 2005). However, there are no studies testing if microglial cells induce presynaptic differentiation of developing neurons.

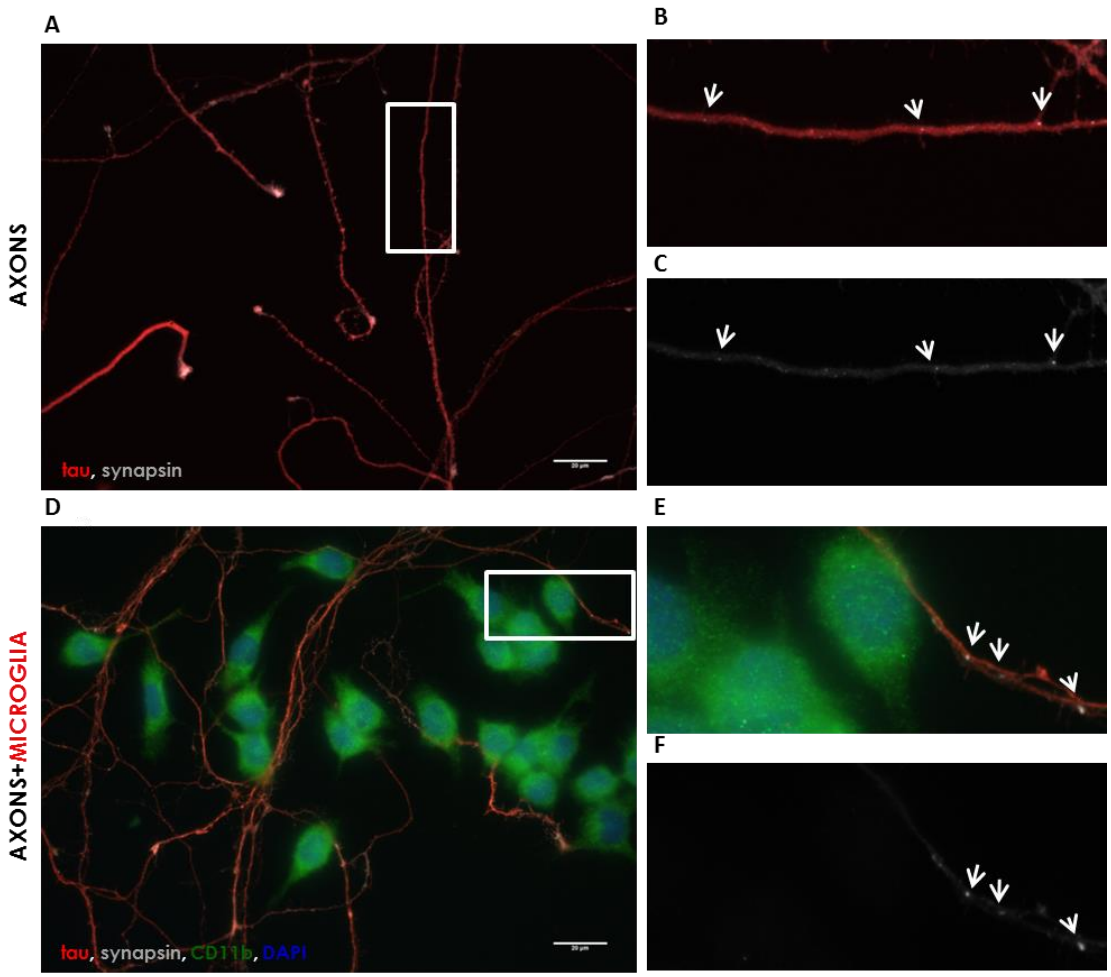
The process of presynaptic differentiation includes several events, among which the clustering of SVs (Jin and Garner, 2008). They are associated with specific proteins, linked to the cytoplasmatic surface, which are part of the synapsin family (a family consisting of at least 10 isoforms encoded by three distinct genes) (De Camilli *et al.*, 1983; Huttner *et al.*, 1983; Fornasiero *et al.*, 2010). Synapsins control important developmental processes that precede the

formation of mature nerve terminals, thus being involved in synapse formation (Fornasiero *et al.*, 2010).

In this work, the effect of microglial cells on embryonic hippocampal neurons was assessed by measuring synapsin puncta per axonal length as a marker of SVs, which clustering is a hallmark of presynaptic differentiation.

4.2.1. MICROGLIAL CELLS TEND TO INCREASE THE NUMBER OF SYNAPSIN PUNCTA

The number of synapsin puncta/µm tends to increase when growing axons are co-cultured with microglia, as compared with isolated growing axons, although this does not reach statistical significance (axons: 0.055 ± 0.016; axons plus microglia: 0.085 ± 0.025, n=7, $p > 0.05$) (Figure 4.2.).



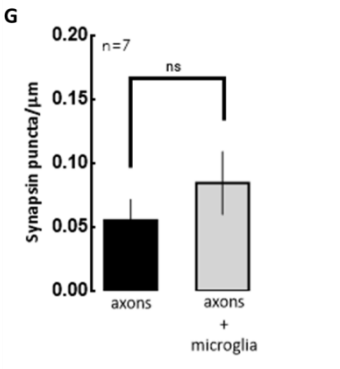


Figure 4.2. – Microglial cells tend to increase the clustering of synaptic vesicles. (A-F) Representative images of axonal/microglial compartment at DIV7, in microfluidic chambers stained for CD11b (microglia marker, green), DAPI (nuclei, blue), tau (axonal marker, red) and synapsin I (marker of synaptic vesicles, white). (B, C) Magnification of an axonal segment of A (white rectangle). (E, F) Magnification of an axonal segment of D (white rectangle). White arrows indicate puncta of synapsin. Scale bars, 20µm. (G) Quantification of the number of synapsin puncta per axonal length. Results are expressed as mean ± SEM of *n* (as indicated above) independent experiments ($p > 0.05$, compared with isolated axons, using paired Student's *t* test).

4.2.2. LPS-ACTIVATED MICROGLIAL CELLS INCREASED SYNAPSIN PUNCTA

The concentration of LPS, 100 ng/mL (at six hours), was previously reported to induce changes in A_{2A}R density in microglial cells (Gomes *et al.*, 2013). In the presence of LPS, the number of synapsin puncta per axonal length was increased when compared with control conditions (axons plus microglia: 0.060 ± 0.010; axon plus microglia in the presence of LPS: 0.125 ± 0.015, n=3) (Figure 4.3.); in isolated axons, no alterations were observed in the number of synapsin puncta per axonal length (axons: 0.044 ± 0.016; axons in the presence of LPS: 0.070 ± 0.028) (Figure 4.3.). The present results show that microglial 'activation' using a bacterial antigen changes the number of synapsin clusters along the axon; thus, even without a contact with the cell body, is sufficient to induce these changes in synaptic protein.

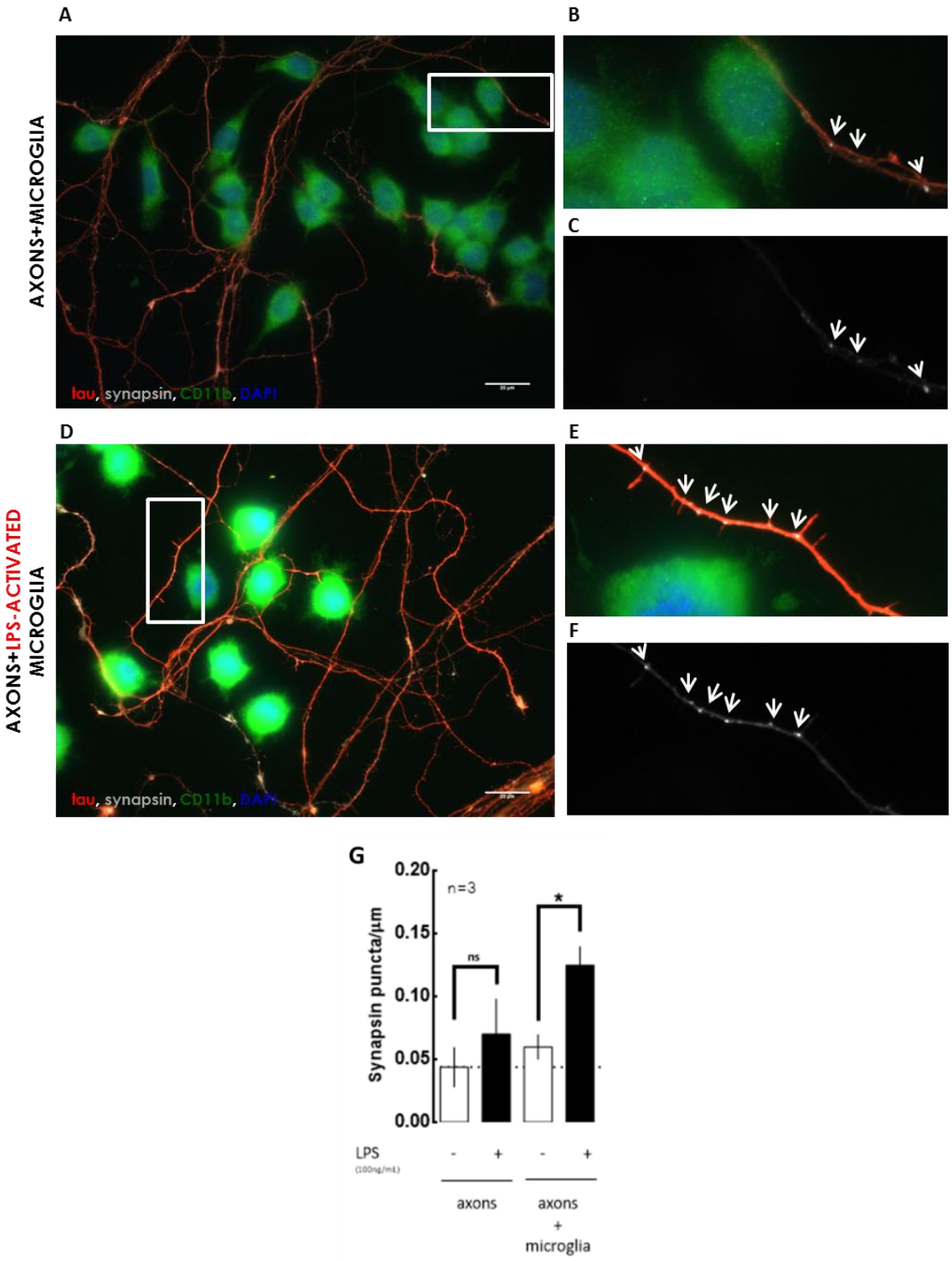


Figure 4.3. – LPS-activated microglial cells increased synapsin puncta. (A-F) Representative images of axonal/microglial compartment at DIV7, in microfluidic chambers stained for CD11b (microglia marker, green), DAPI (nuclei, blue), tau (axonal marker, red) and synapsin I (marker of synaptic vesicles, white). (B, C) Magnification of an axonal segment of A (white rectangle). (E, F) Magnification of an axonal segment of D (white rectangle). White arrows indicate puncta of synapsin. Scale bars, 20µm. (G) Quantification of the number of synapsin puncta per axonal length. Results are expressed as mean ± SEM of *n* (as indicated above) independent experiments (*$p < 0.05$, two-way ANOVA followed by Bonferroni *post-hoc* test).

4.2.3. $A_{2A}R$ BLOCKADE DID NOT PREVENT LPS-INDUCED INCREASE IN SYNAPSIN PUNCTA

Several studies suggest that $A_{2A}R$ antagonists can control neuroinflammation. For example, the tonic activation of $A_{2A}R$ is required for LPS-induced increase of BDNF secretion by microglia in inflammatory-like conditions (Gomes *et al.*, 2013). In order to clarify if a selective $A_{2A}R$ antagonist, SCH58261, is able to regulate the microglia-induced increase of synapsin puncta reported above, we investigated the ability of SCH58261 (50 nM) to modulate LPS effect upon synapsin puncta.

The selective $A_{2A}R$ antagonist SCH58261 did not prevent LPS induced increase of synapsin puncta (axons in the presence of LPS: 0.070 ± 0.028; axons in the presence of SCH 58261: 0.0715 ± 0.032; axons in the presence of LPS and SCH 58261: 0.076 ± 0.031; axons plus microglia in the presence of LPS: 0.125 ± 0.015; axons plus microglia in the presence of SCH 58261: 0.085 ± 0.030; axons plus microglia in the presence of LPS and SCH 58261: 0.128 ± 0.032) (Figure 4.4.).

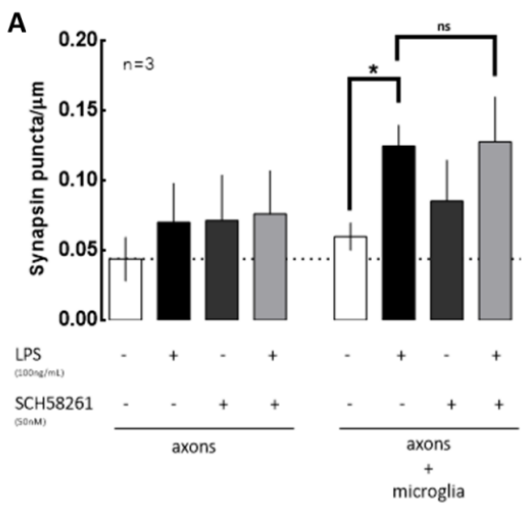


Figure 4.4. – Microglia $A_{2A}R$ blockade did not prevent LPS-induced increase in the clustering of SVs. (A) Quantification of the number of synapsin puncta per axonal length. At DIV4, microglial cells were exposed to pharmacological treatments, lipopolysaccharide (LPS, 100 ng/mL), and/or the selective $A_{2A}R$ antagonist SCH58261 (50 nM) for 6 hours before their wash out. Results are expressed as mean ± SEM of *n* (as indicated above) independent experiments (*p < 0.05, two-way ANOVA followed by Bonferroni *post-hoc* test).

*

One of the main goals of the present work was to evaluate if microglia affect developing neurons, namely by regulating the density of synapsin I, a pre-synaptic molecule causally related with pre-synaptic maturation (Ziv and Garner, 2004).

Several factors can be involved in the regulation of synapsin puncta along the axon, among which diffusible mediators released by microglial cells emerge as good molecular candidates. Additionally, axon-microglia direct physical contact may influence synapsin puncta. To dissect these possibilities, experiments with microglia-conditioned medium (instead of microglia themselves) need to be done.

Further experiments are required in order to identify the mechanism by which microglial cells or microglial mediators control synapsin puncta. One possibility is the regulation of protein synthesis (by the activation of genes encoding synaptic proteins) or trafficking along the axon.

It is also crucial to clarify the physiological role of the LPS-induced increase of synapsin puncta and to test whether these puncta become or not functional synapses. To address this question, the whole-cell patch clamp approach complemented by fluorescence imaging (e.g. by using the styryl dye FM1-43) could be used. The use of FM1-43 allows the investigation of synaptic vesicle exocytosis and endocytosis, a good tool to understand if microglial cells could promote the functional maturation of synapsin puncta, turning them into functional synapses.

A very recent work from Ueno and colleagues (2013) proposes a novel role for microglia during development, besides the well-known phagocytic role: a supportive role for developing neurons. Using cortical neuronal cultures (E18), the authors observe that layer V neurons require microglial support to survive during postnatal development. The observed increase in synapsin puncta in the presence of activated microglia is in line with the study by Ueno and co-workers, further suggesting that microglia, besides phagocytosis, are involved in trophic actions. Guidepost cells have been proposed to provide information for synapse stabilization (Sanes and Yamagata, 1999; Shen and Bargmann, 2003); for example, studies in *Caenorhabditis elegans*

neurons have identified epidermal or neuronal guiding cells determining synapse positioning (Ding *et al.*, 2007). Our results may suggest that microglial cells work as guide cells for synapse positioning in the early phases of embryonic development, by controlling synapsin clustering, accepted as a readout of pre-synaptic maturation (Ziv and Garner, 2004).

The fact that only activated microglia induces an increase of synapsin puncta led us to hypothesize that microglial priming by exogenous factors (such as an infectious agent) during early development could result in alterations of brain wiring subsequent to aberrant changes in the number of synapses and associated with disease in the adulthood.

4.3. LENGTH OF THE AXONAL NETWORK

During brain development, neurons establish neural circuits that require trophic support (Waites *et al.*, 2005; O'Donnell *et al.*, 2009). Total axonal length is controlled by a variety of extracellular and intracellular signals that can be repulsive or permissive molecules (O'Donnell *et al.*, 2009). Repulsive (ephrins, netrins and semaphorins, and repulsive guidance molecule a, RGMa) and permissive molecules (e.g. growth factors) (O'Donnell *et al.*, 2009; Kitayama *et al.*, 2011; Kolodkin and Tessier-Lavigne, 2011) are produced and secreted by glial cells. For this reason, it was considered interesting to analyse if microglia could affect total axonal length, in particular if activated by LPS in the presence and the absence of A_{2A}R antagonist.

4.3.1. MICROGLIAL CELLS DID NOT AFFECT THE AXONAL LENGTH

Kitayama and co-workers (2011) reported that it is necessary the activation of microglia (by LPS) to inhibit axonal and neurite outgrowth. It was our goal to analyze the impact of microglia upon axonal length by the direct contact of these cells with the growing axon, without affecting the neuronal soma. In our hands, and in these particular conditions, the presence of microglia

did not affect the axonal length (axons: 5445 ± 2121; axons plus microglia: 6122 ± 1769, n=6) (Figure 4.5.).

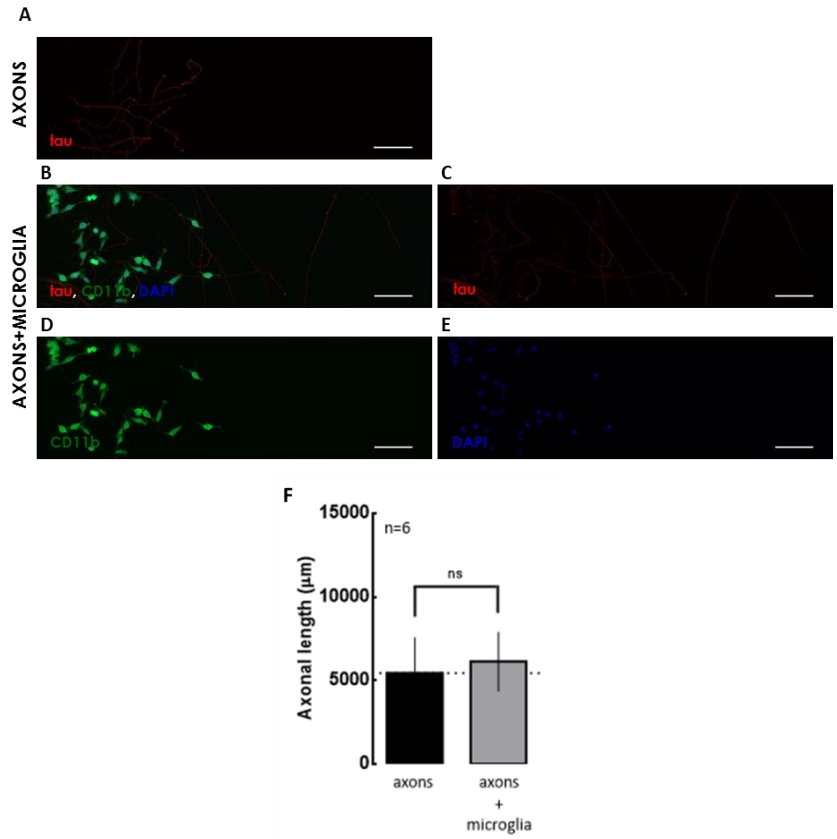


Figure 4.5. – Microglial cells did not affect the axonal length. (A-E) Representative images of axon/microglia compartment at DIV7, in microfluidic chambers stained for tau (axonal marker, red) (A, C), CD11b (microglia marker, green) (D) and DAPI (stains nuclei, blue) (E). Scale bars, 100 µm. (F) Quantification of the total axonal length. Results are expressed as mean ± SEM of *n* (as indicated above) independent experiments ($p > 0.05$, compared with isolated axons, using paired Student's *t* test).

4.3.2. LPS-ACTIVATED MICROGLIAL CELLS DID NOT AFFECT THE AXONAL LENGTH

Considering that microglial regulation of growing axons is highly dependent on their 'activation' state (as seen for synapsin puncta, compare 4.2.1. with 4.2.2.), we next tested the ability of microglia to regulate axonal length in the presence of LPS (100 ng/mL).

Conversely to what was expected from the previous results on synapsin puncta, LPS-activated microglia did not affect the total axonal length (axons: 8815 ± 4105; axons in the presence of

LPS: 9789 ± 4683; axons plus microglia: 5727 ± 2254; axons plus microglia in the presence of LPS, n=3) (Figure 4.6.).

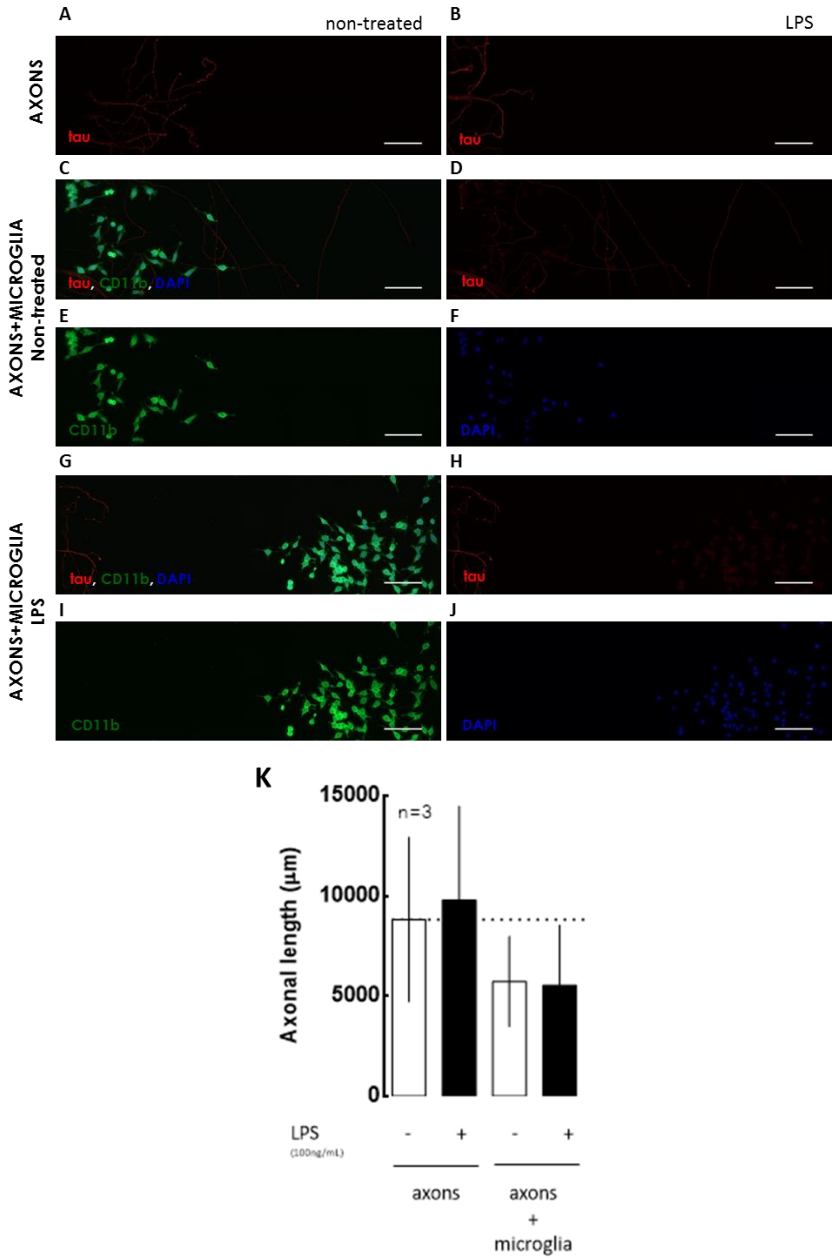
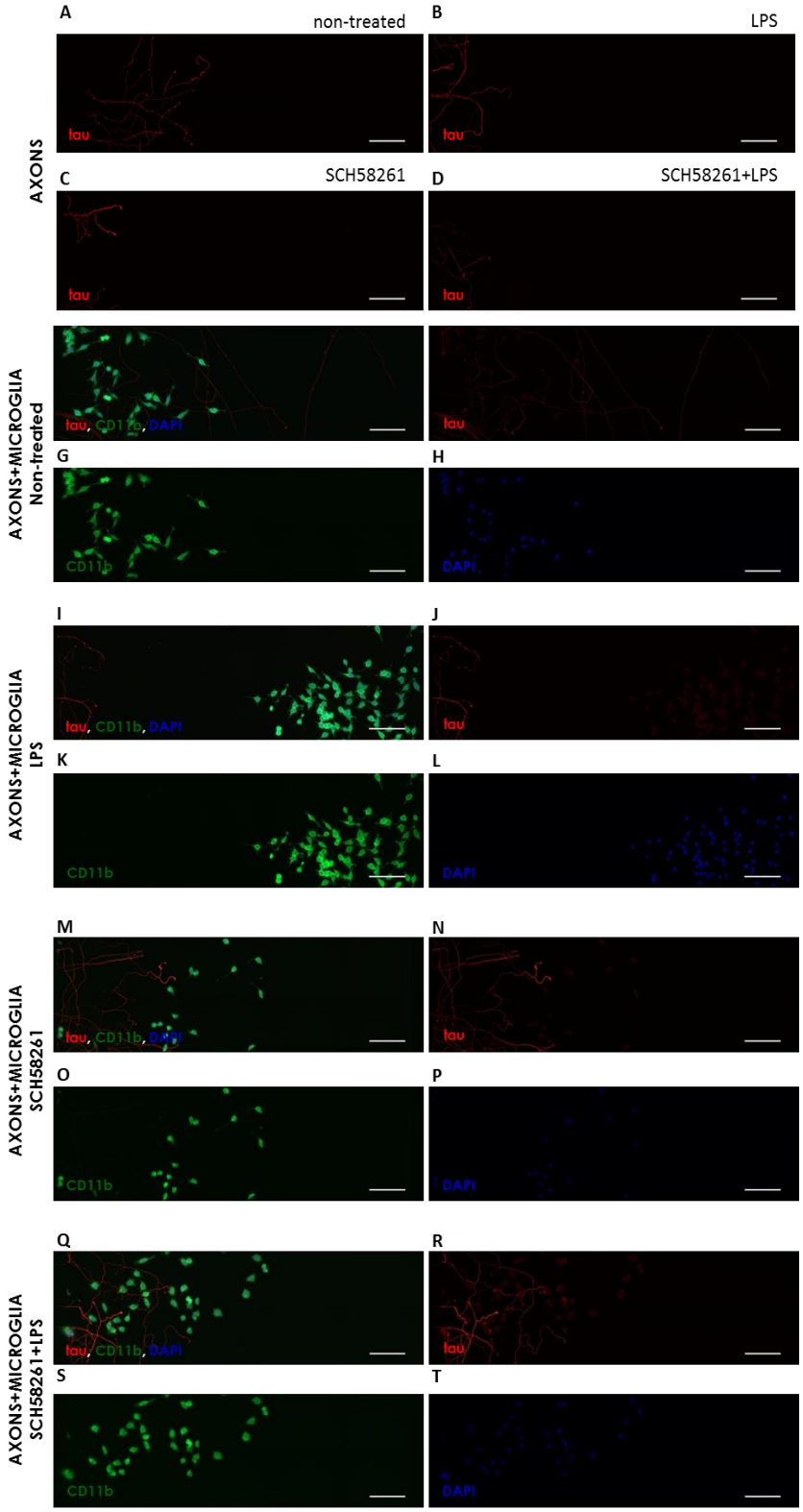


Figure 4.6. – LPS-activated microglia did not affect the total axonal length. (A-J) Representative images of axon/microglia compartment at DIV7, in microfluidic chambers stained for tau (axonal marker, red) (A, B, D, H), CD11b (microglia marker, green) (E, I) and DAPI (nuclei, blue) (F, J). Scale bars, 100 µm. (K) Quantification of the total axonal length. Results are expressed as mean ± SEM of *n* (as indicated above) independent experiments ($p > 0.05$, one-way ANOVA followed by Dunnett's *t* test).

4.3.3. MICROGLIAL AND NEURONAL A_{2A} RECEPTOR EXERT A DUAL ROLE IN THE CONTROL OF THE AXONAL LENGTH

Given that adenosine, through the activation of $A_{2A}R$, is able to control several microglial functions, in particular when these cells are in the presence of activators, such as LPS (e.g. Saura *et al.*, 2005; Gomes *et al.*, 2013), we tested the ability of the selective $A_{2A}R$ antagonist (SCH58261, 50 nM) to modulate the total axonal length in the presence and in the absence of LPS (100 ng/mL).

Blocking microglial $A_{2A}R$ prior to neuronal $A_{2A}R$ did not affect the total axonal length, iregardless the 'activation' state of microglia (although we consider the present results as preliminary data, taking into consideration the high variability obtained in these experiments) (axons plus microglia: 5727 ± 2254; axons plus microglia in the presence of SCH 58261: 6241 ± 2833). Intriguingly, SCH58261 seems to directly control total axonal length: in the absence of microglia, $A_{2A}R$ blockade tends to decrease total axonal length (axons: 8815 ± 4105; axons in the presence of SCH 58261: 3130 ± 1173, n=3) (Figure 4.7.), although not interfering with total axonal length in the presence of LPS (axons: 8815 ± 4105; axons in the presence of LPS: 9789 ± 4683; axons in the presence of LPS and SCH 58261: 5310 ± 3442; axons plus microglia: 5727 ± 2254; axons plus microglia in the presence of LPS: 5529 ± 3005; axons plus microglia in the presence of LPS and SCH 58261: 6173 ± 1929, n=3).


A
non-treated
B
LPS
AXONS
tau
C
SCH58261
D
SCH58261+LPS
tau
AXONS+MICROGLIA Non-treated
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H
CD11b
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AXONS+MICROGLIA LPS
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tau, CD11b, DAPI
tau
K
L
CD11b
DAPI
AXONS+MICROGLIA SCH58261
M
N
tau, CD11b, DAPI
tau
O
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AXONS+MICROGLIA SCH58261+LPS
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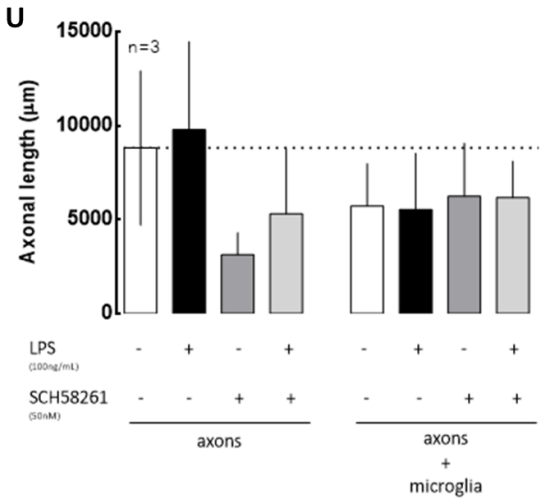

Figure 4.7. – Microglial and neuronal $A_{2A}R$ exert a dual role in the control of the axonal length. (A-T) Representative images of axon/microglia compartment at DIV7, in microfluidic chambers stained for tau (axonal marker, red) (A-D, F, J, N, R), CD11b (microglia marker, green) (G, K, O, S) and DAPI (stains nuclei, blue) (H, L, P, T). Scale bars, 100 µm. (U) Quantification of the total axonal length. Results are expressed as mean ± SEM of *n* (as indicated above) independent experiments ($p > 0.05$, compared with isolated axons, using one-way ANOVA followed by Dunnett's *t* test).

*

Axonal growth is critical for establishing neuronal circuits during developmental period. Purines, such as adenosine 5'-triphosphate (ATP) and adenosine induce alterations in axonal growth (Diaz-Hernandez *et al.*, 2008; Diez-Zaera *et al.*, 2011). Extracellular ATP negatively controls axonal growth and branching in cultured hippocampal neurons (Diaz-Hernandez *et al.*, 2008) and the opposite was observed with extracellular adenosine in human neuroblastoma cells (Abbracchio *et al.*, 1989). Another study (Diez-Zaera *et al.*, 2011) reports an absence of effect of adenosine in the first steps of axonal growth, but the authors use adenosine deaminase (ADA), a drug that converts adenosine to its inactive metabolite, inosine (Diez-Zaera *et al.*, 2011). Furthermore, in this study, both the soma and the axon contacted microglial cells. Given that we are interested in adenosine effects mediated by $A_{2A}R$, we studied the effect of a selective antagonist, SCH58261 (50 nM), upon the length of the axonal network in the presence and in the absence of LPS.

Although it was not expected any result of the application of SCH58261 when hippocampal neurons are cultured in the absence of microglia (at this timepoint axons are not growing

through the channels), a decrease was observed. An acceptable justification for this may be related to the properties of the devices, namely with the main component of microfluidic chambers – PDMS. This polymer, with the chemical formula $CH_3[Si(CH_3)_2O]_nSi(CH_3)_3$ – *n* is the number of repeating monomer $[SiO(CH_3)_2]$ –, have $–CH_3$ groups that make the surface very hydrophobic (Sia and Whitesides, 2003). Consequently, the chambers surface becomes susceptible to nonspecific adsorption of hydrophobic contaminants (Sia and Whitesides, 2003), for example SCH58261. Thus, it may be available to interact with the axons even after drug washout.

Our observations are controversial with a study, showing that adenosine is not involved in the control of axonal growth (Diez-Zaera *et al.*, 2011). A possible explanation for this controversy is related with the experimental conditions used in our study, where microglia is plated in physical contact with the growing axon, in the absence of the influence of the neuronal soma (allowed by device compartmentalization). $A_{2A}R$ are key controllers of the synapse morphology and/or function (Costenla *et al.*, 1999; Canas *et al.*, 2009) and we anticipate that somal and axonal receptors may be involved in the differential regulation of specific cell functions.

It is important to emphasize that the presence of LPS, used as a classical ‘activator´ of microglia, did not affect the axonal network, although it was able to regulate synapsin clustering (see 4.2.). Altogether, our data suggest that the ´activation’ state of microglia is selectively involved in the control of particular neuronal properties of the growing axon.

4.4. GROWTH CONE

Neuronal connectivity in the early phases of development is established by the precise regulation of axon guidance, which is controlled by extracellular cues that attract or repel axons (O'Donnell *et al.*, 2009). Growth cone morphology and motility are controlled by guidance receptors at the plasma membrane and downstream signalling pathways that regulate actin/microtubule cytoskeleton organization (Vitriol and Zheng, 2012). Actin is one of the major cytoskeletal components of growth cones (F-actin forms a meshwork throughout lamellipodia and is densely bundled in filopodia) (Vitriol and Zheng, 2012); thus, the regulation of actin dynamics is relevant for growth cone motility and guidance. Phalloidin, which binds to F-actin, is used to investigate whether microglial cells are implicated in the regulation of growth cone.

The presence of microglial cells did not interfere with growth cone phalloidin area (axons: 32.14 ± 8.27; axons plus microglia: 39.63 ± 7.88, n=4) (Figure 4.8.).

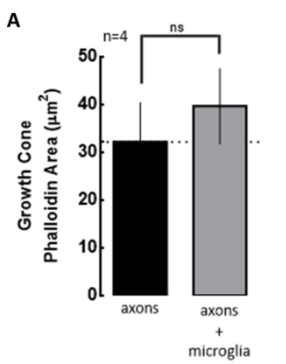

Figure 4.8. – Microglial cells did not affect the growth cone phalloidin area. (A) Quantification of the growth cone phalloidin area. Results are expressed as mean ± SEM of *n* (as indicated above) independent experiments ($p > 0.05$, compared with isolated axons, using paired Student's *t* test).

However, visually, it appeared that when microglial cells were closer to the axons, the growth cone "looked" higher in its area. Thus, we next examined the influence of microglial cells on the phalloidin-stained area, according to the distance between axons and microglial cells: growth cones were divided into two populations, according to the distance to microglia (< or >50 µm). Performing this analysis, we were able to observe that when the growth cone was closer to

microglial cells, the phalloidin area tended to be higher, although without a statistically significant difference (axons: 29.78 ± 5.68; axons plus microglia <50 µm: 44.38 ± 3.21; axons plus microglia >50 µm: 35.24 ± 2.69, n=3) (Figure 4.9.).

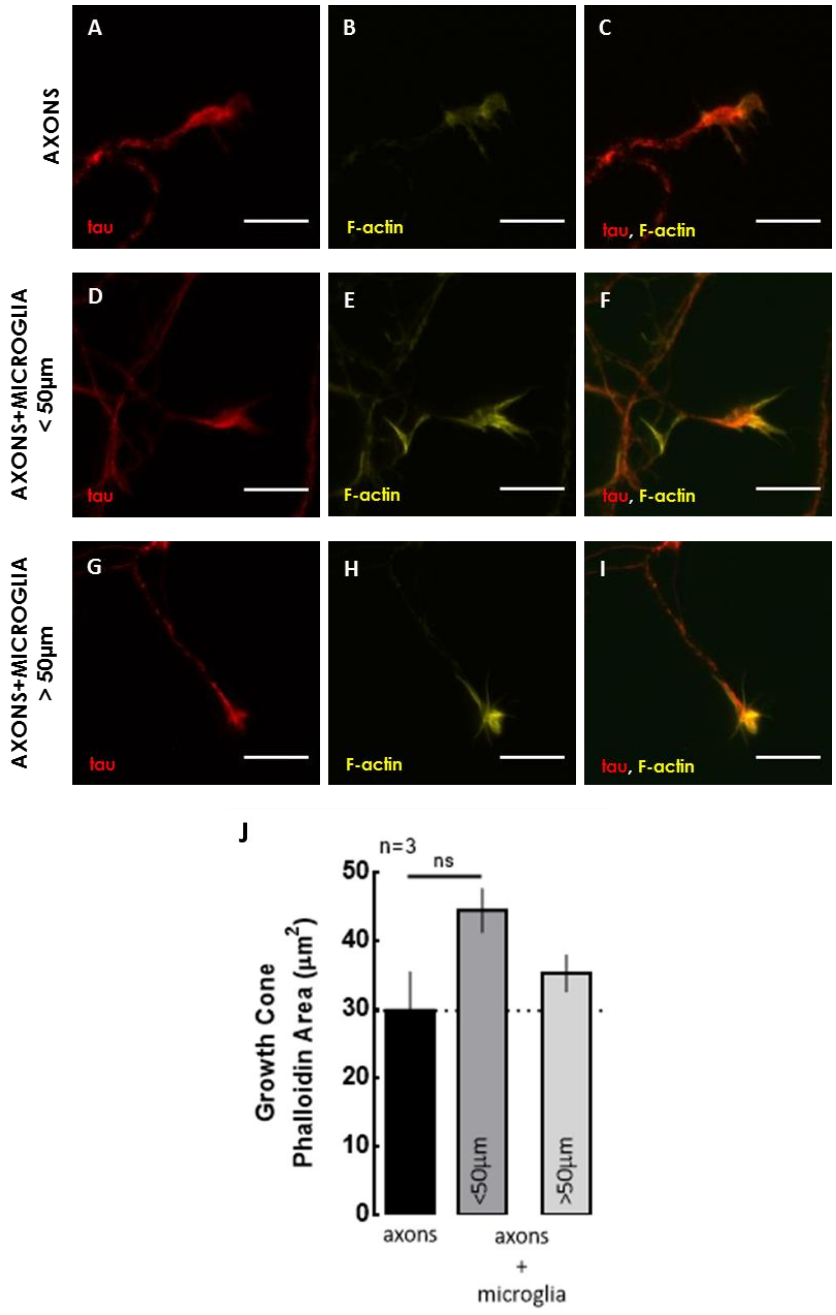

Figure 4.9. – Microglial cells effects upon growth cone phalloidin area. (A-I) Representative images of axon/microglia compartment at DIV7, in microfluidic chambers stained for tau (axonal marker, red) (A, D, G) and phalloidin (marker of F-actin, yellow). Image has been merged. Growth cone image without microglial cells (A-C) and with microglial cells (< 50 µm) (D-F) and (> 50 µm) (G-I), respectively. Scale bars, 10µm. (J) Quantification of the growth cone phalloidin area in two distinct populations: the ones that are within a 50 µm range from microglia and the remaining ones. Results are expressed as mean ± SEM of *n* (as indicated above) independent experiments ($p > 0.05$, Student's *t* test).

*

The motility and guidance responses of the growth cone involve the actin cytoskeleton, which is controlled by many signaling pathways (in turn controlled by extracellular signals). Lamellipodia and filopodia, two distinct F-actin structures that are present at the growth cones have a function in growth cone movement and environmental sensing, respectively (Vitriol and Zheng, 2012).

The present results suggest that growth cones may be affected by factors secreted by microglial cells and that the distance to these cells is important, likely by a dilution effect of mediators released far from the growth cone, an issue that deserves further clarification. Microglial cells may be a source of several regulatory molecules that control the actin network (and possibly their dynamics).

Kitayama and collaborators (2011) showed that, when neurons are cultured with LPS-activated microglia, growth cones are collapsed; non-activated microglia do not induce growth cone collapse. Growth cone collapse has been used for the identification and purification of molecules that are repulsive to growth cones. Nevertheless, future studies are needed to investigate the impact of the selective pharmacological modulation of microglial cells (by LPS in the presence and absence of a selective $A_{2A}R$ antagonist) in the interaction with growth cones.

CHAPTER 5

– CONCLUSION –

OPEN QUESTIONS AND NEW DIRECTIONS

5. CONCLUSION – OPEN QUESTIONS AND NEW DIRECTIONS

The main observations of the present work are (1) LPS-activated microglia increase synapsin puncta, a pre-synaptic molecule causally related to the onset of pre-synaptic maturation, an effect not prevented by $A_{2A}R$ blockade; (2) neuronal and microglial $A_{2A}R$ blockade differentially modulate total axonal length; (3) microglial cells tend to positively regulate growth cone area and this eventual influence is affected by the distance to microglial cells.

Other issues arise as future hypothesis: during development, could microglial cells guide the axons to the appropriate target cell? Is it possible that microglial cells exert a guiding role in the formation of synapses? Could microglial cells provide spatial information for synapse positioning? Could microglia exert a dual role in the control of synapses: formation of presynaptic '*boutons'* and elimination of synapses? Could an infection of the CNS in the early phases of development determine neuropathological alterations in the adulthood?

Understanding the functions and the molecular pathways underlying microglia-synapse interactions in the developing brain and their exact role in the navigation of growing axons is mandatory to elucidate the pathophysiology of neurodevelopmental diseases. This work helped to better understand the physical relationship between microglia and growing axons, suggesting a novel role for microglia, besides the well-known phagocytic role. Importantly, this study highlights the impact of the selective modulation of $A_{2A}R$ in neurons and microglial cells upon developing neurons.

CHAPTER 6

– REFERENCES –

6. REFERENCES

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