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Ciências

Role of Striatal-Enriched Protein Tyrosine Phosphatase (STEP) in the control of microglial reactivity

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"Remember to look up at the stars and not down at your feet. Try to make sense of what you see and wonder about what makes the universe exist. Be curious. And however difficult life may seem, there is always something you can do and succeed at.
It matters that you do not just give up."

Stephen Hawking

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A todos os que me acompanham em alcançar as metas e a cumprir os meus sonhos.
Durante 5 anos.
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Resumo

As proteínas tirosina fosfatases (PTPs) são fatores reguladores nas vias de sinalização inflamatórias. O sistema nervoso central tem como células imunes residentes as células microgliais, as quais participam no início e na propagação da resposta inflamatória. A proteína fosfatase de resíduos de tirosina enriquecida no estriado (STEP), é uma fosfatase específica do sistema nervoso central codificada pelo gene PTPN5. O mRNA STEP origina por splicing alternativo a $STEP_{61}$, isoforma associada à membrana e a $STEP_{46}$, isoforma citosólica. A desregulação da STEP está implícita na base molecular de vários distúrbios neuropsiquiátricos. Os estudos publicados sobre a STEP centram-se na expressão e papel a nível neuronal. Tanto quanto é do nosso conhecimento não existem dados publicados sobre a expressão da STEP pela microglia ou sobre a regulação da reatividade microglial por esta fosfatase.

Os nossos dados da análise de PCR mostraram a expressão do RNAm da STEP em culturas primárias da microglia obtidas a partir de cérebro de rato. A presença da proteína STEP foi também verificada por análise imunocitoquímica e Western blot. Por outro lado, os níveis de expressão da STEP parecem ser regulados por exposição a estímulos inflamatórios. Culturas de microglia expostas ao agente pró-inflamatório lipopolissacarídeo (LPS) apresentaram níveis aumentados de $STEP_{61}$ e $STEP_{46}$. Como forma de avaliar o papel da STEP no controlo da reatividade da microglia induzida por LPS, recorremos ao inibidor específico da STEP (TC-2153). O tratamento com este inibidor reduziu significativamente o número de células microgliais que expressam a sintase do óxido nítrico induzida (iNOS) e também o número de células fagocitícas quantificadas após a exposição ao LPS. Contudo, a inibição da STEP não afetou significativamente a libertação de óxido nitríco nem a expressão da interleucina-1beta (Il-1ß) ou do fator de necrose tumoral alfa (TNFα). Em suma, nossos resultados mostram que a STEP é expressa por células que não pertencem à linhagem neuronal, e sugerem que a STEP participa em algumas das vias envolvidas na reatividade da microglial.

Palavras-chave

STEP, proteína fosfatase de resíduos de tirosina, microglia, neuroinflamação

Abstract

Protein tyrosine phosphatases (PTPs) are key regulatory factors in inflammatory signaling pathways. The central nervous system has as resident immune cells the microglial cells, which participate in the initiation and propagation of the inflammatory response. STriatal-Enriched Protein Tyrosine Phosphatase (STEP) is a central nervous system specific phosphatase encoded by the PTPN5 gene. STEP mRNA is alternatively spliced into the membrane-associated $STEP_{61}$ and the cytosolic $STEP_{46}$. Accumulating evidence implicates STEP dysregulation in the molecular basis of several neuropsychiatric disorders. Published data about STEP, focus on expression and role at the neuronal level. To our knowledge, there are no published data on the expression of STEP by microglial cells or on the regulation of microglial reactivity by this phosphatase.

Our results from PCR analysis show the expression of STEP mRNA in primary cultures of microglia obtained from rat brain. STEP expression was further confirmed by the immunocytochemical analysis and Western blot. On the other hand, STEP expression levels appear to be regulated by exposure to inflammatory stimuli. Microglia cultures exposed to the pro-inflammatory agent lipopolysaccharide (LPS) showed increased levels of $STEP_{61}$ and $STEP_{46}$. To examine the role of STEP in the control of microglia reactivity induced by LPS, we used a specific pharmacological inhibitor of STEP (TC-2153). STEP inhibition significantly reduced the number of microglial cells expressing inducible nitric oxide synthase (iNOS) and the number of phagocytic cells quantified after LPS stimulation. On the other hand, inhibition of STEP did not significantly affect the nitric oxide (NO) release and the expression of Interleukin 1 beta (IL-1ß) or tumor necrosis factor alpha (TNFα). Taken together, our results show that STEP is expressed by cells that do not belong to the neuronal lineage and suggest that STEP participates in some of the pathways involved in microglia reactivity

Keywords

STEP, protein tyrosine phosphatase, microglia, neuroinflammation

Resumo alargado

As proteínas tirosinas fosfatases (PTPs) são fatores reguladores chave nas vias de sinalização inflamatória. A proteína fosfatase de resíduos de tirosina enriquecida no estriado (STEP), é uma fosfatase específica do sistema nervoso central codificada pelo gene PTPN5. O mRNA de STEP origina por splicing alternativo quatro isoformas baseadas na mobilidade eletroforética, as isoformas mais abundantes são a $STEP_{61}$, isoforma membranar e a $STEP_{46}$, isoforma citosólica, e duas isoformas menos comuns que incluem a $STEP_{38}$ e a $STEP_{20}$, sendo estas últimas inativas cataliticamente. A outra isoforma conhecida, $STEP_{33,}$ é obtida através da clivagem da calpaína.

Em condições fisiológicas, as células microgliais apresentam uma estrutura ramificada e são as principais intervenientes na resposta inflamatória inata, participando na primeira linha de defesa em resposta a vários estímulos. Num contexto de lesão as células microgliais tornam-se reativas e adquirem uma morfologia ameboide responsável pelos processos de migração em direção ao local de lesão e ativação de mecanismos de fagocitose para remoção de dendritos celulares. A ativação microglial em resposta a um estímulo neurotóxico está geralmente associada a um aumento da libertação de fatores pró-inflamatórios e vários mediadores neurotóxicos, tais como fator de necrose tumoral-α (TNF-α), interleucina-1ß (IL-1ß), óxido nítrico (NO), espécies reactivas de oxigênio (ROS) e proteases, capazes de provocar degeneração neuronal (microglia M1). Por outro lado, dependendo da natureza e da intensidade do estímulo, as células da microglia podem libertar citocinas anti-inflamatórias e factores neurotróficos envolvidos em mecanismos celulares de protecção e reparação neuronal (microglia M2).

As PTPs podem ser o alvo para a modulação da ativação microglial e ser uma boa estratégia para desenvolver terapias em doenças neurodegenerativas e neuroinflamatórias. Actualmente não existem dados publicados sobre a expressão da STEP pelas células da microglia ou sobre a regulação da reatividade microglial por esta fosfatase. Com este trabalho pretendemos determinar se STEP exerce algum papel no controlo da neuroinflamação. Os nossos dados da análise de PCR mostraram a expressão do RNAm da STEP em culturas primárias da microglia obtidas a partir de cérebro de rato. A presença da proteína STEP foi também verificada por análise imunocitoquímica e Western blot. Por outro lado, os níveis de expressão da STEP parecem ser regulados por exposição a estímulos inflamatórios. Culturas de microglia expostas ao agente pró-inflamatório lipopolissacarídeo (LPS) apresentaram níveis aumentados de $STEP_{61}$ e $STEP_{46}$.

Para examinar o papel da STEP no controle da reatividade da microglia, utilizamos como ferramenta um inibidor farmacológico específico da STEP (TC-2153) e analisamos sua influência na reatividade da microglia que pode ser induzida por uma resposta pró-inflamatória, como a exposição ao LPS. A inibição da STEP reduziu significativamente o número de células microgliais que após a exposição ao LPS expressam óxido nítrico sintase induzida (iNOS, em cerca de 60%),

e que têm atividade fagocitíca (em cerca de 65%). A inibição da STEP não afetou significativamente nem a libetração de óxido nitríco nem a expressão da interleucina-1beta (Il-1ß) ou do fator de necrose tumoral alfa (TNFα).

Os nossos resultados sugerem que a reatividade microglial está associada a um aumento nos níveis de STEP e que a atividade STEP contribui ainda mais para o processo neuroinflamatório

É sabido que a neuroinflamação é um dos principais desencadeadores da neurodegeneração, uma vez que a reatividade da microglia pode iniciar ou amplificar o dano neuronal, ou ser uma consequência disso. Portanto, a inibição da hiperativação microglial pode ser uma boa estratégia para desenvolver terapias para doenças neurodegenerativas.

Os resultados obtidos no presente trabalho sugerem que a inibição STEP pode ser uma estratégia terapeutica em processos neuroinflamatórios e, em consequência, ter potencial terapêutico em processos neurodegenerativos.

Palavras-chave

STEP, proteína fosfatase de resíduos de tirosina, microglia, neuroinflamação

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List of Abbreviations

AD	Alzheimer's disease	HD	Huntington's disease
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor	HRP	Horseradish peroxidase-conjugated
Aß	ß-amyloid peptide	Iba1	Ionized calcium-binding adapter molecule 1
Bcl-2	B-cell lymphoma 2	IFNγ	Interferon gamma
bp	Base pair	IL	Interleukin
BSA	Bovine Serum Albumine	iNOS	Inducible nitric oxide synthase
cAMP	Cyclic adenosine monophosphate	KIM	Kinase-interacting motif
CD	Cluster of differentiation	KO	Knockout
cDNA	Complementary deoxyribonucleic acid	LBP	LPS binding protein
CNS	Central nervous system	Leu	Leucine
CREB	cAMP response element-binding protein	LPS	Lipopolysaccharide
CT	Cycle time	MAPK	Mitogen activated protein kinases
CTR	Control	MEM	Minimum Essential Medium Eagle
CyPA	Cyclophilin A	mGluR	Metabotropic glutamate receptors
Cys	Cysteine	MHCII	Major histocompatibility complex II
D1R	Dopamine receptor D1	Mhtt	Mutant huntingtin
DARPP-32	cAMP-regulated phosphoprotein of 32 kDa	mRNA	Messenger ribonucleic acid
DEPC	Diethylpyrocarbonate	NED	N-1-naphthylethylenediamine
DHPG	S-3,5-dihydroxyphenylglycine	NMDA	N-methyl-D-aspartate
DNA	Deoxyribonucleic acid	NO	Nitric oxide
ECM	Extracellular matrix	p38	Stress-activated Protein Kinase p38
Elk1	ETS domain-containing protein	PBS	Phosphate buffer saline
ERK 1/2	Extracellular Signal-regulated Kinase 1 and 2	PBS-T	PBS containing 0.1% Tween-20
FBS	fetal bovine serum	PCP	Phencyclidine
FXS	Fragile X Syndrome	PCR	Polymerase chain reaction
Fyn	Fyn kinase tyrosine	PDL	Poly-D-lysine

PEST	Proline-glutamic acid-serine-threonin	SFK	Src-family of kinases
PFA	Paraformaldehyde	STEP	STriatal-Enriched Protein Tyrosine Phosphatase
PG	Prostaglandins	SZ	Schizophrenia
PKA	Protein kinase	TBS	Tris-buffered saline
PP	Polyproline	TBS-T	0.1% Tween 20 in Tris-buffered saline
PP1	Protein phosphatase-1	TC-2153	8-(trifluoromethyl)-1,2,3,4,5-benzopentathiepin-6-amine hydrochloride
PP2B	Protein phosphatase 2B	TEMED	Tetramethylethylenediamine
PSA	Ammonium persulfate	TGF-β	Transforming growth factor β
PTP	Protein tyrosine phosphatase	Thr	Threonine
PVDF	Polyvinylidene difluoride	TLR4	Toll-like receptor-4
Pyk2	Proline-rich tyrosine kinase 2	TM	Transmembrane domains
RNA	Ribonucleic acid	TNF-α	Tumor necrosis factor α
ROS	Reactive oxygen species	Tyr	Tyrosine
SDS	Sodium dodecy lsulfate	UPS	Ubiquitin proteasome system
Ser	Serine	WT	Wild-type

Chapter 1
Introduction

1 Introduction

The protein tyrosine phosphatase (PTP) superfamily of enzymes functions in a coordinated manner with protein tyrosine kinases to control signalling pathways that underlie a broad spectrum of fundamental physiological processes including gene regulation, cell growth, differentiation, migration, and synaptic plasticity (Lee et al. 2015; Lombroso et al. 2016; Tonks 2006). Unlike the protein kinases, which are derived from a common ancestor, the protein phosphatases have evolved in separate families that are structurally and mechanistically distinct (Tonks 2006). In the human genome, 107 genes encode the four families of protein tyrosine phosphatases (Figure 1). Specifically, PTPs are either tyrosine-specific or dual-specificity phosphatases, and the tyrosine-specific PTPs are further subdivided into receptor-like or intracellular PTPs. Receptor-like PTP's have an extracellular receptor domain, transmembrane domain and usually two catalytic domains. In contrast, the nonreceptor cytoplasmatic PTP's contain a single catalytic domain (Goebel-Goody et al. 2012; Johnson and Hunter 2005; Zhang 2002). Dysregulation in tyrosine phosphorylation play a role in the pathogenesis of numerous inherited or acquired human diseases from cancer to neuropsychiatric diseases. Consequently, PTPs are prime targets for drug discover (Cohen and Alessi 2013; He et al. 2014). One PTP of particular promise for the treatment of neuropsychiatric disorders is striatal-enriched protein tyrosine phosphatase (STEP) (Goebel-Goody et al. 2012).

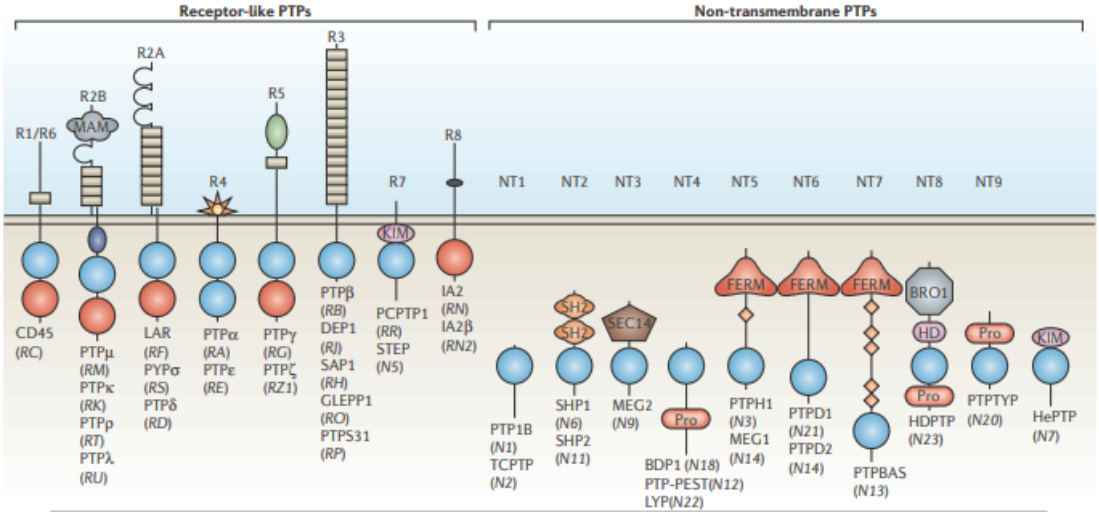


Figure 1. **The class of PTPs**. PTPs are either tyrosine-specific or dual-specificity phosphatases, and the tyrosine-specific PTPs are further subdivided into receptor-like or intracellular PTPs. Receptor-like PTP's have an extracellular receptor domain, transmembrane domain and usually two catalytic domains. In contrast, the nonreceptor cytoplasmatic PTP's contain a single catalytic domain. STEP is a member of the family of intracellular tyrosine-specific phosphatases. From (Tonks 2006).

1.1 STriatal-Enriched Protein tyrosine phosphatase

The STriatal-Enriched Protein Tyrosine Phosphatase, designated as STEP, is a central nervous system (CNS) specific phosphatase encoded by the Ptpn5 gene(Boulanger et al. 1995; Goebel-Goody et al. 2012; Lombroso et al. 1991) and first described by Paul Lombroso (Lombroso et al. 1991). The human STEP locus maps to chromosome 11p15.2-p15.1, the rat STEP gene to chromosome 1q22 and the murine STEP gene to chromosome 7B3-B5 (Figure 2) (Li et al. 1995).

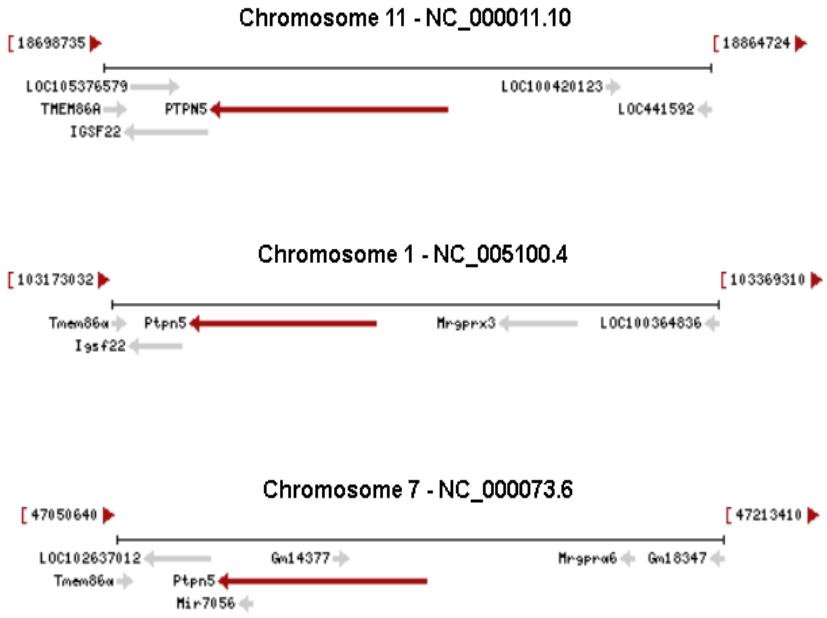


Figure 2. STEP localization. The human STEP locus maps to chromosome 11p15.2-p15.1, the rat STEP gene to chromosome 1q22 and the murine STEP gene to chromosome 7B3-B5. From (human: https://www.ncbi.nlm.nih.gov/gene/84867;mus:https://www.ncbi.nlm.nih.gov/gene/19259; rat: https://www.ncbi.nlm.nih.gov/gene/29644).

1.1.1 STEP structure

STEP mRNA is alternatively spliced into four variants (Figure 3) based on their electrophoretic mobility (Boulanger et al. 1995; Goebel-Goody et al. 2012; Sharma et al. 1995).

The most abundant isoforms are $STEP_{61}$ and $STEP_{46}$ (Bult et al. 1996; Goebel-Goody et al. 2012; Li et al. 1995; Lombroso et al. 1993, 2016) and these two STEP isoforms are differentially expressed in brain regions (Boulanger et al. 1995; Lorber et al. 2004) and during development (Okamura et al. 1997; Raghunathan et al. 1996). Both isoforms are expressed in the striatum, central nucleus of the amygdala and the optic nerve, whereas other brain areas such as hippocampus, neocortex, spinal cord and lateral amygdala only express the $STEP_{61}$ isoform (Boulanger et al. 1995; Goebel-Goody et al. 2012; Lorber et al. 2004; Xu, Kurup, Nairn, et al. 2012).To date studies on STEP focuses only the expression of STEP by neuronal cells (Kamceva et al. 2016) and to date, there are no data on the expression of STEP by glial cells.

Regarding the stages of development in rat $STEP_{61}$ is abundantly expressed at birth and its expression continues throughout adulthood, while $STEP_{46}$ is expressed from the sixth day after birth and progressively increases its expression until the twenty-fourth day post-birth, at which it reaches a maximum levels, remaining then constant throughout adult life (Goebel-Goody et al. 2012; Okamura et al. 1997; Raghunathan et al. 1996; Xu, Kurup, Nairn, et al. 2012).

$STEP_{46}$ is primarily a cytosolic protein, whereas $STEP_{61}$ is targeted to membrane compartments (Bult et al. 1996; Goebel-Goody et al. 2012; Lombroso et al. 1993, 2016). The $STEP_{61}$ isoform contain an additional sequence of 172 aminoacids at its N-terminus which include two polyproline (PP) rich regions, two hydrophobic transmembrane domains (TM) and two proline-glutamic acid-serine-threonin (PEST) sequences. The function of the PP regions is to confer substrate specificity for the STEP isoforms. The Fyn affinity (STEP substrate) to associate with $STEP_{61}$ is ten times greater than with $STEP_{46}$, since this shorter isoform does not contain the PP regions. PEST sequences act as a signal peptides for proteolytic degradation (Boulanger et al. 1995; Bult et al. 1996; Goebel-Goody et al. 2012; Lombroso et al. 2016). Both $STEP_{61}$ and $STEP_{46}$ contains a highly conserved domain at their C-terminus, the consensus sequence of PTP ([I/V] HCxAGxxR [S/T] G)[1] which is required for the catalytic function, sharing an active site pattern consisting of a cysteine and an arginine which are essential for catalysis enzymatic activity. The presence of cysteine in the active site is responsible for the common characteristic of the protein phosphatases of tyrosine residues, which is to be inhibited by pervanadate, p-chloromercuribenzoate, and other oxidizing agents. Upstream of the catalytic domain is a kinase-interacting motif (KIM) required to associate STEP with its substrates and which plays an essential role in many important biological processes such as the stress response,

[1] I-isoleucine, V-valine, H-histidine, C-cysteine, A-alanine, G-glycine, R-arginine, S-serine, T-threonine and X- any amino acid

cell proliferation, apoptosis and tumorigenesis (Bult et al. 1996; Goebel-Goody et al. 2012; Lombroso et al. 2016; Nguyen et al. 2002; Paul et al. 2003; Xu, Kurup, Nairn, et al. 2012).

Two minor alternatively spliced variants of STEP include $STEP_{38}$ and $STEP_{20}$. Both are truncated isoforms which do not contain PTP consensus domain and are therefore catalytically inactive. The functions of these isoforms are not yet completely clarified, although it was suggested they may compete with the catalytically active isoforms for binding to the substrates thus preventing tyrosine dephosphorylation (Goebel-Goody et al. 2012; Lombroso et al. 2016; Sharma et al. 1995).

$STEP_{33}$ is the only isoform resulting from post-tranlational processing mediated by calpains (Braithwaite et al. 2008; Goebel-Goody et al. 2012; Gurd et al. 1999; Lombroso et al. 2016).

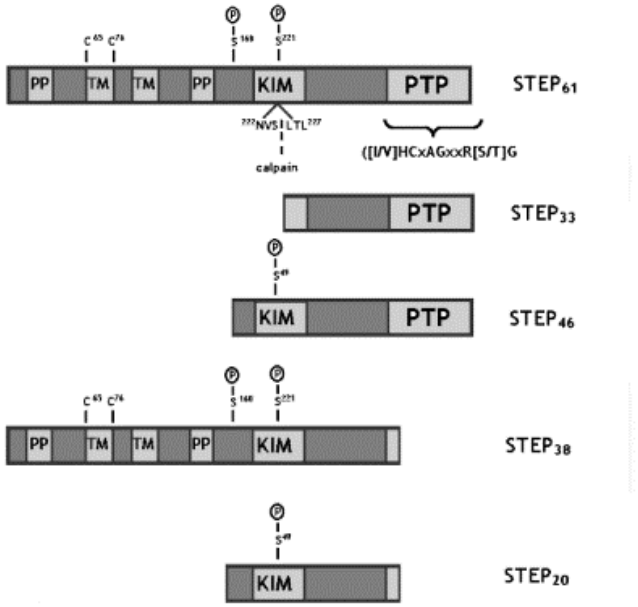


Figure 3. **STEP structure.** There are four alternatively spliced variants of STEP ($STEP_{61}$, $STEP_{46}$, $STEP_{38}$, and $STEP_{20}$), and one calpain cleavage product ($STEP_{33}$). Adapted from (Goebel-Goody et al. 2012).

1.1.2 STEP substrates

The discovery of STEP substrates (Figure 4) was a major advance in understanding the possible role of STEP in the regulation of signaling pathways. STEP dephosphorylates and inactivates proteins that include the mitogen-activated protein kinase family, glutamate receptors, Fyn kinase tyrosine and proline-rich tyrosine kinase 2 (Pyk2)(Goebel-Goody et al. 2012; He et al. 2014; Lombroso et al. 2016).

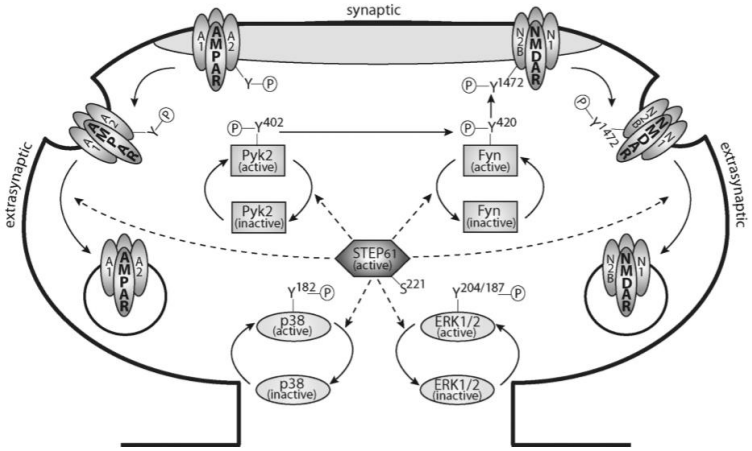


Figure 4. STEP substrates. STEP dephosphorylates ERK1/2, p38, Fyn, Pyk2, and glutamate receptors leading to their inactivation. From (Goebel-Goody et al. 2012).

A. Mitogen-activated protein kinase family

Extracellular Signal-regulated Kinase 1 and 2 (ERK1/2) and Stress-activated Protein Kinase p38 (p38) are two members of the mitogen-activated protein kinase family (MAPK). ERK1/2 and p38 have opposing actions: ERK1/2 promotes synaptic strengthening (Goebel-Goody et al. 2012; He et al. 2014; Paul et al. 2003; Sweatt 2004) and is an essential component in signaling pathways that regulate behavioral memory formation whereas p38 activates cell death pathways (Borders et al. 2008; Cuenda and Rousseau 2007; Goebel-Goody et al. 2012; He et al. 2014).

STEP dephosphorylates regulatory tyrosine residues in the activation loop of ERK1/2 (Tyr^{204} in ERK1 and Tyr^{187} in ERK2) and p38 (Tyr^{182}), leading to inactivation of these proteins (Goebel-Goody et al. 2012; Robinson and Cobb 1997). Activation of ERK1/2 is achieved by the phosphorylation of Thr^{202} and Tyr^{204} in ERK1 and Thr^{185} and Tyr^{187} in ERK2 through double-specificity kinases. One function of ERK1/2 is to initiate transcription through activation of the transcription factors cAMP response element-binding protein (CREB) and ETS domain-containing protein (Elk-1) in the nucleus. Inactivation of ERK requires the dephosphorylation of $Tyrosine^{204/187}$ by STEP, thus limiting its activity (Goebel-Goody et al. 2012; Paul et al. 2007; Robinson and Cobb 1997; Sweatt 2004). The protein p38 is responsible for cellular responses under conditions of stress and inflammation and similarly to ERK, its activation is achieved by

the phosphorylation of Threonine^{180} and Tyrosine^{182}. p38 initiates cascades of cell death signaling by B-cell lymphoma 2 (Bcl-2) phosphorylation and by the regulation of pro-apoptotic proteins and transcription factors. STEP dephosphorylates Tyrosine^{182} and consequently inactivates p38 (Cuadrado and Nebreda 2010; Goebel-Goody et al. 2012; Muñoz et al. 2003; Xu et al. 2009).

The ability of STEP to regulate two opposing mitogen activated protein kinases (MAPKs), ERK1/2 and p38 depends on whether synaptic or extrasynaptic N-methyl-D-aspartate receptors (NMDARs) are activated (Xu et al. 2009). Prolonged glutamate stimulation results in activation of extrasynaptic NMDARs and cleavage of $STEP_{61}$ by calpains, resulting in a nonfunctional isoform, $STEP_{33}$. $STEP_{33}$ lacks an intact KIM domain and does not interact with its substrates, including p38. This leads to the sustained activation of p38 and favors p38-mediated cell death pathways. In contrast, synaptic NMDAR stimulation leads to ubiquitination and degradation of $STEP_{61}$ and subsequent ERK1/2 activation, favoring the development of synaptic plasticity and neuronal survival. Both promote the reduction of $STEP_{61}$ levels. The synaptic stimulation leads to phosphorylation of ERK1/2, whereas extrasynaptic stimulation leads to phosphorylation of p38. These events seem mechanistically distinct because extrasynaptic NMDAR-mediated cleavage and inhibition of $STEP_{61}$ fail to activate ERK1/2. Perhaps the answer to this conundrum lies in the subcellular localization of p38 and ERK1/2. Specifically, p38 is more concentrated in extrasynaptic membranes compared with synaptic membranes, so extrasynaptic stimulation could lead to the selective cleavage of the extracellular pool of STEP (Goebel-Goody et al. 2012; Xu et al. 2009). Alternatively, it is possible that extrasynaptic NMDAR stimulation promotes dominant ERK-inactivating signals that supersede STEP cleavage (Hardingham and Bading 2010). Nonetheless, STEP role in regulating ERK1/2 and p38 after synaptic and extrasynaptic NMDAR activation, respectively, probably explains why extrasynaptic stimulation promotes cell death, whereas synaptic stimulation promotes cell survival (Goebel-Goody et al. 2012; Xu et al. 2009).

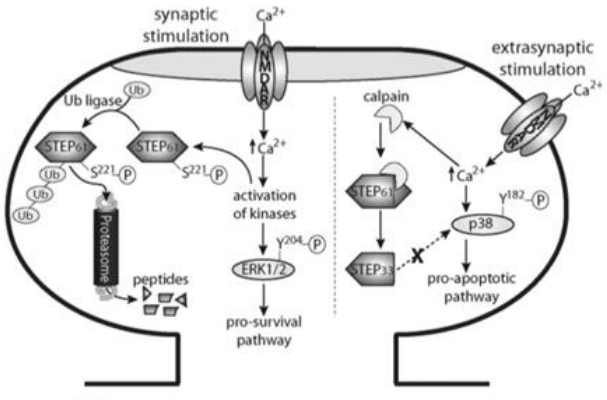


Figure 5. Ability of STEP to regulate two opposing MAPKs. The synaptic stimulation promotes synaptic strengthening, while extrasynaptic stimulation promotes cell death pathway. From (Goebel-Goody et al. 2012).

B. Glutamate Receptors

STEP regulates the phosphorylation and surface expression of two glutamate receptor subtypes, N-methyl-D-aspartate receptor (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) (Braithwaite et al. 2008; Goebel-Goody et al. 2012).

The NMDA receptor is a glutamate receptor and ion channel protein found in nerve cells (Schorge and Colquhoun 2003). NMDARs are heteromeric ligand gated ion channels assembled from two subunit families: NR1, which consists of eight recognized isoforms that are generated from alternative splicing of a single gene, and NR2, composed of NR2A, NR2B, NR2C and NR2D, encoded by four distinct genes. The NR1 subunit is essential for functional NMDAR channels, whereas the four NR2 subunits modulate channel activity and functional properties of the receptors (Jiang et al. 2011). $STEP_{61}$ regulates the phosphorylation of NR2B by two parallel mechanisms: by direct dephosphorylation of NR2B Tyr^{1472} (Snyder et al. 2005; Xu et al. 2010) and indirectly via dephosphorylation and inactivation of the SFK Fyn(Nguyen et al. 2002). Phosphorylation of Tyr^{1472} tightly and dynamically regulates the surface expression of NR2B (Roche et al. 2001). Tyr^{1472} resides within a conserved tyrosine-dependent endocytic motif. When dephosphorylated, the tyrosine residue in this motif binds to clathrin and adapter protein 2 via strong hydrophobic interactions and promotes endocytosis. Corroborating this hypothesis, the surface expression of NR1/NR2B receptor complexes is elevated in STEP knockout (KO) mice (Venkitaramani et al. 2011; Zhang et al. 2010).

The AMPA receptor is an ionotropic transmembrane receptor for glutamate that mediates fast synaptic transmission in the central nervous system. AMPARs are ligand-gated ion channels composed of four types of subunits, designated as GluR1, GluR2, GluR3, and GluR4 (Mayer and Armstrong 2004). Although the molecular mechanisms underlying tyrosine-dependent internalization of AMPARs remains incompletely understood, $STEP_{61}$ seems to promote the endocytosis of AMPARs in a manner similar to that in which it promotes NMDARs, by dephosphorylating a key tyrosine residue in the GluR2 subunit (Gladding et al. 2009; Moult et al. 2006) (Chen et al. 2013; Zhang et al. 2008). Whether internalized NMDAR and AMPAR complexes are recycled or degraded is not yet known (Lombroso et al. 2016). S-3,5-dihydroxyphenylglycine (DHPG, a potent agonist of group I metabotropic glutamate receptors [mGluR]) leads to internalization of GluR1/GluR2. DHPG stimulation of mGluRs increases the local translation of STEP, resulting in the subsequent dephosphorylation and endocytosis of GluR1/GluR2. Moreover, neuronal cultures from STEP knockout mice display increased surface expression of AMPARs and do not undergo DHPG-mediated AMPAR endocytosis; however, internalization of AMPARs can be restored with the re-introduction of STEP into the knockout mouse culture (Zhang et al. 2008).

C. Tyrosines Kinases

Two other STEP substrates are Fyn kinase tyrosine and proline-rich tyrosine kinase 2 (Pyk2). Dephosphorylation of the regulatory tyrosines in their activation loops inactivates these kinases (Nguyen et al. 2002; Xu, Xu et al. 2012). $STEP_{61}$ has two polyproline-rich regions that, in addition to the KIM domain, are involved in substrate binding and contribute to substrate specificity; the first polyproline domain facilitates binding to Fyn (Nguyen et al. 2002), while the second polyproline domain is necessary for binding to Pyk (Xu et al. 2012). Of note, Fyn phosphorylates NR2B at Tyr^{1472}, the same site that is dephosphorylated by STEP. Thus, STEP dephosphorylates NR2B directly and at the same time dephosphorylates and inactivates the kinase that phosphorylates NR2B (Nakazawa et al. 2001; Nguyen et al. 2002; Snyder et al. 2005).

Current studies suggest that PTPα, an activator of Fyn kinase, is a new STEP substrate dephosphorylated by STEP at Tyr^{789} (Xu et al. 2015). In contrast to STEP, which dephosphorylates the activation loop and thereby inactivates Fyn, PTPα dephosphorylates a distinct inhibitory pTyr residue in Fyn (Engen et al. 2008; Ingley 2008). Notably, inactivation of STEP contributes to the increased tyrosine phosphorylation of PTPα and subsequent translocation into lipid raft fractions, leading to the activation of Fyn (Xu et al. 2015). Thus, STEP has a two modes of inactivating Fyn: it directly inactivates Fyn and concomitantly prevents activation of Fyn by PTPα by blocking its translocation to the membrane (Lombroso et al. 2016).

1.1.3 STEP regulation

Expression and ability of STEP to bind to and dephosphorylate its substrates is regulated by several known mechanisms: phosphorylation, proteolytic cleavage, dimerization and ubiquitination. Some of these mechanisms are disrupted in animal models with neuropsychiatric disorders leading to changes in STEP activity(Goebel-Goody et al. 2012).

A. Phosphorylation

Phosphorylation is an important form of posttranslational modification that regulates various intracellular signaling pathways. $STEP_{61}$ and $STEP_{46}$ contain a kinase interacting motif, which regulates the interactions of STEP with its substrates. Both $STEP_{61}$ and $STEP_{46}$ isoforms are phosphorylated by cyclic adenosine monophosphate (cAMP) dependent protein kinase (PKA), (Goebel-Goody et al. 2012; Paul et al. 2000). PKA-mediated STEP phosphorylation is promoted by activation of the dopamine receptor D1 (D1R). D1R activation stimulates cAMP synthesis and activates PKA, which in turn leads to phosphorylation of both $STEP_{61}$ and $STEP_{46}$ at a conserved serine residue (designated Ser^{221} in $STEP_{61}$ and Ser^{49} in $STEP_{46}$) (Valjent et al. 2005). The phosphorylation of the serine residues prevents STEP from interacting with its substrates.

Activation of PKA stimulates dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) phosphorylation at Thr^{34} and thereby converts DARPP-32 into a potent inhibitor of protein phosphatase-1 (PP1), the phosphatase that dephosphorylates STEP Ser^{221} thus promoting the interaction of $STEP_{61}$ with its substrates. Conversely, neuronal stimulation triggers calcium influx and activation of calcineurin to dephosphorylate and inactivate DARPP-32, thereby reducing DARPP-32-mediated inhibition of PP1 and increasing $STEP_{61}$ activity by reducing phosphorylation of Ser^{221} (Goebel-Goody et al. 2012; Svenningsson et al. 2004; Valjent et al. 2005).

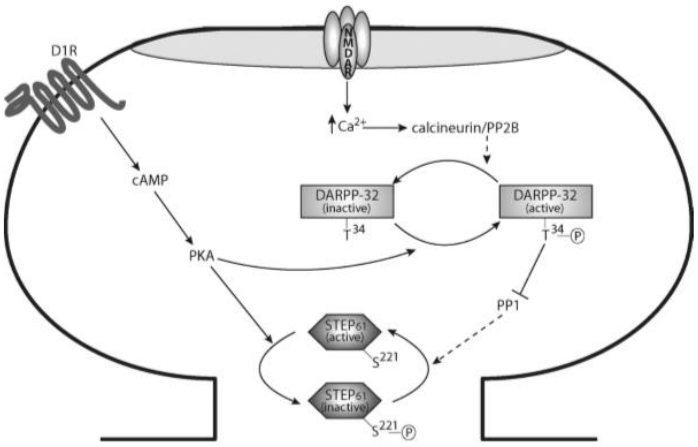


Figure 6. **Regulation of STEP phosphorylation.** D1R activation stimulates cAMP synthesis and activates PKA, which phosphorylates $STEP_{61}$ at Ser^{221}. Phosphorylation inhibits the binding of STEP to its substrates. PKA also leads to phosphorylation and activation of DARPP-32. When phosphorylated, DARPP-32 inhibits PP1 activity, which is the phosphatase that dephosphorylates STEP and promotes the interaction with its substrates. Conversely, NMDAR stimulation initiates calcium influx and activation of calcineurin/PP2B to dephosphorylate and inactivate DARPP-32, thereby reducing DARPP-32-mediated inhibition of PP1 and increasing STEP activity by reducing phosphorylation of Ser^{221}. From (Goebel-Goody et al. 2012).

B. Proteolytic cleavage

$STEP_{61}$ is proteolytically cleaved by a calcium-dependent mechanism involving calpains. This cleavage occurs between residues Ser^{224} and Leu^{225} in the KIM domain, resulting in a nonfunctional isoform, $STEP_{33}$. $STEP_{33}$ can not associate with or dephosphorylate its substrates (Xu et al. 2009). This cleavage frequently occurs after strong glutamatergic stimulation such as during excitotoxic or ischemic insult. A peptide that spans the $STEP_{61}$ cleavage site blocks the proteolysis of $STEP_{61}$ and is neuroprotective against glutamate excitotoxicity and oxygen-glucose deprivation (Xu et al. 2009). These findings denote that treatments preventing the cleavage of $STEP_{61}$ may be useful in stroke/ischemia (Braithwaite et al. 2008; Goebel-Goody et al. 2012; Gurd et al. 1999; Xu et al. 2009).

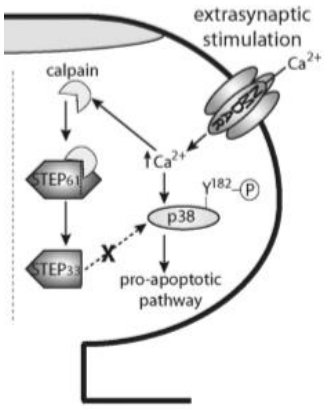


Figure 7. <u>Proteolysis of $STEP_{61}$</u>. Extrasynaptic NMDAR stimulation invokes calpain-mediated proteolysis of $STEP_{61}$ producing the truncated cleavage product $STEP_{33}$. The p38 is preferentially activated by extrasynaptic NMDAR stimulation, and the cell death pathways are subsequently initiated. From (Goebel-Goody et al. 2012).

C. Dimerization

Dimerization of $STEP_{61}$ occurs within the hydrophobic region of the amino terminus of $STEP_{61}$ and involves intermolecular disulfide bond formation between two cysteine residues (Cys^{65} and Cys^{76}). Only dimerization of $STEP_{61}$ occurs, not $STEP_{46}$, leading to decreased of phosphatase activity. Under conditions of oxidative stress, induced by hydrogen peroxide, $STEP_{61}$ dimers are formed leading to further decrease in phosphatase activity. Although $STEP_{46}$ does not undergo basal dimerization, the exposure to oxidative stress may induce the formation of $STEP_{46}$ oligomers and moderate loss of activity (Deb et al. 2011). Since oxidation of the cysteine residue at the catalytic site also leads to inhibition of tyrosine phosphatase activity, these studies may suggest a class of noncompetitive STEP inhibitors(Goebel-Goody et al. 2012).

D. Ubiquitination

The ubiquitination of target proteins involves the covalent attachment of ubiquitin to the substrate and often leads to proteasomal degradation of the protein. Data from the literature associates impairement of the ubiquitin proteasome system (UPS) to cancer and neurodegeneratives diseases (Hegde 2010). $STEP_{61}$ is a target for ubiquitin-mediated proteasomal degradation. STEP ubiquitination is differentially regulated by synaptic and extrasynaptic NMDARs. When synaptic NMDARs are stimulated, $STEP_{61}$ is ubiquitinated and rapidly degraded from synaptic sites by the UPS pathway presumably to diminish STEP activity and permit enhanced signaling of STEP substrates that promote synaptic plasticity (Goebel-Goody et al. 2012; Xu et al. 2009). STEP degradation is required for sustained ERK activation. Activated ERK phosphorylates several synaptic and cytoplasmic proteins, and is translocated to the nucleus where it phosphorylates and activates transcription factors such as CREB and Elk-1 (Thiels and Klann 2001; Xu et al. 2012). These events can have lasting effects on the formation and stabilization of dendritic spines and therefore contribute to long-term information storage(Xu et al. 2012).

Although the molecular mechanisms underlying ubiquitination of $STEP_{61}$ are still misunderstood, the amino-terminal region of $STEP_{61}$ contains two PEST sequences (Bult et al. 1996) and these are often found in proteins degraded by the UPS. Current investigations are aimed at addressing whether phosphorylation of residues adjacent to the PEST sequences and/or other mechanisms are responsible for initiating $STEP_{61}$ ubiquitination(Goebel-Goody et al. 2012). Kurup et al, demostrate that parkin is an E3 ubiquitin ligase that regulates $STEP_{61}$ levels through the ubiquitin proteasome system. In cellular models, parkin ubiquitinates $STEP_{61}$, whereas clinically relevant parkin mutants fail to do so. In Parkinson's disease, in which parkin function is compromised, $STEP_{61}$ accumulates and the increase in $STEP_{61}$ is associated with a decrease in the phosphorylation of its substrate ERK1/2 and the downstream target of ERK1/2, pCREB, due to down-regulation of synaptic proteins required for neuronal plasticity.These studies suggest that $STEP_{61}$ is a substrate of parkin (Kurup et al. 2015).

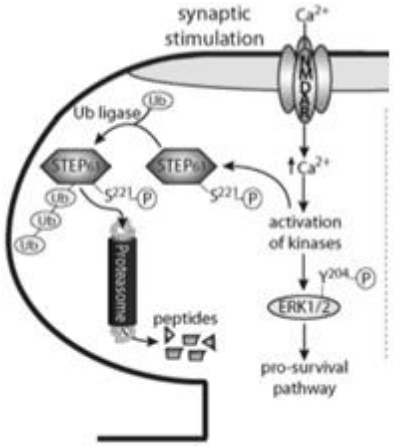


Figure 8. <u>STEP ubiquitination</u>. Synaptic NMDAR stimulation results in the degradation of $STEP_{61}$ by ubiquitin proteasome system, leads to an increase in ERK1/2 activation and promotes neuronal survival. From (Goebel-Goody et al. 2012).

1.1.4 STEP inhibition

Neuropsychiatric disorders might benefit from the development of STEP inhibitors. Xu et al, identified the benzopentathiepin 8-(trifluoromethyl)-1,2,3,4,5-benzopentathiepin-6-amine hydrochloride (known as TC-2153) as a novel inhibitor of STEP activity with an IC_{50} of 24.6nM (Figure9). TC-2153 represents a novel class of PTP inhibitors based upon a cyclic polysulfide pharmacophore. The mechanism by which TC-2153 inhibits STEP activity involves the formation of a covalent bond with a cysteine residue within the catalytic domain of STEP (Xu et al. 2014). The oxidative attack and addition of a sulfur to the cysteine promotes the loss of STEP catalytic activity and increased tyrosine phosphorylation of STEP substrates in neuronal cultures and WT mice. Mass spectrometry confirmed the modifications of cysteine in the active site, suggesting that a sulfur from the benzopentathiepin ring is retained (Boivin et al. 2010; Hertog et al. 2008). Current studies showing that oxidative regulation of PTPs is an important regulatory mechanism occurring in cells link tyrosine phosphorylation signaling and redox status (Boivin et al. 2010; Hertog et al. 2008).

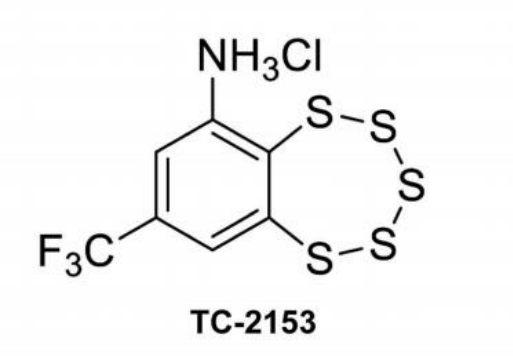


Figure 9. **STEP inhibitor.** The benzopentathiepin 8-(trifluoromethyl)-1,2,3,4,5-benzopentathiepin-6-amine hydrochloride (known as TC-2153) inhibits STEP activity by the formation of a covalent bond with a cysteine residue within the catalytic domain of STEP.

1.1.5 STEP and neurodegenerative diseases

Many studies focused on understanding how STEP dysregulation contributes to the pathophysiology of neuropsychiatric disorders. In cases, such as in Alzheimer's disease (AD), Schizophrenia (SZ) and Fragile X Syndrome (FXS), epileptogenesis, and alcohol-induced memory loss, STEP protein level and activity are up-regulated. In others, such as Huntington's disease (HD), drug abuse, stroke/ischemia, and inflammatory pain STEP protein and/or activity is down-regulated (Goebel-Goody et al. 2012).

Table 1. Neuropsychiatric disorders associated with changes in STEP. From (Goebel-Goody et al. 2012).

Disorder	STEP Abnormalities Associated with the Disorder	Remarks	References
Alzheimer's disease	Phosphorylation ↓ Protein levels ↑	Aβ binds to α7nAChRs and triggers activation of PP2B/calcineurin and PP1 to dephosphorylate $STEP_{61}$; increased $STEP_{61}$ protein levels are due to Aβ-induced inhibition of the ubiquitin proteasome system; genetic reduction of STEP attenuates cognitive, biochemical, and electrophysiological deficits in AD mice	Snyder et al., 2005; Kurup et al., 2010; Zhang et al., 2010
Schizophrenia	Protein levels ↑	STEP KOs are resistant to PCP-induced hyperlocomotion and cognitive deficits; neuroleptics lead to $STEP_{61}$ phosphorylation and promote phosphorylation and activation of STEP substrates	Correa et al., 2009; Carty et al., 2010
Fragile X syndrome	Protein levels ↑	STEP mRNA associates with the RNA-binding protein FMRP; absence of FMRP increases translation of $STEP_{61}$; STEP deletion reduces audiogenic seizures in *Fmr1* KO mice	Goebel-Goody et al., 2010; Darnell et al., 2011
Epileptogenesis	Protein expression in inhibitory hilar interneurons—High	Expression in inhibitory interneurons prevents ERK1/2 activation and promotes cell death; STEP KOs are more resistant to pilocarpine-induced SE	Choi et al., 2007; Briggs et al., 2011
Alcohol-induced memory loss	Activity ↑	STEP contributes to the ethanol-induced inhibition of NMDAR function, LTP, and fear conditioning	Alvestad et al., 2003; Wu et al., 2010; Hicklin et al., 2011
Huntington's disease	mRNA ↓ Protein ↓ Phosphorylation ↑	STEP increases vulnerability of neurons to undergo cell death	Saavedra et al., 2011
Drugs of abuse	Phosphorylation ↑	Increased $STEP_{61}$ phosphorylation is associated with greater ERK1/2 phosphorylation; infusion of TAT-$STEP_{C\text{-}S}$ into VLS prevents amphetamine-induced behavioral stereotypies	Valjent et al., 2005; Tashev et al., 2009
Stroke/ischemia	mRNA in CA1/2 after global ischemia ↓ mRNA post-tMCAO ↓ mRNA in DG ↑	$STEP_{61}$ is cleaved into $STEP_{33}$ after tMCAO; use of competitive peptide spanning cleavage site attenuates cell death after glutamate excitotoxicity or oxygen-deprivation models	Braithwaite et al., 2008; Xu et al., 2009
Inflammatory pain	Interaction with Fyn ↓	Reduced $STEP_{61}$-Fyn association leads to phosphorylation and activation of Fyn as well as phosphorylation of GluN2B Tyr^{1472}	Yang et al., 2011

A. Disorders associated with up-regulation of STEP protein levels or activity

- Alzheimer's disease (AD) is a debilitating neurodegenerative disorder associated with memory impairments. It is characterized by hyperphosphorylation of tau protein, accumulation of ß-amyloid peptide (Aß) and the formation of amyloid plaques, all of which have been implicated in synaptic loss and cognitive decline (Paul et al. 2007; Xu et al. 2012).

In AD, Aß peptide through α7nAChRs binding and synaptic NMDAR stimulation leads to Ca^{2+} influx into neurons and evokes activation of PP2B/calcineurin and PP1 to dephosphorylate $STEP_{61}$ at Ser^{221}, thereby increasing the affinity of $STEP_{61}$ for its substrates (Snyder et al. 2005). Aß modulates STEP through another parallel pathway. Aß peptide inhibits the ubiquitin proteasome system and prevents degradation of $STEP_{61}$. The result is an accumulation of unphosphorylated and active $STEP_{61}$ protein in AD, which leads to inappropriate dephosphorylation of NR2B Tyr^{1472} and internalization of NR2B-containing NMDARs (Goebel-Goody et al. 2012; Xu et al. 2012).

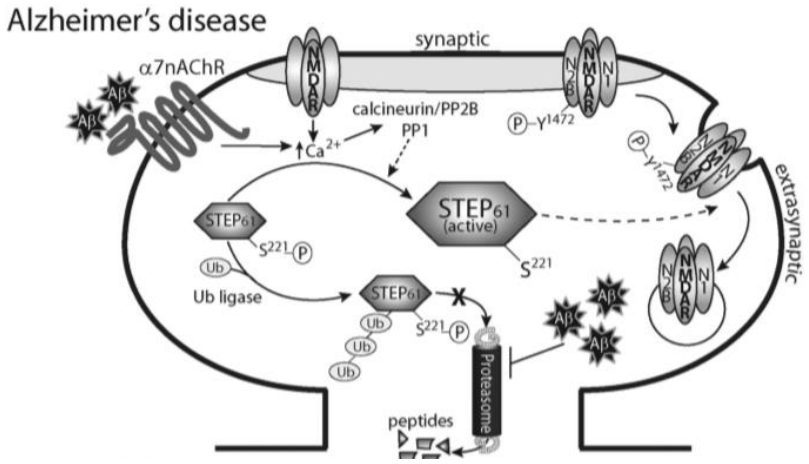


Figure 10. STEP in Alzheimer´s disease. Aß modulates STEP via two parallel pathways: Aß peptide binding to α7nAChRs and synaptic NMDAR stimulation leading to a Ca^{2+} influx into neurons and invoke activation of PP2B/calcineurin and PP1 to dephosphorylate $STEP_{61}$, thereby increasing the affinity of $STEP_{61}$ for its substrates. The other pathway, Aß peptide inhibits the ubiquitin proteasome system and prevents degradation of $STEP_{61}$. The result is an accumulation of unphosphorylated and active $STEP_{61}$ protein levels in AD, which leads to inappropriate dephosphorylation of NR2B Tyr^{1472} and internalization of NR2B-containing NMDARs. From (Goebel-Goody et al. 2012).

-Schizophrenia (SZ) has a complex etiology, in which genetics, the environment, and neuronal dysfunction are all contributing factors. SZ is a mental disorder with behavioral and cognitive deficits, in part due to the disruption of glutamatergic signaling (Owen et al. 2016). Patients with schizophrenia present higher STEP expression in the anterior cingulated cortex and dorsolateral prefrontal cortex (Barksdale et al. 2014; Carty et al. 2012). Similar effects were observed in mice treated with the psychotomimetics MK-801 and phencyclidine (PCP) (Carty et al. 2012). In contrast, STEP knockout mice are less sensitive to acute and chronic PCP administration in terms of their locomotor and cognitive effects. Furthermore, several typical and atypical antipsychotic medications, which are D2R antagonists, for schizophrenia treatment

can act through STEP. Treatment of mice with antipsychotics induces protein kinase A-mediated $STEP_{61}$ phosphorylation and inactivation and increases NR1/NR2B receptor surface expression(Carty et al. 2012; He et al. 2014). This suggests that the beneficial effects of neuroleptics are mediated, at least in part, through restoration of NMDAR levels at synaptic sites. It has been suggested that proteasome degradation of STEP is blocked in both AD and schizophrenia, leading to STEP accumulation and glutamate receptor internalization, which further contribute to AD and schizophrenia pathogenesis. (Carty et al. 2012; Zhang et al. 2010).

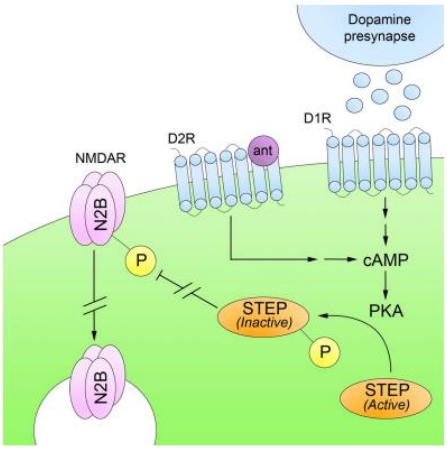


Figure 11. STEP in Schizophrenia´s disease. Deficits in glutamatergic neurotransmission are believed to underlie the pathophysiology of SZ. Administration of typical and atypical antipsychotics, which are D2R antagonists, increase PKA-mediated phosphorylation of STEP. Inactivation of STEP results in increased trafficking of NMDARs to synaptosomal surface membranes. From (Fitzpatrick and Lombroso 2011).

B. Disorders associated with down-regulation of STEP protein levels or activity

- STEP is highly expressed in striatal projection neurons, the neuronal population most affected in Huntington's disease(Saavedra et al. 2011).

HD is a progressive, fatal, neurodegenerative disorder characterized by poor muscle coordination, mood alterations, and dementia (Ross and Tabrizi 2011). The cause of HD is an abnormal CAG expansion in exon-1 of the huntingtin (htt) gene (MacDonald et al. 1993) in striatal projection neurons, which constitute the vast majority of striatal neurons (Reiner et al. 1988). R6/1 mice expressing mutant huntingtin (mhtt), displayed reduced STEP protein levels in the striatum and cortex whereas its phosphorylation was increased in the striatum, cortex and hippocampus, which correlated with enhanced PKA activity and reduced calcineurin activity and futher contributes to an enhancement of STEP phosphorylation and inactivation (Saavedra et al. 2011). In accordance with decreased STEP expression and activity, the phosphorylation of both ERK1/2 and p38, two targets of STEP, are elevated in R6/1 mice striatum later in life (20–30 weeks). Decreased STEP protein levels and hyperphosphorylation of STEP are also observed in several other mouse models of HD such as R6/2, Tet/HD94, and HdhQ7/Q111, confirming the results from R6/1 mice (Saavedra et al. 2011).

1.1.6 Protein tyrosine phosphatases and neuroinflammation

Protein tyrosine phosphatases (PTPs) are key regulators in inflammatory signaling pathways and microglial cells are resident immune cells in the central nervous system that participate in the initiation and propagation of an inflammatory response. Therefore, PTPs can be the target for modulation of microglial activation and to develop therapeutics for neurodegenerative and neuroinflammation diseases (Perry and Holmes 2014; Song et al. 2016). Yet, little is known about their role in neuroinflammation and, to our knowledge, there are no data on the expression of STEP by microglial cells or on the regulation of microglial reactivity by this phosphatase. The expression of 6 different PTPs (PTP1B, TC-PTP, SHP2, MEG2, LYP2, and RPTPβ) was correlated with glial activation and neuroinflammation(Song, Jung, et al. 2016; Song, Kim, et al. 2016).

Song et al. demonstrated that all PTPs described are expressed in the brain and glia (Song, Kim, et al. 2016) and expression of PTP1B, SHP2 and LYP are increasead in the inflamed brain. The expression of PTP1B, TC-PTP and LYP increases in microglial cells exposed to the pro-inflammatory agent, lipopolysaccharide (LPS). Specific pharmacological inhibitors of PTPs were used to assess the role of PTPs in microglial activation and neuroinflammation. Inhibition of PTP1B, TC-PTP, SHP2, LYP and RPTPβ suppressed the production of nitric oxide, a microglial reactivity parameter, in LPS-treated microglial cells in a dose-dependent manner. In addition, intracerebroventricular injection of inhibitors of PTP1B, PTP-TC, SHP2 and RPTPβ decreased microglial activation in a model of LPS-induced neuroinflammation (Song, Kim, et al. 2016). These results suggest that multiple PTPs, with different expression patterns and specific functions, are involved in the regulation of microglial activation and neuroinflammation (Song, Kim, et al. 2016).

1.2 Microglia

Microglial cells were discovered in 1919 by Pio del RioHortega, while using a silver carbonate staining method (Wang and Wei 2012). Microglia represent the endogenous brain defence and immune system, responsible for CNS protection against various types of pathogenic factor (Kim and de Vellis 2005). The origin of microglia relates to the early colonization of the CNS by mesodermal progenitors (Kaur et al. 2001) that arise from the yolk sac (Figure 12) (Alliot et al. 1999; Ginhoux et al. 2010).

It is now recognized that monocytes and tissue macrophages are not, as had been previously proposed (Furth and Cohn 1976), microglia progenitors in either health or disease (Kierdorf et al. 2013; Neumann and Wekerle 2013) and that, in adulthood, microglia are an independent self-renewing population(Askew et al. 2017; Bruttger et al. 2015; Tay et al. 2017). Human microglia turn over at a yearly median rate of 28% and live, on average, for 4.2 years. It is described that most of the microglial population is renewed several times over the course of a lifetime (Réu et al. 2017). In support of the importance of microglial self-renewal, a recent study demonstrated that the repopulated microglia that rapidly replenish the adult brain microglial population after microglial depletion are solely derived from the proliferation of residual microglia and not from newly generated progenitors (Huang et al. 2018).

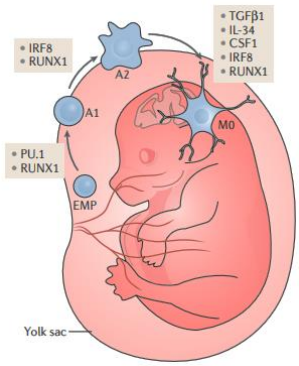


Figure 12. The origin of microglia. Microglia arise early during development from progenitors in the embryonic yolk sac. Adapted from (Butovsky and Weiner 2018)

1.2.1 Morphological states of microglia

Microglia exist in resting or activated states depending on the milieu, which differs in the healthy CNS and in various disease states. In physiologic conditions, the immune responses are fine-regulated in order to maintain tissue homeostasis. In the pathological condition, however, the immune responses are uncontrolled to either extreme of the immune balance, which is highly related with cell loss or cell dysfunction occurring during the inflammatory processes (Bolós et al. 2017; Tang and Le 2016). Under physiological conditions microglia has a resting morphology. When the microglial cells detect an insult the cells alter their ramified resting morphology and become ameboid (Figure 13)(Bolós et al. 2017). This process is generally called "microglial activation" and proceeds through several steps. The M1 (neurotoxicity)/M2 (neuroprotection) paradigm (Figure 14) is a simplified model to decipher the two polars of the inflammatory responses (Colton 2009; Colton and Wilcock 2010; Franco and Fernández-Suárez 2015). Classical activation originally respond to the injury and infection, and generally act in the first line to protect the tissue and promote the destruction of invading pathogens. However, they also induce neurotoxicity due to the release of pro-inflammatory factors and various neurotoxic mediators such as such as tumor necrosis factor-α (TNF-α), interleukin-1ß (IL-1ß), nitric oxide (NO), reactive oxygen species (ROS), and proteases, and often setup a vicious cycle between dying neurons and acute inflammation. Microglia in this state is termed “M1 microglia" (Block et al. 2007; Le et al. 2001; Li et al. 2004). After the onset of classical activation, an anti-inflammatory and repair phase is rapidly initiated and leads to wound healing and brings back tissue homeostasis promoted by “M2 microglia”. Alternative activation is limited to the activation state triggered by anti-inflammatory cytokines IL-4 or IL-13 and is closely associated with M2 genes that promote anti-inflammation, tissue repair, and extracellular matrix (ECM) reconstruction (Colton 2009; Ponomarev et al. 2007). While acquired deactivation is another state to alleviate acute inflammation and is induced primarily by uptake of apoptotic cells or exposure to anti-inflammatory cytokines such as IL-10 and transforming growth factor-ß (TGF-ß). During the progression of neurodegenerative diseases, there is an imbalance of M1/M2 populations with the M1 phenotype being more predominant at late stages (Colton and Wilcock 2010; Sawada et al. 1999).

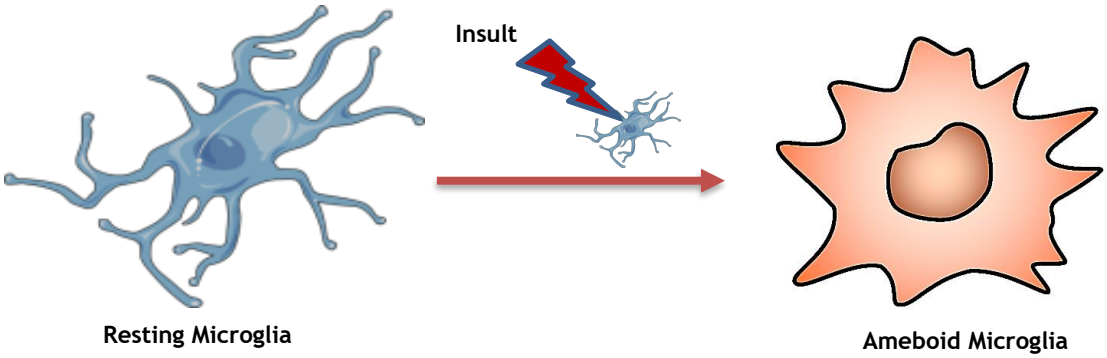


Figure 13. Morphologies of microglia. Under physiological conditions the microglia in the central nervous system has a resting morphology and when detect an insult in the brain microglial cells become ameboid.

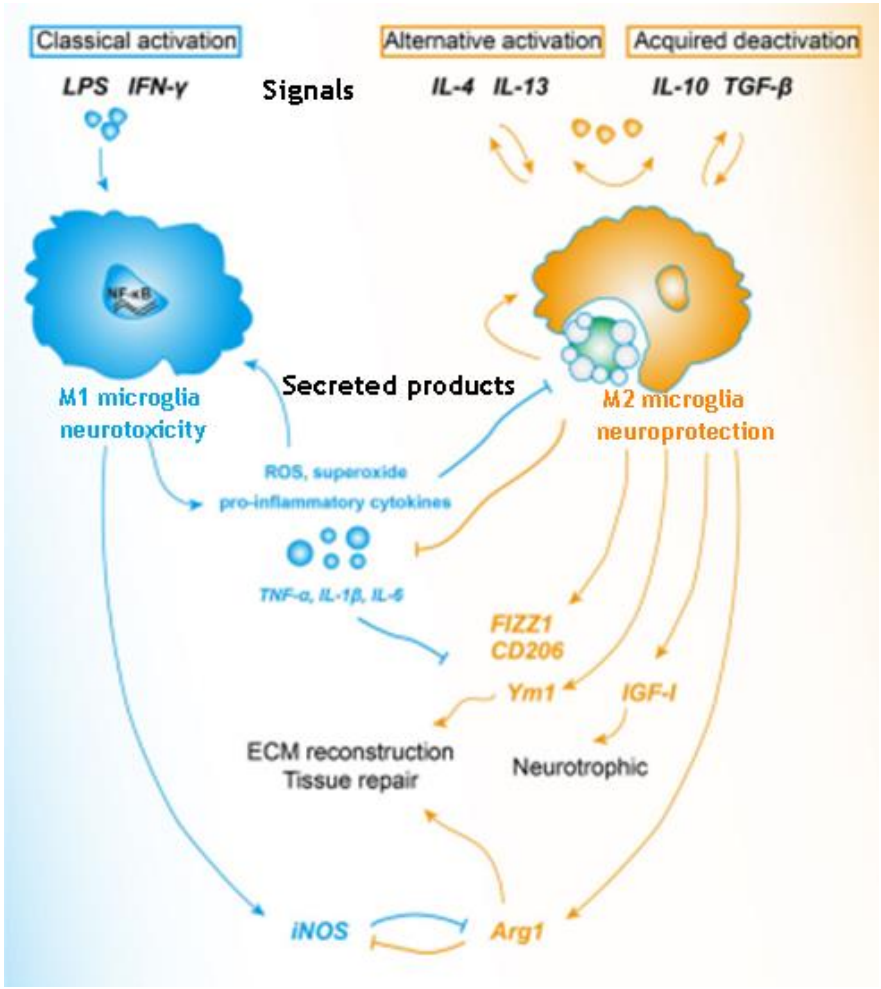


Figure 14. M1/M2 microglia. Microglia possess states of "classical activation," "alternative activation," and "acquired deactivation," depending on the milieu in which they become activated and the factors they are stimulated. M1 microglia is neurotoxicity and induce iNOS and NF-κB pathways and produce various pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, as well as superoxide, ROS and NO. M2 microglia is neuroprotective and include the states of both alternative activation and acquired deactivation, which are induced by IL-4/IL-13 and IL-10/TGF-β, respectively. M2 microglia facilitate phagocytosis of cell debris and misfolded proteins, promote ECM reconstruction and tissue repair, and support neuron survival by neurotrophic factors. From (Tang and Le 2016).

1.2.2 Microglial markers

Microglia express macrophage markers, including adhesion G protein coupled receptor E1 (ADGRE; also known as cell surface glycoprotein F4/80), crystallizable fragment (Fc) receptors, αM integrin (ITGAM; also known as CD11b) (Akiyama and McGeer 1990; Perry et al. 1985) and the calcium binding protein allograft inflammatory factor 1 (AIF1; also known as IBA1) (Ginhoux and Prinz 2015). It is described that Iba1 will stain both ramified and ameboid microglia, although expression levels will increase with increased reactivity. Microglia phenotypes can also be distinguished by cluster of differentiation 68 (CD68), since this protein express in only expressed at high levels in macrophages and activated microglia (Hendrickx et al. 2017). However, several proteins have been proposed as specific markers to differentiate between M1 and M2 states (according to Table 2) (Bolós et al. 2017; Franco and Fernández-Suárez 2015).

Table 2. Specific markers to differentiate microglia M1/M2. Arg1, arginase1; CCL7, chemokine (C-C motif) ligand 7; CD, cluster of differentiation; FIZZ1, found in inflammatory zone 1; Iba1, ionized calcium-binding adapter molecule 1; IFN-γ, interferon γ; IGF-1, insulin growth factor 1; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MHC II, major histocompatibility complex II; TGF-ß, transforming growth factor ß; TNF-α, tumor necrosis factor α; YM1, chitinase-like 3. Adapted from (Bolós et al. 2017).

M1 neuroxicity	M2 neuroprotection
CCL7	Arg1
CD68	CD23
CD80	CD68
CD86	CD163
Iba1	CD204
iNOS	CD206
MHCII	FIZZ1
TNFα	Iba1
IL-1ß	IGF-1
	YM1
	TGFß

1.2.3 Functions of microglia

In the normal brain the main function of microglia is 'surveillance' (Figure 15)(Chen and Trapp 2016). However, microglia exhibits widely differing functions at different stages in life and in physiological or pathological situations such as capability for migration and motility, phagocytosis and antigen presentation (figure 16 and Table 3) (Tang and Le 2016). In the pre-natal CNS, microglia regulates the outgrowth of dopaminergic axons in the forebrain and shape the fasciculation of axonal tracts in the corpus callosum by providing trophic support to neurite formation (Pont-Lezica et al. 2014; Squarzoni et al. 2014). Microglial cells exhibit two types of movement activity: in the ramified form they actively move their processes without translocation of the cell body. Whereas In the amoeboid form microglial cells move their processes, but in addition the whole cell migrate through the brain tissue. Microglial migration occurs also during development, when invading monocytes spread through the brain. Another type of migration is triggered by a pathological insult when the branched microglia undergoes activation, transforms into the amoeboid form and migrates to the lesion site (Kettenmann and Verkhratsky 2011). Besides, microglia is also important for regulating the migration and positioning of new neurons to ensure that the laminar structure of the cortex is correctly shaped (Squarzoni et al. 2014).

Microglial cells are the innate phagocytes of the CNS tissue. This function is important for the normal brain, during brain development, and in pathology and regeneration (Kettenmann and Verkhratsky 2011). During fetal neurogenesis, more neurons are produced than are required for the development of cerebral cortex. To maintain homeostasis, microglia limit the over-production of neurons by phagocytizing neural precursor cells (Cunningham et al. 2013). During CNS development microglial phagocytosis is instrumental in removing apoptotic cells and may be involved in synapse removal during development and also in pruning synapses in the postnatal brain. In response to a lesion, microglial cells accumulate at the damaged site and remove cellular debris or even parts of damaged cells. Through phagocytosis microglial cells can also remove pathological factors such as beta-amyloid in Alzheimer's disease or myelin fragments in demyelinateing disease (Kettenmann and Verkhratsky 2011).

Microglia are the dominant antigen presenting cells in the central nervous system. Under resting conditions, the expression of the molecular complex for presenting antigen, the major histocompatibility complex II (MHCII) and co-stimulatory molecules such as CD80, CD86 and CD40 are below detection. Upon injury, microglial cells scavenge cellular debris as part of wound healing and tissue repair (Chen and Trapp 2016) and and as a consequence of microglial activation the expression of MHCII and co-stimulatory molecules increase. This has been described in Multiple Sclerosis witch microglial cells phagocytose myelin, degrade it and present peptides of the myelin proteins as antigens. By releasing cytokines such as CCl2 microglial cells promote the recruitment of leucocytes to the CNS. Microglia interact with infiltrating T lymphocytes and mediate the immune response in the brain by stimulating the proliferation of both TH1- and TH2-CD4 positive T cells. (Kettenmann and Verkhratsky 2011).

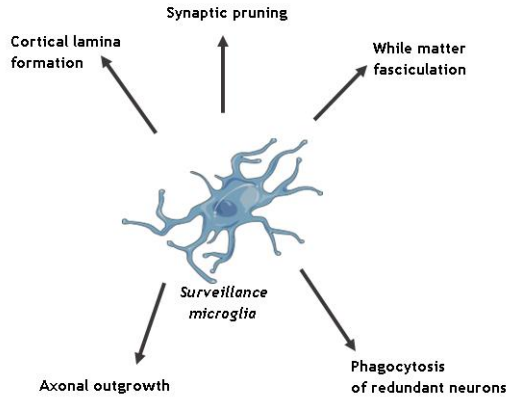


Figure 15. Functions of microglia in physiological conditions. Microglia maintain tissue homeostasis during brain development by pruning synapses or phagocytizing redundant neurons. They also participate in the proper formation of CNS structures, including cortical lamina formation and axon bundle fasciculation. Adapted from(Chen and Trapp 2016)

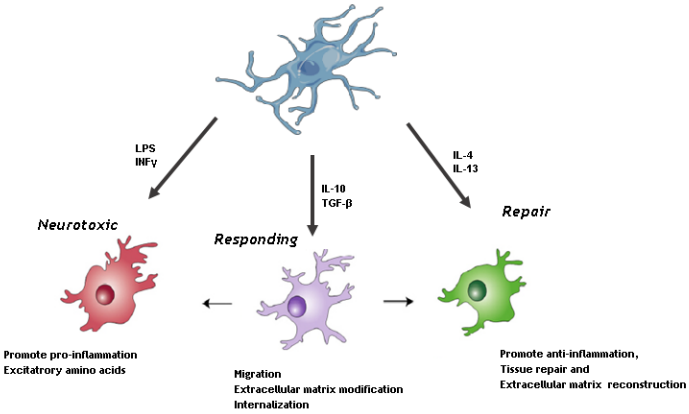


Figure 16. M1/M2 functions. M1 Microglia promote a pro-inflammatory state, while an anti-inflammatory and repair phase mediated by M2 microglia leads to wound healing and brings back tissue homeostasis. Adapted from (Tang and Le 2016).

Table 3. Functions of microglia. From (Boche et al.2013).

Function	*Examples*
CNS development	• Phagocytic activity during neuronal/synaptic development likely represents 'pruning' of redundant neurons and connections • Development influenced by secretion of cytokines, neurotrophins and growth factors
Recognition of pathogens (innate immune function)	• Receptors (e.g. Toll-like receptors, TLRs) recognize evolutionarily conserved antigens on surface of pathogens known as pathogen-associated molecular patterns (PAMPs) such as the endotoxin lipopolysaccharide (LPS) • Similar mechanisms possibly also involved in response to extracellular protein accumulations (e.g. amyloid plaques)
Phagocytosis	Ingestion and destruction by digestive enzymes in lysosomes of: • Multiple types of damaged cells (e.g. infarct) • Neurons (e.g. neuronophagia, Wallerian degeneration, tract degeneration) • Micro-organisms (e.g. abscess) • Virally infected cells (e.g. herpes encephalitis) • Erythrocytes and haemoglobin breakdown products (e.g. haemosiderin) following haemorrhage
Antigen presentation	• Presentation of pathogens (e.g. in bacterial, fungal, viral infections) bound to MHC for activation of T lymphocytes • Possibly relevant also in autoimmune disease
Recognition of bound antibody (adaptive immune function)	• Respond to antibodies bound to pathogens (opsonization) • Possibly also relevant to autoimmune disease (e.g. demyelination, paraneoplastic syndromes
Cytotoxicity	• Reactive oxygen species/respiratory burst (H_2O_2, NO) • Cytokines (e.g. IL, TNF, interferons, TGF, CSF) • Secretion of glutamate, aspartate
Extracellular matrix remodelling	• Proteases (MMPs degrade extracellular matrix)
Modulation of inflammation/ immune responses	• Chemokines (attract other inflammatory cells) • CD200 receptor (CD200 secreted by neurons has anti-inflammatory role) • Interferon-γ (promotes further microglial activation)
Repair	• Removal of cell debris facilitates plasticity and synaptogenesis
Stem cells	• Regulation of stem cell proliferation (e.g. granule cell neurons of hippocampus)
Tumours	• Response to neoplastic cells, possible regulation of tumour cell proliferation
Lipid transport	• Secretion of lipoprotein particles which deliver lipids to neurons for maintenance of cell membranes and synapses, facilitating synaptic plasticity
Viral entry into CNS	• CCR5 and CD4 are receptors for entry of HIV into macrophages and hence into CNS
Support mycobacteria	• Permits intracytoplasmic survival of mycobacteria (e.g. tuberculosis)
Demyelination	• Myelin destruction/phagocytosis (e.g. multiple sclerosis)

CNS, central nervous system; MHC, major histocompatibility complex; IL, interleukin; TGF, transforming growth factor; CSF, colony-stimulating factor; MMP, matrix metalloproteinase; HIV, human immunodeficiency virus; CCR5, C–C chemokine receptor 5.

1.2.4 Neuroinflammation

Neuroinflammation is self-defense reaction of the living brain against a wide range of stimuli (CNS trauma, ischemia, infection, toxic insult and autoimmunity) that disrupt physiological homeostasis (Gao and Hong 2008). Neuroinflammation is a complex set of processes in brain that may begin with microglial activation and may yield both beneficial (anti-inflammatory) or a detrimental (pro-inflammatory) effects (Cai et al. 2014). For long time the brain was considered a privilaged local because of its limited inflammatory response and lack of lymphatic infiltration (Hurley and Tizabi 2013). When a trauma or brain injury occurs, an inflammatory response is activated, which may be acute, or may become chronic, in which the activation of microglia amplifies its destructive effects, further aggravating the disease. Acute neuroinflammation refers mainly to activation of microglia that result in a phagocytic phenotype and the release of inflammatory mediators such as cytokines and chemokine. Acute neuroinflammatory response executes reparative actions to the benefit of the organism, as it tends to engulf dead cells and debris to minimize injury. In contrast, chronic neuroinflammation is a long-standing and self-perpetuating neuroinflammatory response that persists long after the initial injury or insult. Chronic neuroinflammation is detrimental to nervous tissue and may contribute significantly to the progression of neurodegenerative diseases (Cai et al. 2014; Hurley and Tizabi 2013; Kraft and Harry 2011; Streit et al. 2004).

1.2.5 Lipopolysaccharide-induced Microglial Activation

Lipopolysaccharide (LPS) has been used to promote the direct activation of glia, in particular microglia. LPS is a macromolecular complex consisting of lipid A (consisting of a unique diglucosamine backbone to which six fatty acid chains are attached) and a polysaccharide (O-antigen with multiple repeating units of monosaccharides and a polysaccharide core with an unusual sugar (2- keto-3-deoxyoctonate)), linked by a covalent bond. It is found in the outer membrane of Gram-negative bacteria and is a potent inducer of inflammation, strongly activating microglial cells (Dutta et al. 2008). This endotoxin binds to specific receptors in order to induce the release of cytokines and other inflammatory mediators. Toll-like receptor-4 (TLR4) is up-regulated upon brain inflammation and, together with CD14, is considered the major LPS receptor mediating LPS-induced neuroinflammation (Leulier and Lemaitre 2008; Lu et al. 2008). LPS associates with soluble LPS binding protein (LBP) and CD14 which is anchored outside of the plasma membrane of the microglia. Signal transduction occurs through the interaction of the LPS-CD14 complex with the extracellular accessory protein MD-2 and the transmembrane TLR4 (Figure 17; Dutta et al. 2008; Liu and Bing 2011; Lu et al. 2008). This association trigger the activation of kinases of various intracellular signaling pathways and a positive regulation of gene transcription for proinflammatory factors and free radical-generating enzymes. Thus, when microglia is activated by LPS, the release of cytokines such as

IL-1β and TNFα and of prostaglandins is increased, iNOS expression is induced, with consequent increase of NO, and ROS production is also increased (Banati et al. 1998; Block and Hong 2005; Gibbons and Dragunow 2006; Kim and Joh 2006; Rubio-Perez and Morillas-Ruiz 2012).

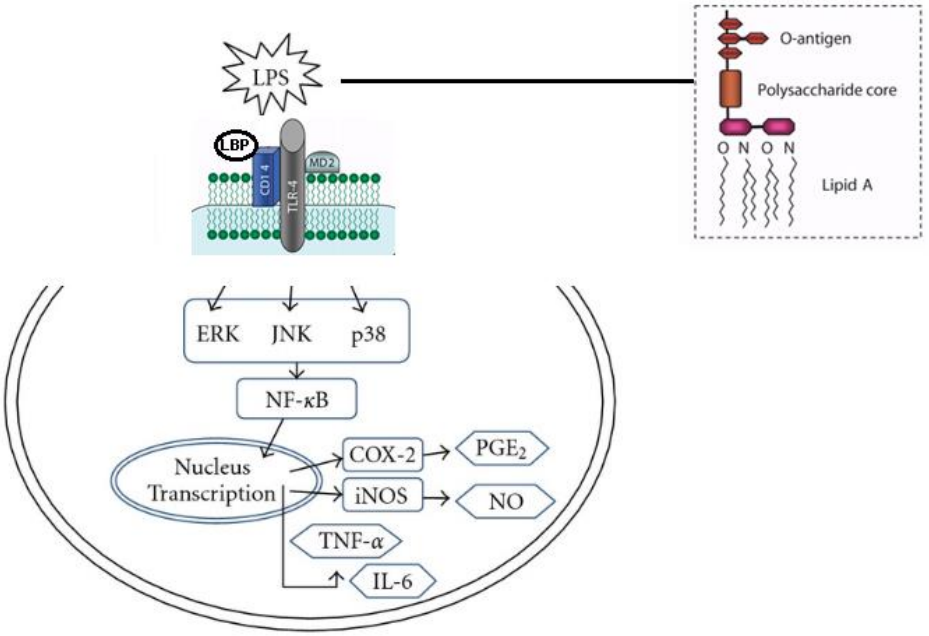


Figure 17. Lipopolysaccharide-induced microglia activation. LPS binding protein (LBP) works as a chaperon that enhances the binding of LPS to its intermediate receptor CD14. Association of the LPS-CD14 complex with Toll like receptor-4 (TLR-4), together with the accessory adaptor protein MD2, initiates downstream signaling events that involve mitogen activated protein kinases (MAPK) and transcription factors such as nuclear factor-kappa B (NF-κB). Upregulation of gene transcription leads to the production and release of cytokines such as TNFα and IL-1β. Induction of COX-2 and iNOS expression results in the biosynthesis and release of prostaglandins (PGs) and nitric oxide (NO). Activation of the multi-subunit phagocyte oxidase complex (PHOX), also called NADPH oxidase generates superoxide anion that combines with NO from iNOS form the more damaging peroxynitrite free radical (ONOO-). Adapted from (Dutta et al. 2008; Kiernan et al. 2016).

Chapter 2
Objectives

2 Objectives

The STEP protein is a central nervous system specific phosphatase. Accumulating evidence implicates STEP dysregulation in the molecular basis of several neuropsychiatric disorders. Almost all the data concerning STEP focuses on its expression and effects on neurons, with a documented role in the postsynaptic modulation of synaptic strength. To our knowledge there are no data on the expression of STEP by microglial cells or on the regulation of microglial reactivity by this phosphatase. However, it is known that PTPs are key regulatory factors in inflammatory signaling pathways and the microglial cells are involved in neuroinflammation.

This project aims to investigate the contribution of STEP to the regulation of microglial reactivity. Different specific objectives were defined for this work:

1. To characterize the expression of different STEP isoforms by primary cultures of microglia.

2. To determine if STEP expression is altered by neuroinflammation.

3. To determine the ability of STEP to regulate microglia reactivity.

Chapter 3

Materials and Methods

3 Materials and Methods

3.1 Primary cultures of microglia

All experiments with animals were performed in accordance with the national ethical requirements for animal research (Decreto-Lei n.º 113/2013) and with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Directive 2010/63/EU).

Postnatal Wistar rat pups with 2 to 5 days were sacrificed in order to obtain primary cultures of microglia from rat brain (Figure 18). Initially, wistar rat pups were placed on ice until they remain in the state of hypothermia. After decapitated, the brain was removed and transferred to phosphate buffer saline (PBS)[2]. The meninges were carefully removed and the resulting tissue was mechanically dissociated using sequentially pipette tips of smaller diameter. After centrifugation at 405 g for 3 minutes at 37°C (3K18C BioblockScientific; Sigma Laboratory Centrifugues), the supernatant was removed and the cells were resuspended in M10C-G[3]. Viable cells were assessed by the trypan blue exclusion method using a neubauer chamber. This method is based on the principle that viable cells have intact membranes, which prevent entry of the dye, while compromised membranes allow the incorporation of the dye into the cells. For cell counting a 1:1 dilution of the cell suspension with trypan blue was performed. Then, the cells were plated at a density of $0.352x10^6$ cells/3.60 cm^2 in multiwells (Orange scientific), coated with poly-D-lysine (P1024-100MG Sigma) which simulates the extracellular matrix, and maintained at 37ºC in a 5% CO_2 and 95% air atmosphere. After 7 days of culture, the microglia was obtained by removal of astrocytes with a trypsin solution[4] diluted in a ratio of 1:4 in MEM for 30 minutes. Microglia was then mantained in M10C-G at 37ºC in a 5% CO_2 and 95% air atmosphere, for 5 to 7 days, to alow the cells to acquire a resting state.

Figure 18. Rat brain of postnatal Wistar rat pups (postnatal day 3).

[2] PBS: 1.4M, KCl 27mM, KH_2PO_4 15mM e Na_2HPO_4 81mM, pH 7.2
[3] M10C-G consisting of Minimum Essential Medium Eagle (MEM; M0268-1L Sigma) supplemented with 2.2g/l sodium bicarbonate ($NaHCO_3$), 0.75% glucose 45%, 0.12% antibiotic (penicillin and streptomycin, Sigma), 0.02% insulin (I5500-50MG Sigma) and 10% fetal bovine serum (FBS; S0615 Biochrom AG).
[4] Trypsin solution: 0.5g/l Trypsin (Sigma, T7409-10G) and 0.2g/l EDTA.4Na dissolved in PBS

3.2 Microglia stimulation

Before starting cell treatments, the culture medium of microglial cells was renewed with M10C-G. The specific pharmacological inhibitor of STEP (TC-2153, 1µM) was added 1 hour before adding the pro-inflammatory agent, lipopolysaccharide (LPS 2µg/ml, L4391-1MG Sigma). The culture was then maintained at 37ºC in a 5% CO_2 and 95% air atmosphere for further 24 hours. A scheme of the experimental procedure is represented in figure 2. Supernatants were collected for measurement of NO, while the cells were used to evaluate the phagocytic activity and the expression of STEP, iNOS, IL-1ß and TNFα.

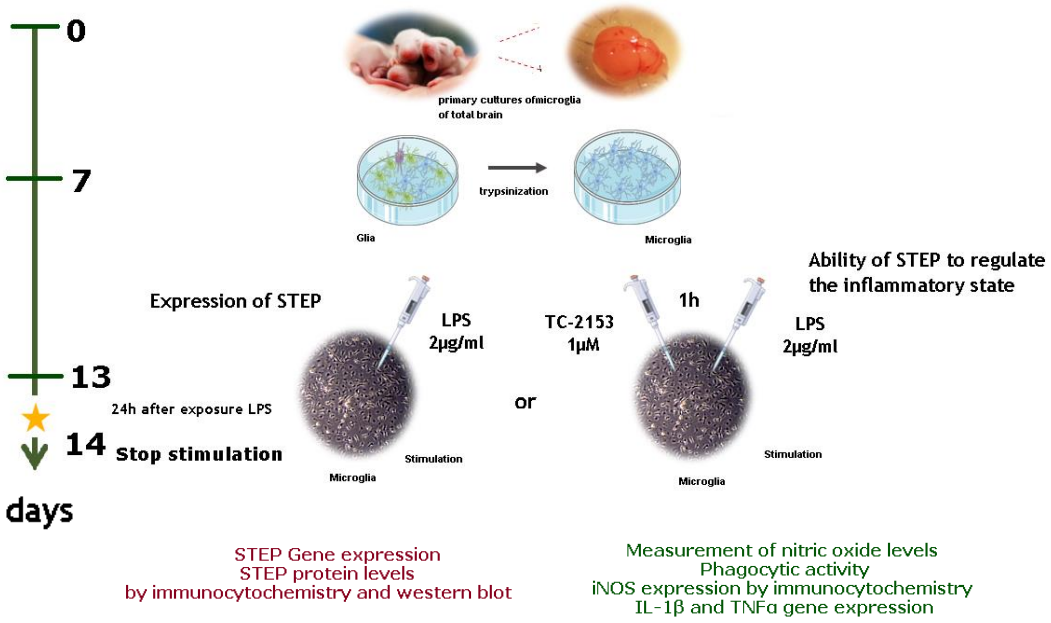


Figure 19. Schematic representation of the experimental procedures used to study the role of STEP in neuroinflammation.

3.3 Measurement of nitric oxide

Twenty-four hours after stimulation with LPS, the supernatants were collected and the amount of NO was determined by quantification of the accumulated nitrite (NO_2^-). Nitrite is a primary NO product which upon reaction with sulfanilamide and N-1-naphthylethylenediamine (NED) results in a colored azo compound under acidic conditions. This is a diazotization reaction originally described by Griess in 1879 (Figure 20). To perform this assay, 50µl of each sample were placed in a 96-well plate and then the same amount of the sulfanilamide solution (1% sulfanilamide and 5% phosphoric acid diluted in water) was added. The mixture was incubated for 8 minutes at room temperature before adding 50µl of a solution containing NED (0.1% N-1-naphthylethylenediamine diluted in water). After an 8 minutes incubation the absorbance was spectrophotometrically quantified at 550 nm (xMark™BIO-RAD) (Figure 21). The concentration of nitrite was then determined through the standard curve of sodium nitrite ($NaNO_2$).

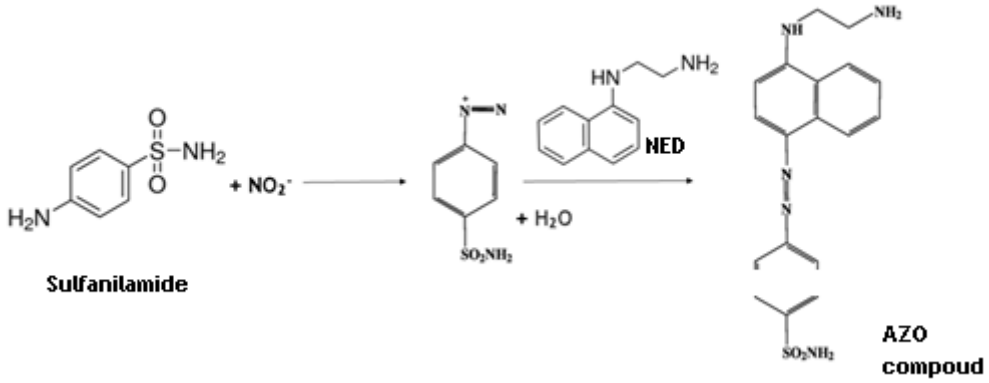


Figure 20. Chemical reactions involved in the measurement of NO_2^- with the Griess Reagent System.

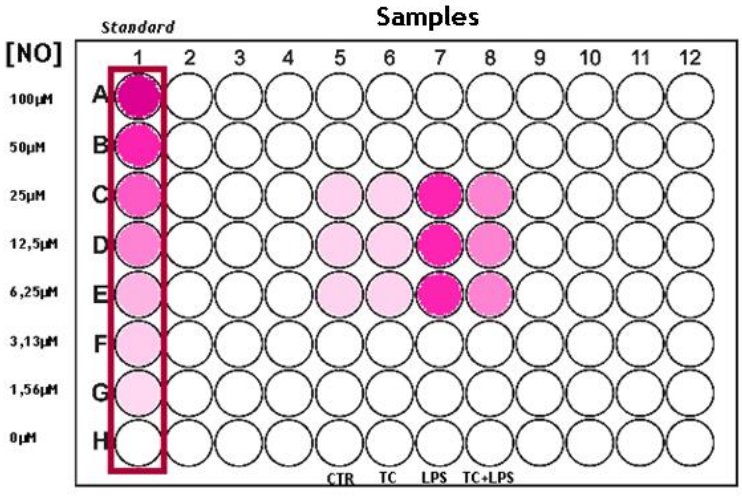


Figure 21. Multiwell format for the Nitrite Standard reference curve which is spectrophotometrically quantified at 550 nm (xMark™BIO-RAD).

3.4 Evaluation of phagocytic activity

Cell cultures were incubated at 37ºC for 20 minutes with 500µl /1.9cm^2 of a solution containing fluorescent microspheres (L1030-1L Sigma) diluted in M10C-G (0.5 µl fluorescent microspheres in 25ml M10C-G). After two washes with MEM, to remove the non-engulfed microspheres, the cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes. Subsequently, the cells were permeabilized by incubation with 1% Triton X-100 in PBS for 5 minutes. To prevent non-specific binding of the antibodies, PBS containing 0.1% Tween-20 (PBS-T) and 20% FBS was added and allowed to incubate for 1 hour at room temperature. After the blocking period the cells were washed with PBS-T and incubated for 1 hour at room temperature with the primary antibody, anti-Iba1 (1:2000; 019-19741WAKO) diluted in PBS-T 1% FBS. At the end of this incubation the coverslips were washed 6x during 15 minutes with PBS-T, and then incubated with the secondary antibody, anti-rabbit conjugated to Alexa 546 (1:1000; Invitrogen) diluted in PBS-T with 1% FBS, during 1 hour at room temperature. Lastly the cells were incubated with 2µM Hoechst 33342 (H1399 Invitrogen), a nuclear marker, prepared in PBS-T, for 10 minutes. After three washes with PBS-T, the coverslips were mounted on microscope slides with DAKO mounting medium (Glostrup, Denmark). The quantification of the phagocytic cells was performed by analyzing 25 fields on each coverslip, acquired with a fluorescence microscope Axiobserver Z1 (Zeiss), with a 63x objective. In each independent experiment 2 coverslips/experimental condition were analyzed.

3.5 Immunocytochemistry

STEP and iNOS expression were determined by immunocytochemistry.

After removal of the culture medium, cells were washed with MEM, fixed with 4% PFA for 10 minutes and then permeabilized by incubation with 1% Triton X-100 in PBS, for 5 minutes. To prevent non-specific binding of the antibodies, PBS-T with 20% FBS and 0.3% albumin was added and allowed to incubate for 1 hour (iNOS) or 2 hours (STEP) at room temperature. After the blocking period the cells were washed with PBS-T and incubated for one or three nights, depending on the antibody, at 4ºC, with the primary antibody diluted in PBS-T 1% FBS. Antibodies were used according to Table 1. After incubation with the primary antibody the coverslips were washed 6x during 15 minutes with PBS-T, and then were incubated with the corresponding secondary antibodies conjugated to Alexa 488 or Alexa 546 fluorophores diluted in PBS-T with 1% FBS during 1 hour at room temperature or overnight at 4ºC. After incubation with the secondary antibodies, cells were washed again 6x with PBS-T for 15 minutes and then incubated for 10 minutes with 2µM Hoechst 33342 (H1399 Invitrogen) prepared in PBS-T. After 3 further washes, the coverlips were assembled on microscope slides using DAKO mounting medium (Glostrup, Denmark). Fluorescent images were acquired on a fluorescence microscope (Axiobserver Z1, Zeiss) with a 63x magnification. In each experiment 20 fields per coverlip and 3 coverlips per experimental condition were analyzed.

Table 4. Primary and secondary antibodies used in the immunocytochemistry assay.

Protein	Primary Antibody source	Dilution	Incubation time	Reference/ Company	Secondary Antibody	Dilution	Incubation time	Company
STEP	mouse	1:1000	60h	23E5 Cell SIgnaling Technology	goat anti-mouse IgG conjugated to Alexa 488	1:1000	14h	Invitrogen
Iba1	rabbit	1:2000	60h	019-19741 WAKO	goat anti-rabbit IgG conjugated to Alexa 546	1:1000	14h	Invitrogen
iNOS	mouse	1:500	14h	610431 BD Bioscience	goat anti-mouse IgG conjugated to Alexa 488	1:1000	1h	Invitrogen

3.6 Western Blot

STEP protein levels were determined by western blotting of cell extracts.

Protein extracts obtained after cell disruption with 30µl of lysis buffer[5] were collected and the total protein present in the samples was quantified by the Bradford method using Bovine Serum Albumine (BSA) as protein standard. This method is based on the change in absorbance of the Coomassie bright blue dye which under acidic conditions binds to the positive residues of the proteins acquiring its anionic form. Absorbance was determined at 595nm.

The sample volume corresponding to 30µg of denatured protein was added to a denaturing solution[6] and heated at 100°C for 5 minutes. The reducing agent ß-mercaptoethanol was added to prevent oxidation of the cysteines and to break the disulfide bridges. Bromophenol blue allows visualization of the sample in the well and allows following the migration front during the sample run through the gel; glycerol, as it is denser than water, allows the samples to deposit at the bottom of the well, preventing them from rising and mixing with the electrophoresis buffer. SDS is ionic detergent that binds to proteins in the samples giving them a global negative charge. This inherent loading on all proteins after binding to SDS allows them to have the same charge/mass ratio. In these conditions the mobility of proteins depends exclusively on their molecular weight.

After denaturation of samples they were added to the stacking gel[7], a gel with a high pore size. Polymerization of acrylamide was initiated by a peroxide chemical method, in which PSA was the initiating peroxide as a source of free radicals and the TEMED quaternary amine was the polymerization catalyst for the stabilization of these free radicals. Proteins in the sample were separated according to their molecular weight on the resolving gel[8] under a voltage of 130V, for approximately 1 hour at room temperature (BIO-RAD system). Before assembling the sandwich for the electrotransfer, the polyvinylidene difluoride (PVDF, GE Healthcare, Amersham, UK) membranes were activated by dipping the membranes for 5 seconds in methanol 100%, followed by immersion for 5 minutes in water and then 10 minutes in electroblotting buffer[9] PVDF membranes have a strong affinity for proteins. After the electrophoresis was finished, the stacking gel was removed. In order to make the proteins accessible to antibody detection they were transfered from the resolving gel onto a PVDF membranes by an electrotransference carried out at 25V for 45 minutes.

In order to avoid non-specific antibody bindings, the membranes were blocked with 5% milk powder diluted in TBS-T[10] for 1 hour at room temperature. After 1 hour in blocking solution,

[5] Lysis buffer: 1% Triton X-100, TrisHCl 25mM, EGTA 2.5mM, EDTA 2.5mM, protease inhibitor cocktail (04693132001 ROCHE, Sigma) and phosphatase inhibitor (Na_3VO_4,2mM)

[6] Denaturing solution : 62.5 mM Tris-HCl pH6.8, 2% sodium dodecy lsulfate (SDS), 10% glycerol, 140 mM ß-mercaptoethanol and 0.1% bromophenol blue

[7] Stacking gel: containing 4% acrylamide, 0.5M Tris-HCl pH6.8, 10% SDS, 0.05% ammonium persulfate (PSA) and 0.1% tetramethylethylenediamine (TEMED)

[8] Resolving gel: consisting of 12% acrylamide in 1.5M Tris-HCl pH8.8, 10% SDS, 0.05% PSA and 0.05% TEMED

[9] Electroblotting buffer: 10mM Ciclohexilamin Acid propanosulphonic (CAPS) and 10% metanol pH11

[10] TBS-T: 0.1% Tween-20 in 20mM Tris, 137mM NaCl and 0.38% 1M HCl

the membranes were incubated with the primary antibodies diluted in TBS-T (according to Table 2) overnight at 4ºC. The next day, after washed three times in TBS-T, for 10 minutes each, the membranes were incubated overnight at 4ºC with the horseradish peroxidase-conjugated (HRP) secondary antibody (according to Table 2) diluted in TBS-T.

The proteins were detected by chemiluminescence by exposure of Luminata Crescendo Western HRP Substrate (Milipore) during 5 minutes at room temperature. Detection of the bands was performed on a ChemiDoc™ MP Imaging System (Bio-Rad). The fluorescence intensity of bands is directly proportional to the amount of protein present in the sample. The image is viewed using Image Lab™ software (Bio-Rad). In order to normalize the results obtained, the membranes were incubated for 1 hour with antibody directed against a housekeeping protein, mouse anti-GAPDH antibody (1:5000, Millipore) diluted in TBS-T. Subsequently, the membrane was incubated with the corresponding secondary antibody (anti-mouse HRP - Santa Cruz Biotechnology, 1:20000), during 1 hour. Unbound antibody was removed by 3 washes, for 10 each, minutes with TBS-T. Subsequently, the membranes were again revealed by chemiluminescence by exposure of HRP Substrate during 1 minute at room temperature.

Table 5. Primary and secondary antibodies used in Western Blot.

Protein	Source	Dilution	Incubation	Reference /Company	Secondary Antibody	Dilution	Incubation	Company
STEP	Mouse	1:1000	14h	23E5/ Cell Signalling Technology	Anti-mouse IgG conjugated to HRP	1:20000	14h	Santa Cruz Biotech
GAPDH	Mouse	1:5000	1h	MAB374/ Milipore	Anti-mouse IgG conjugated to HRP	1:20000	1h	Santa Cruz Biotech

3.7 mRNA expression analysis

Total RNA was extracted from microglial cells using Trizol (TRI, 2302700 5prime) consisting of a monophasic solution of phenol and guanidinium isothiocyanate which during extraction breaks the cells and maintains RNA integrity. Trizol (600 µl) was added and incubated for 5 minutes at room temperature. Then, 120 µl of chloroform was added per 600 µl of Trizol used for RNA extraction, then samples were homogenized by vigorous inversion and incubated for 10 minutes at room temperature. Subsequently, samples were centrifuged at 12,000 g for 15 minutes at 4°C (Mikro 200R, HettichZentrifugen) in order to separate the mixture into three phases: a red lower organic phase containing the proteins, the whitish interface containing DNA and a colorless upper aqueous phase where the RNA of interest is present. In order to recover total RNA by precipitation the aqueous phase was transferred to a new centrifuge tube in which were 300µl of isopropanol was added per 600µl of Trizol used. After a 10 minutes incubation, at room temperature, the sample was centrifuged at 12,000g for 10 minutes at 4°C (Mikro 200R, HettichZentrifugen), the supernatant was removed, and the RNA pellet was washed through the addition of ethanol 75% in water treated with 0.01% diethylpyrocarbonate (DEPC) at -20°C to remove any impurities (300 µl per 600 µl of Trizol used). After a final centrifugation at 7500 g for 5 minutes at 4°C (Mikro 200R, HettichZentrifugen), the supernatant was rejected again and ethanol in excess was removed. The sediment containing the RNA was resuspended and rehydrated in 20 µl of DEPC water.

The concentration of total RNA (µg/µl) were quantified using the UV/Vis NanoPhotometer™ spectrophotometer (Implen). To determine integrity and purity of RNA, the ratio should be between 1.8 and 2.1, with a value lower than 1.8 indicating contamination with proteins or with phenol and greater than 2.1 indicating contamination with DNA.

RNA integrity was analyzed in a 1% agarose gel with 0.05% Green Safe, a nucleic acid intercalator. Samples were prepared with 2µl of sample, 8µl of sterile water and 1µl of loading buffer 10x. The gel was visualized on an UVITEC transilluminator (UVitec Cambridge, United Kingdom). The integrity of the RNA was confirmed by the existence of two distinct RNA bands, 18S and 28S, in which the last band should present twice the intensity of the first. All RNA samples were stored at -80ºC.

3.8 cDNA synthesis

Complementary deoxyribonucleic acid (cDNA) was synthesized from 1 µg total RNA by the action of the enzyme reverse transcriptase. The mixtures were prepared with reagent volumes for n+1 reactions. Thus, for each 20 µl final volume cDNA synthesis reaction, the appropriate volume was added to 1 µg of RNA, 1 µl of Random Primers (50 ng/µl, MB12901 Nzytech), 1 µL of deoxynucleotidostriphosphate-dNTPs (10 mM, R0181 Thermo Scientific) to make up a volume of 17µl with sterile water free of nucleases. This mixture was incubated in the thermal cycler (T100™ ThermalcCycler, Biorad) for 5 minutes at 65°C. Subsequently, another mixture was added with the following reagents: 2 µl 10x Buffer (Nzytech) and 1 µl Moloney Murine Leukemia Virus reverse transcriptase (20000U M-MLV RT Nzytech). Tgis was followed by an incubation at 37°C for 50 minutes and finally at 70°C for 15minutes to inactivate the enzyme.

All cDNA samples were stored at -20 ° C.

3.9 Conventional PCR

Through conventional polymerase chain reaction (PCR) it was possible to confirm the expression of the STEP gene in primary cultures of microglia and to optimize the annealing temperature of primers used in RT-PCR. In this procedure Taq DNAPolymerase was used to amplify DNA fragments. To each sample tested a MIX[11] was prepared. The primers were chosen through PrimerBLAST-NCBI-NIH program and were commissioned by STAB VIDA (according to Table 3). For each reaction, with the exception of negative control, 1 µl of cDNA was added. To proceed to gene amplification the MultiGene™ OptiMax Thermal Cycler (Labnet International) was used. Samples were heated at 95ºC for 3 minutes, followed by 40 cycles of 95ºC for 30 seconds, then 45 seconds at optimal annealing temperature for each primer, 1 minute at 72ºC and, at finally, 5 minutes at 72ºC. PCR products were run out on a 2% agarose gel in the presence of 0.05% GreenSafe. For this, 9µl of each PCR product and 1µl of loading buffer 10x were deposited in the agarose gel. In the first well was added 5µl of NZYDNA Ladder VI (MB08901, Nzytech). The gel was visualized on an UVITEC transilluminator (UVitec Cambridge, United Kingdom). By comparison with NZYDNA Ladder VI the size of fragments obtained were confirmed.

[11] MIX: 2.5µl 10x Buffer which includes 20 mM $MgCl_2$ and 0.625µl Taq DNAPolymerase (EP0702, Nzytech), 0.5µl dNTPS (10mM, R0181 Thermo Scientific), 0.75µl Forward primer (10mM) and 0.75 µL Reverse primer (10mM), and completed with sterile water to a volume of 24µl

Table 6. Primers used in PCR.

Gene	Primers (5'- 3')	Fragment size (bp)	Annealing Temperature
$STEP_{61}$	FW 5'TCACCAGACCCTGAAGATCC3' RV 5'ATCTCTACGCCATCGTGGAC3'	245	62°C
IL-1β	Fw 5′AGGCTGACAGACCCCAAAAG3′ RV 5′CTCCACGGGCAAGACATAGG3′	178	60°C
TNFα	FW 5′GATCGGTCCCAACAAGGAGG3′ RV 5′TTTGCTACGACGTGGGCTAC3′	129	62°C
Cyclophilin A CyPA	FW 5'CAAGACTGAGTGGCTGGATGG3' RV 5'GCCCGCAAGTCAAAGAAATTAGAG3'	163	60ºC (IL-1β) 62ºC ($STEP_{61}$ and TNFα)

3.10 Real time RT-PCR

Real-time RT-PCR allowed the evaluation of the relative expression of the STEP gene. The relative expression of IL-1ßα and TNFα was also evaluated.

First, the procedure was optimized by assessing primer efficiency for all genes with 4 different cDNA dilutions (1: 1, 1: 5, 1: 25 and 1: 125) in which the concentration of primers to be used was adjusted. The more cDNA in the sample, the earlier it amplifies. The primers are efficient if the slope is comprised between of -3.93 to -2.92. For each assay, cDNA (1 µl) was mixed in 20 µl of a solution containing 10µl Luminaris HiGreen Fluorescein qPCR Master Mix (Thermo Fisher Scientific), 0.2 µM (L-1ß and TNFα) or 0.3 µM (STEP) of each primer and sterile water. Real time PCR reactions were settled according to table 8 in a thermocycler CFX ConnectTM (Bio-Rad). To normalize the levels of gene expression the cyclophilin A (CyPA) gene was used as control (housekeeping gene).

mRNA expression was determined using the cycle time (CT) values normalized to those of cyclophilin A. The results are expressed as $2^{-\Delta\Delta CT}$ relative to control in experiments with cell cultures based on the Pfaffl method.

Table 7. Primers used in Real-time RT-PCR.

Gene	Primers (5'- 3')
$STEP_{61}$	FW 5'TCACCAGACCCTGAAGATCC3' RV 5'ATCTCTACGCCATCGTGGAC3'
IL-1ß	Fw 5'AGGCTGACAGACCCCAAAAG3' RV 5'CTCCACGGGCAAGACATAGG3'
TNFα	FW 5'GATCGGTCCCAACAAGGAGG3' RV 5'TTTGCTACGACGTGGGCTAC3'
CyPA	FW 5'CAAGACTGAGTGGCTGGATGG3' RV 5'GCCCGCAAGTCAAAGAAATTAGAG3'

Table 8. Real-time RT-PCR protocol.

Gene	Initial denaturation	Denaturation	Annealing	Extension	Cycles
$STEP_{61}$/ CyPA	95ºC 3 min	95ºc 10 s	62°C 45 s	72ºC 10 s	40x
IL-1ß/ CyPA	95ºC 3 min	95ºc 10 s	60°C 45 s	72ºC 10 s	40x
TNFα/ CyPA	95ºC 3 min	95ºc 10 s	62°C 45 s	72ºC 10 s	40x

3.11 Statistical Analysis

Data are expressed as percentages of values obtained in control conditions, as percentage induced by LPS or as percentage of the total number of cells (according to the text and legend of the figures), and are presented as mean ± S.E.M. of the number of independent experiments indicated in the legends of the figures. Comparisons of means between two groups was performed using Student's t test, and between three or more groups by one-way ANOVA followed by Bonferroni's Multiple Comparison Test. Results were considered statistical significant when $p < 0.05$. All statistical analysis were performed using GraphPad Prism 5 (GraphPad Sotware Inc., San Diego, CA).

Chapter 4

Results

4 Results

4.1 Characterization of microglial culture

Primary cultures of microglia from total brain were obtained from a glial cell culture from which astrocytes were removed by trypsinization. Microglial culture was characterized by immunocytochemistry. For evaluation of culture purity, microglial cells were labeled for Iba-1, a calcium-binding protein specifically expressed in macrophage/microglia, and with Hoechst to stain the nuclei of all cells (Fig 22A). About 92% of the cells present in the culture expressed the microglial marker Iba1 (Figure 22B.).

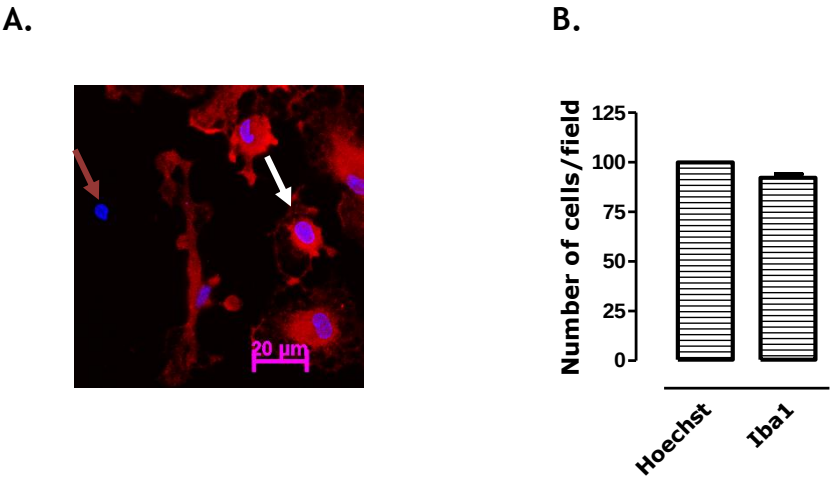


Figure 22. **Characterization of purity of the microglia primary culture of the total brain.** (A) Representative images show the immunocytochemistry performed against Iba1 (red) with nuclei were stained with Hoechst 33342(blue). The white arrow indicates Iba+ cells and the red arrow indicates Iba- cells (B) Quantification of the percentage of cells labeled for Iba1. Each bar represents the mean ± SEM of three independent experiments, performed in triplicate.

4.1.1 Promoting microglia reactivity trough LPS exposure

Previous results from our group showed treatment with LPS 0.1 µg/ml for 24h induced the activation of ventral midbrain microglial cells. In order to ascertain whether the concentration used with midbrain cultures was also effective in promoting activation of the microglia from total brain microglial cells were stimulated with the pro-inflammatory agent LPS 0.1 µg/ml and 2µg/ml for 12h, 18h and 24h. Our results indicate that exposure to LPS 2µg/ml for 24h induces a strong increase in the release of nitric oxide, a well established marker of microglia reactivity(Bolós et al. 2017). In subsequent studies those were the stimulation conditions used to induce microglia reativity (Figure 23).

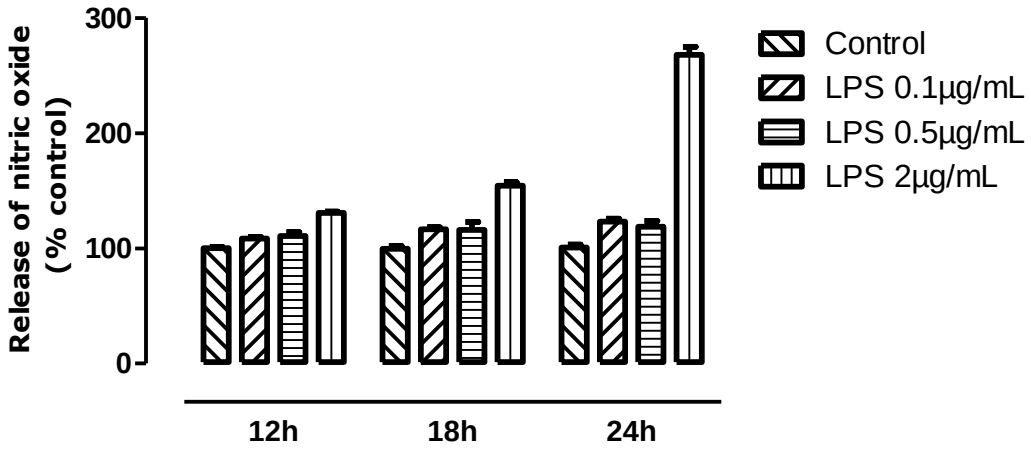


Figure 23. **Adjustment of experimental conditions required to induce microglial reactivity.** Primary cultures of microglia were exposed to different concentrations of LPS (0.1 µg/ml, 0.5 µg/ml and 2 µg/ml) and during different periods (12h,18h and 24h). Data represent the mean ± SEM of one experiment.

4.2 Does microglia express STEP?

Taking into account that PTPs are key regulators of inflammatory signaling pathways and microglial cells are main players in the neuroinflammatory process with this project we aimed to unveil the role of STEP in the control of microglia reactivity. To our knowledge there are no published data on the expression of STEP by microglial cells or on the regulation of microglial reactivity by this phosphatase.

To disclose if STEP is present in primary cultures of microglia we analyzed the STEP mRNA by conventional PCR. PCR results confirm $STEP_{61}$ mRNA expression in microglia, through specific amplification of the nucleotides sequences of STEP gene. The amplified products were run out in a 2% agarose gel stained with GreenSafe, and by comparison with NZYDNA Ladder VI it was confirmed that the size of fragments obtained (245bp) corresponded to the STEP gene. Different temperatures were tested to establish the optimal annealing temperature of STEP primers (62ºC, Figure 24). The presence of the protein and its cellular location was further confirmed by the immunocytochemical analysis and Western blot. The levels of STEP were analyzed through immunocytochemistry using an anti-STEP (Fig 25A., green) and anti-Iba1 (Fig 25A., red) antibody. Western blot analysis showed that both isoforms of STEP, $STEP_{61}$ and $STEP_{46}$, are expressed by microglia, with the band correspondent to the $STEP_{61}$ isoform being much stronger (Figure 25B.).

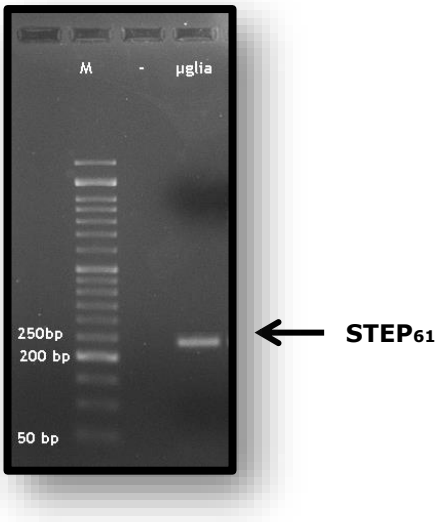
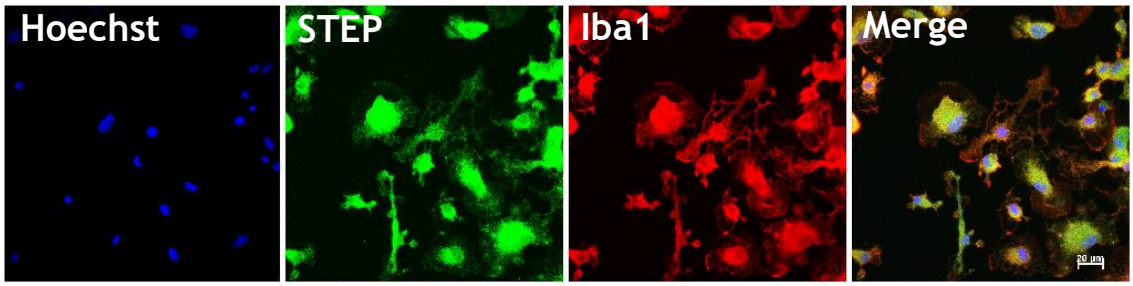


Figure 24. <u>Expression of STEP mRNA in primary cultures of microglia</u>. Representative product of PCR with specific band of 245bp for STEP mRNA. From left to right is represented the molecular weight ladder, the negative control and the fragment obtained at 62ºC.

A.



B.

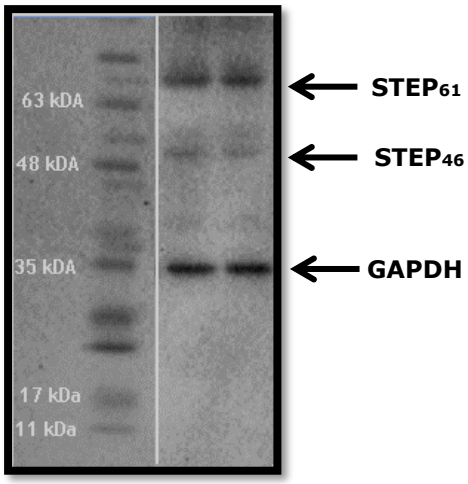


Figure 25. Expression of STEP in primary cultures of microglia. (A) Representative images of immunocytochemical analysis performed against the STEP protein (green) and Iba1 protein (red) followed by nuclei staining with Hoechst 33342 (blue). (B)Representative Western blot probed for STEP (MW: 61kDa and MW:43kDa) protein and GAPDH protein (37 kDa).

4.2.1 Influence of microglia reactivity in STEP expression

After verifying the expression of STEP in primary cultures of microglia, we evaluate STEP expression in microglia exposed to an inflammatory stimulus. Microglial cells were treated with LPS for 24h. $STEP_{61}$ mRNA expression in response to LPS was evaluated by Real-time RT-PCR. LPS treatment induced a significant increase in gene expression to 197% of control levels (Figure 26). After verifying the increase of PTPN5 transcription, STEP expression by microglia was evaluated by immunocytochemistry against STEP and Iba1and Western blot. Quantification of fluorescence intensity corresponding to STEP under control conditions and after by exposure to the LPS revealed a small, not statistically significant, increase of STEP expression after exposure with LPS (Figure 27). Throught Western blot it was verified that both STEP isoforms were increase by LPS stimulation (Figure 28).

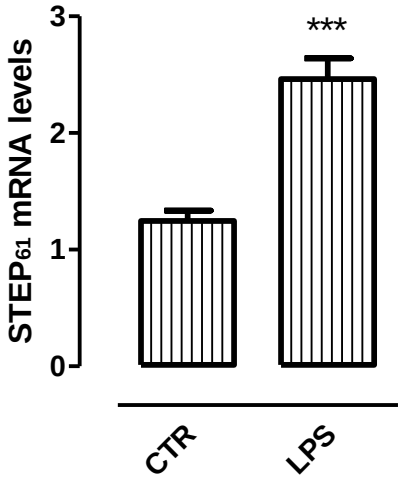
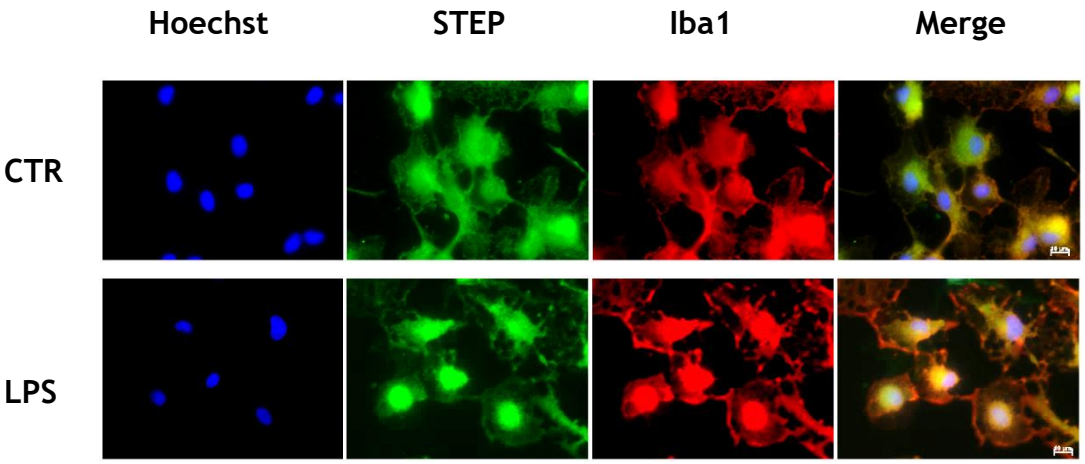


Figure 26. **STEP expression was increased in microglia stimulated with LPS.** STEP61 mRNA levels were measured by real-time PCR in primary microglial cells after incubation with LPS 2µg/ml for 24 hours. Data represent the mean ± SEM of four independent experiments performed in triplicate and expressed as fold change of STEP mRNA levels relative to control (CTR). Statistical analysis was performed using t-Student Test (***p<0.001 compared to CTR).

A.



B.

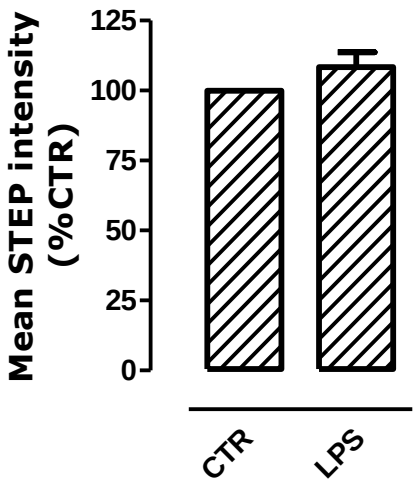


Figure 27. **STEP expression by LPS stimulated microglia.** (A.) Representative images of immunocytochemical analysis that performed against STEP protein (green) and Iba1 protein(red) under control conditions and after exposure to LPS. The image was acquired on a fluorescence microscope with a magnification of 63x. (B.) Comparison of STEP protein levels through measurement of mean STEP fluorescence intensity under control conditions and after exposure to LPS. Data represent the mean ± SEM of four independent experiments performed in duplicate and expressed in percentage of control.

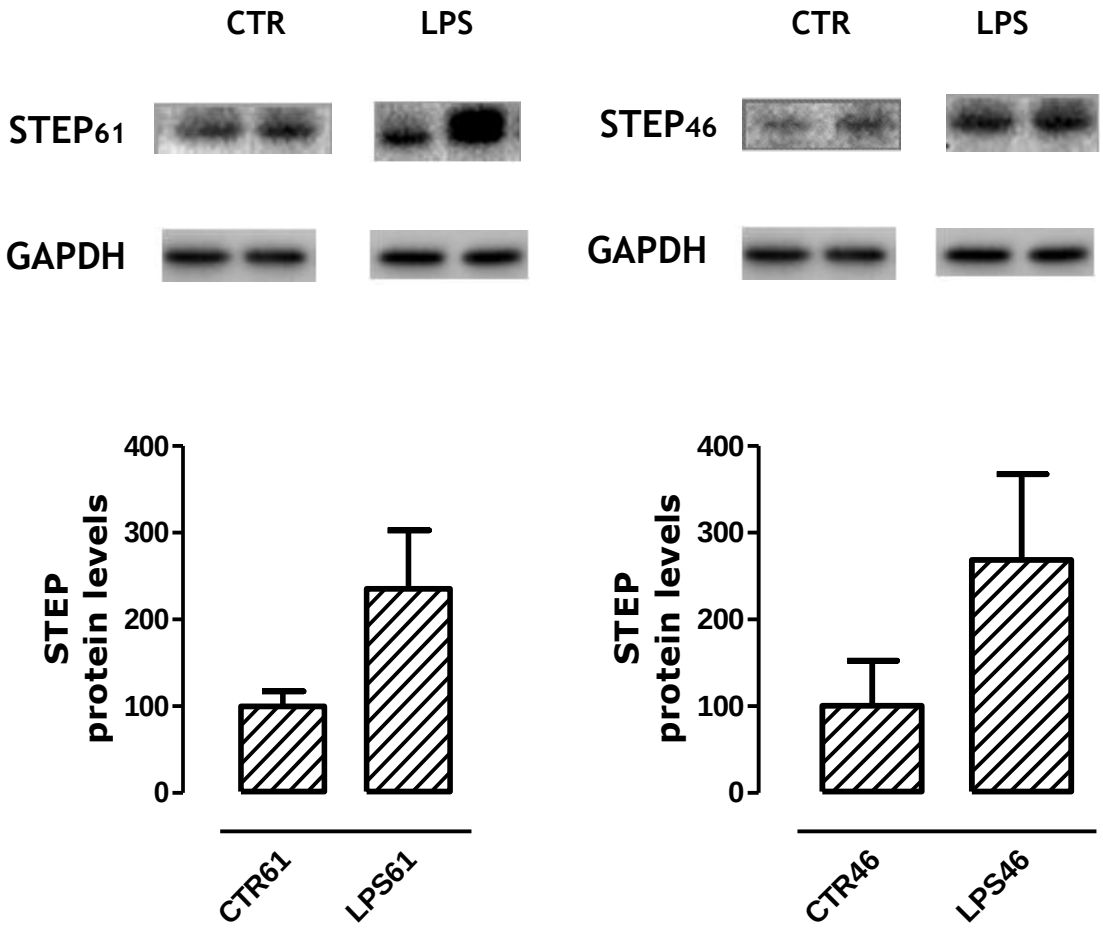


Figure 28. **Both STEP isoforms levels were increased in microglia stimulated with LPS.** Western blotting analysis in primary microglial cells after incubation with LPS 2µg/ml for 24 hours in the presence of TC-2153 (1µM). Representative image of a Western blot probed for STEP (MW: 61kDa or MW: 46kDa) and GAPDH (MW: 37kDa) proteins. Results from the optical densitometry analysis revealed an increase in STEP levels after exposure to LPS, as compared to the control. Data represent the mean ± SEM of one experiment performed in triplicate and results were expressed as STEP/GAPDH ratio.

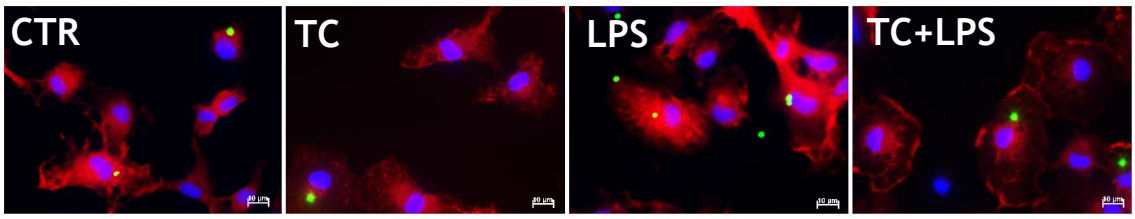
4.3 Evaluate the ability of STEP to regulate microglia reactivity

The thirth part of these chapter focuses on clarifying the involvement of STEP in the control of microglia reactivity. To explore this, we used as a tool a specific pharmacological inhibitor of STEP, TC-2153, and analysed its influence on microglial reactivity. The parameters of microglia reactivity analysed were the release of NO, phagocytic activity and expression of iNOS, IL-1β and TNFα.

4.3.1 Phagocytic activity

To determine the role of STEP in the control of microglia reactivity evoked by LPS cells were treated with TC-2153 1μM 1 h before starting the stimulation with LPS 2μg/ml. Cells were incubated with both compounds for further 24h and at the end of this period were exposed to fluorescent microspheres. Cells that incorporated these microspheres were considered phagocytic. To confirm that the cells incorporating microspheres were microglial cells, in parallel cells were immunolabeled for the microglial marker Iba1 (Figure 29A.). Cells incorporating microspheres were quantified for each condition. Our results demonstrate that in the presence of LPS the percentage of phagocytic cells increased by 85%, as compared to control. TC-2153, *per se*, did not affect the number of phagocytic cells, whereas LPS-induced phagocytosis was reduced by TC-2153 pre-treatment by 65%, as compared with LPS (Figure 29B.).

A.



B.

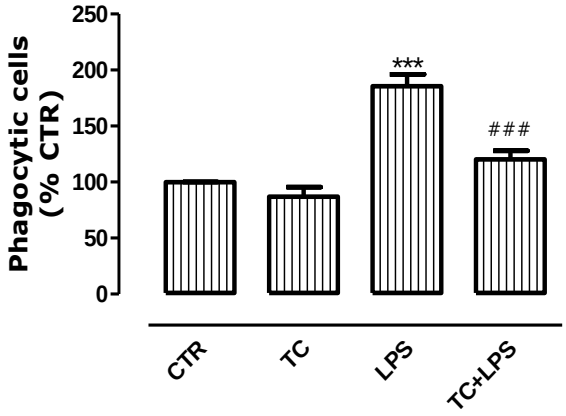
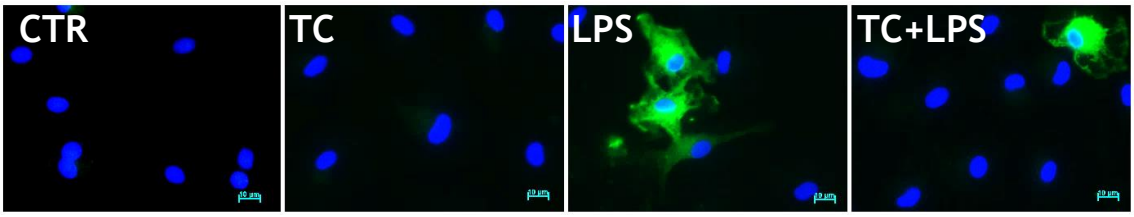


Figure 29. STEP inhibitor reduces LPS-induced phagocytic activity. Microglial cells were exposed to LPS (2 µg/ml) for twenty-four hours in the presence of TC-2153(1µM). Cells were incubated with fluorescent microspheres to evaluate phagocytic activity. (A) Representative photomicrographs (63x magnification) of microglia culture exposed to fluorescent microspheres (green) and iba-1 (red). Nuclei were stained with Hoescht (blue). (B) Quantification of microglial cells that incorporated microspheres (phagocytic cells). Data represent the mean ± SEM of four independent experiments performed in duplicate and expressed in percentage of CTR. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test (*** p <0.001 compared to CTR and ###p <0.001 compared to LPS).

4.3.2 iNOS expression

Inducible nitric oxide synthase (iNOS) is one of three enzymes generating NO from the amino acid L-arginine. Assessement of iNOS expression by immunocytochemistry showed that control microglia does not express detectable levels of iNOS, whereas upon stimulation with LPS (2µg/ml) there was a strong increase in the number of cells immunopositive for iNOS (Figure 30A). Moreover, inhibition of STEP with TC-2153 (1µM) reduces the the number of microglia expressing inducible nitric oxide synthase (iNOS) after LPS stimulation by 60% (Figure 30B).

A.



B.

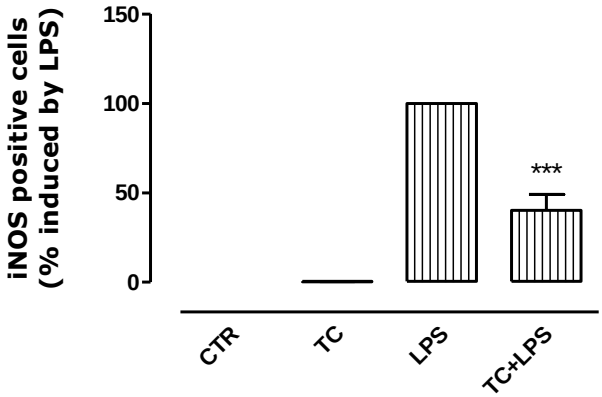


Figure 30. STEP inhibition reduces LPS-induced iNOS expression. Microglial cells were exposed to LPS (2µg/ml) for 24h in the presence of TC-2153(1µM). iNOS expression was evaluated by immunocytochemistry. (A) Representative photomicrographs (63x magnification) of the immunostaining for iNOS (green), in which nuclei were stained with Hoescht (blue). (B) Quantification of iNOS-immunopositive cells for each experimental condition. Data represent the mean ± SEM of three independent experiments performed in triplicate and expressed in percentage induced by LPS. Statistical analysis was performed using using one-way ANOVA followed by Bonferroni's Multiple Comparison Test (*** p <0.001 compared to LPS).

4.3.3 Release of nitric oxide

Nitric oxide (NO) is a small, highly diffusible, gaseous and reactive molecule with a short half life that is synthesized by nitric oxide synthase (NOS) via enzymatic conversion of L-arginine to L-citrulline. Under normal physiological conditions, NO is an important secondary messenger, playing a crucial role in intracellular communication and intracellular signaling in the nervous system. iNOS is produced mainly by microglia in response to microorganisms and tissue damage and is a main step towards successful arrest of the invading offender by the innate immune system. In addition, reactive microglia express iNOS resulting in the biosynthesis of relatively high quantities of NO. Anomalous overproduction of NO leads to neurotoxicity. Our results showed that the low levels of NO released in control conditions were significantly increased by exposure to LPS (2µg/ml) by about 92%, as compared to the control. Surprisingly, TC-2153 pre-treatment did not affect significantly NO release evoked by LPS (Figure 31).

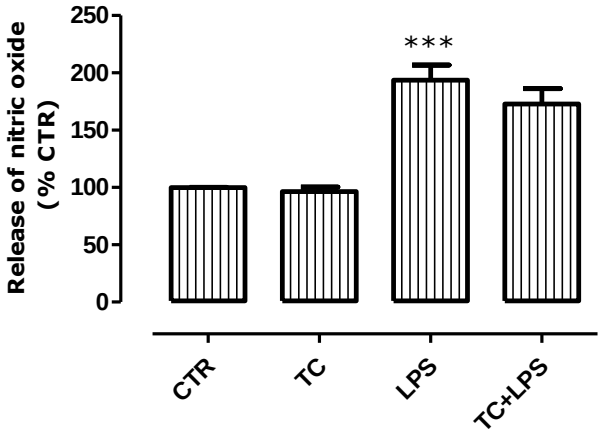


Figure 31. <u>NO release by LPS-stimulated microglia is attenuated by STEP inibhitor.</u> Microglial cells were exposed to LPS 2 µg/ml for 24h in the presence of TC-2153 (1µM). Culture supernatants were collected and NO levels released by microglial cells quantified by the Griess Method. Data represent the mean ± SEM of sixteen experiments performed in triplicate and expressed in percentage of CTR. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test (*** $p < 0.001$ compared to CTR).

4.3.4 IL-1ß and TNFα expression

Interleukin 1 beta (IL-1ß) and tumor necrosis factor (TNFα) are pro-inflammatory cytokines produced by reactive microglia in the M1 phenotype. To further analyse the possible contribution of STEP in the control of microglial reactivity the expression levels of IL-1ß and TNFα were assessed by Real-time RT-PCR. Our results demonstrate that there is a significant increase in gene expression after stimulation with LPS. IL-1ß increased by 361% whereas TNFα was increased by 57%, as compared to the control. Inhibition of STEP did not significantly affect the expression of IL-1ß or TNFα (Figure 32A.). Interestingly, basal expression of TNFα was decreased by TC-2153 treatment (Figure 32B.).

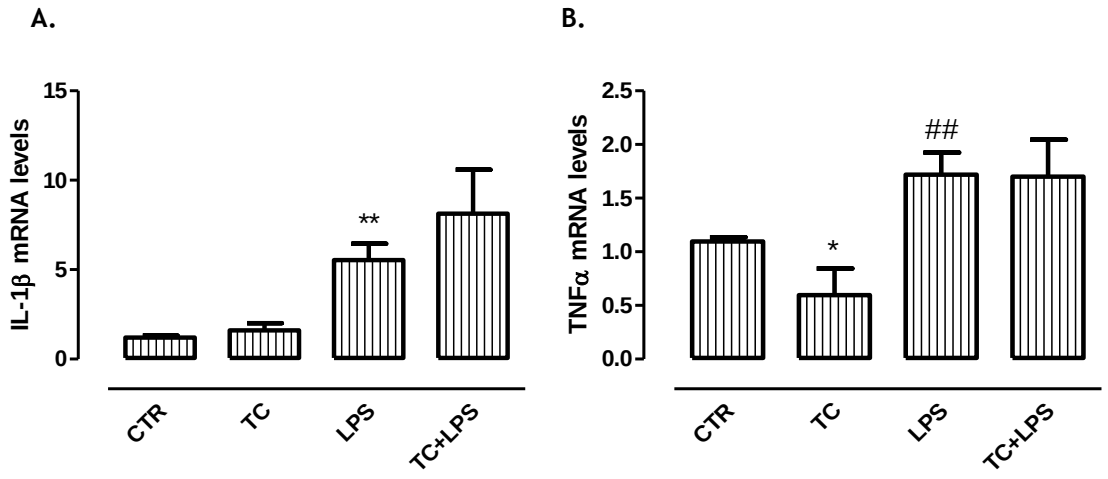


Figure 32. LPS-induced IL-1ß and TNFα mRNA levels in microglia is not affected by STEP inhibition. (A) IL1ß mRNA levels were measured by real-time PCR in primary microglial cells after incubation with LPS 2µg/ml for 24 hours in the presence of TC-2153 (1µM). Data represent the mean ± SEM of three independent experiments performed in triplicate and expressed as fold change of IL-1ß mRNA levels relative to CTR. Statistical analysis was performed using t-Student Test ($^{**}p<0.01$ compared to CTR). (B)TNFα mRNA levels were measured by real-time PCR in primary microglial cells after incubation with LPS 2µg/ml for 24 hours in the presence of TC-2153 (1µM). Data represent the mean ± SEM of three independent experiments performed in triplicate and expressed as fold change of TNFα mRNA levels relative to CTR. Statistical analysis was performed using t-Student Test ($^{\#\#}p<0.01$ and $^{*}p<0.05$, compared to CTR).

Chapter 5

Discussion

5 Discussion

Protein tyrosine phosphatases (PTPs) are key regulatory factors in inflammatory signaling pathways and microglial cells are resident immune cells in the central nervous system that participate in the initiation and propagation of an inflammatory response. Several evidences demonstrate the ability of PTPs to regulate the potentially harmful effects of microglia (Song, Kim, et al. 2016). They can prevent an excessive activation of the microglial cells, reducing several parameters of microglial reactivity, namely NO production and phagocytic activity. On the other hand, Song and collaborators demonstrated that PTP1B is an important positive regulator of neuroinflammation and a promising therapeutic target for neuroinflammatory and neurodegenerative diseases. Therefore, PTPs can be the target to develop therapeutics for neurodegenerative and neuroinflammation diseases (Song, Jung, et al. 2016).

With the present work we aimed to unveil the role of STEP in the control of the neuroinflammatory process. To date, STEP studies focus on STEP expression in neuronal cells (Kamceva et al., 2016), mainly associated with neurodegenerative diseases such as Alzheimer's disease (Snyder et al., 2005) and Huntington's disease (Saavedra et al., 2011), and to date there are no data on the expression of STEP in microglia.

In the first part of this study, we characterized the microglial culture by immunocytochemistry where microglial cells were labeled for Iba-1, a calcium-binding protein specifically expressed in macrophage/microglia. About 92% of the cells present in the culture expressed the microglial marker Iba-1 and the remaining 8% were constituted mainly by astrocytes. In this culture exposure to LPS 2µg/ml for 24 hours induced a strong increase in the release of nitric oxide, indicative of a reactive state of microglia.

In the second part of this study, we characterized the expression of different STEP isoforms in primary cultures of microglia from Wistar rat brain and demonstrated for the first time the expression of STEP by microglial cells. Studies in N9 microglial cell line (supplementary material) are concordant with this data. These results were further supported by data from PCR and Western blot analysis. PCR results confirm the expression of the $STEP_{61}$ mRNA by microglia. Western blot results showed that both $STEP_{61}$ and $STEP_{46}$ isoforms are expressed with the band corresponding to the $STEP_{61}$ isoform presenting a stronger intensity, suggestive of a higher expression. STEP expression in the presence of the inflammatory stimulus LPS was also assessed (Chhor et al. 2013). Immunocytochemistry showed small changes, although not statistically significant, in STEP expression when the cultures were submitted to an LPS stimulus, suggesting an increase of STEP expression by reactive microglia. To confirm these results, a Real-time RT-PCR and Western blot analysis were performed. Western blot analysis showed an increase in the protein levels of both STEP isoforms after LPS stimulation. Data from RT-PCR indicate that the increase in STEP protein levels was coupled to an increase of PTPN5 transcription. Taken together these data showed that microglia express STEP protein and that microglial reactivity is associated with an increase of STEP expression. Currently, there are few

examples of a direct association of PTPs with neuroinflammatory diseases, however there are several studies that evaluate the expression of PTPs after exposure to LPS and that are concordant with the results obtained (Song, Jung, et al. 2016; Song, Kim, et al. 2016; Vieira et al. 2017).

In order to determine the ability of STEP to regulate microglial reactivity we used as a tool a specific pharmacological inhibitor of STEP (TC-2153) and analysed its influence on microglial reactivity induced by LPS. TC-2153 has been proposed as a therapeutic target in human diseases due to the improvement of cognitive function in several neurodegenerative disorders (Xu et al. 2014) or as potential psychotropic activities (Carty et al. 2012). The classical activation of microglia results in an ameboid morphology and are implicated in the pathogenesis of various neuroinflammatory diseases. These morphological changes, which occur during the transition from a typical resting state to an activated phenotype, are associated with increased production of pro-inflammatory mediators, namely, the release of NO, phagocytic activity and expression of iNOS, IL-1ß and TNFα (Bolós et al. 2017). Our model replicated these characteristiscs. A significant increase in NO release, phagocytic activity and expression of iNOS, IL-1ß and TNFα was observed in LPS stimulated microglia. Similar results were obtained with the N9 microglial cell line (supplementary material) in which a significant increase in iNOS expression, NO release and phagocytic activity in LPS-stimulated cultures were reported. These results are similar to other works showing an increase in these inflammatory markers, well-established markers of microglia reactivity (Chhor et al. 2013; Song, Jung, et al. 2016; Song, Kim, et al. 2016; Vieira et al. 2017). The inhibition of the activity of STEP significantly reduced the number of microglial cells expressing iNOS and the number of phagocytic cells after LPS stimulation. However, there was no significant decrease in NO release, suggesting that there is another pathway for NO production, other than through iNOS, which is not regulated by STEP. Studies with the N9 microglial cell line are concordant with the data obtained with primary cultures of microglia. Inhibition of STEP activity with TC-2153 significantly reduced LPS-induced expression of iNOS and phagocytic cells without altering NO release. Regarding the expression of the citokines IL-1ß and TNFα, inhibition of STEP did not significantly impact on its mRNA levels thus suggesting that STEP does not regulate the pathways that control the expression of these pro-inflammatory citokines. The results obtained suggest that STEP activity regulates pathways that control processes associated with the reactive status of microglia such as iNOS expression and phagocytosis but does not regulate the expression of proinflammatory cytokines or the release of NO. Other works suggest that PTP inhibitors can be exploited for therapeutic modulation of microglial activation in neuroinflammatory diseases because suppressed expression of iNOS, IL-1ß, and TNF-α in LPS-stimulated BV-2 microglial cells (Song, Jung, et al. 2016; Song, Kim, et al. 2016). We know that p38 and NF-κB also play an important role in the transcriptional regulation of proinflammatory mediators and p38 is a STEP substrates. We may hypothesize that STEP inhibition increases p38 phosphorylation but may decrease LPS-induced NF-κB activity, which suppresses IL-1ß and TNF-α.

Our results support that neuroinflammation is associated with an increase in STEP levels. Moreover, STEP activity promotes mechanisms associated with the microglia reactive status, further potentiating the inflammatory process.

There is a consensus that neuroinflammation is one of the main triggers in neurodegeneration, with microglial reactivity either initiating or amplifying neuronal damage, or being a consequence of it. When microglia is activated in response to an inflammatory stimulus, it produces several factors, which in excess can lead to neuronal death, such as ROS, NO and proinflammatory cytokines. On the other hand, neuronal damage is coupled to the release of several molecules such as α-synuclein and neuromelanin that potentiate microglia activation. This results in a cycle in which there is a repetition of neurotoxic activation of microglia in response to neuronal injury (Block and Hong 2005; Block et al. 2007). Therefore, inhibition of microglial hyperactivation might be a good strategy to develop therapeutics for neurodegenerative diseases (Perry and Holmes 2014). Since microglial reactivity contributes to the neurodegenerative process, and that our results support that STEP inhibition reduces microglia reactivity, STEP inhibition can be a therapeutic strategy to control the neuroinflammatory processes and in consequence neurodegeneration.

Chapter 6

Conclusions e Futures Perspectives

6 Conclusions and Futures Perspetives

Throughout this work we collected evidence of a significant correlation between STEP and neuroinflammation. Microglial cells express $STEP_{46}$ and $STEP_{61}$ and microglia reactivity is coupled to increased STEP levels. Moreover, Inhibition of STEP significantly reduces various parameters of microglial reactivity, namely phagocytic activity and iNOS expression. These results suggest that PTP inhibitors can be exploited for therapeutic modulation of microglial activation in neuroinflammatory diseases.

The following experiments could help to further confirm the role of STEP in the control of neuroinflammation and to explore if its inhibition could represent a strategy to protect from the neurotoxicity induced by inflammatory conditions:

- The comparison of microglial reactivity in STEP-KO and WT mice, both in control conditions and after exposure to an inflammatory stimulus, would be important to confirm the relevance of STEP in the control of neuroinflammation in vivo.

- To clarify if STEP is involved in the release of IL-1ß and TNFα it would be important to evaluate the release of these cytokines by ELISA.

- In order to explore how the state of microglial reactivity is associated with increased STEP function, it would be important to evaluate the changes of its activity by determining the levels of phosphorylated (pSTEP, non-active) and non-phosphorylated forms (active). This could also be evaluated indirectly by determining the phosphorylated and nonphosphorylated forms of STEP substrates, such as ERK1/2, p38 and the tyrosine kinase Fyn.

- Finally, to confirm that inhibition of STEP activity could be a novel therapeutic strategy for neuroinflammatory and neurodegenerative diseases it would be important to evaluate in an in vivo model, for example mice exposed to LPS, how STEP inhibition impacts on the neuronal loss promoted by LPS.

Chapter 7
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7 Bibliography

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Supplementary Material

Unpublished data on the role of STEP in neuroinflammation in N9 microglial cell line, were performed by Rita Videira and mentioned above in the discussion.

A. STEP expression

Through immunocytochemical analysis using an anti-STEP (Fig 34, green) antibody and Hoechst to nuclei stain (Fig 34, blue) she demonstrated that N9 microglial cell line express STEP.

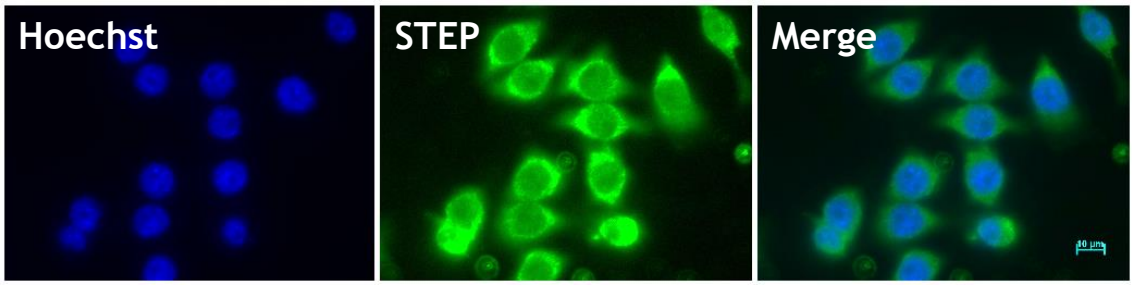

Figure 33. **Expression of STEP in N9 microglial cell line**. Representative images of immunocytochemical analysis that was performed against STEP protein (green) and was followed by nuclei staining with Hoechst (blue). The image was acquired on a fluorescence microscope (Axiobserver Z1, Zeiss) using the 63x objective.

B. Ability of STEP to regulate the inflammatory state

To determine the role of STEP in the control of microglia reactivity evoked by LPS cells were treated with 1μM specific pharmacological inhibitor of STEP (TC-2153) 1 h before starting the stimulation with pro-inflammatory agent LPS 0.1μg/ml. Cells were incubated with both compounds for further 24h and at the end of this period the parameters of microglia reactivity analysed were the phagocytic activity, expression of iNOS and release of NO.

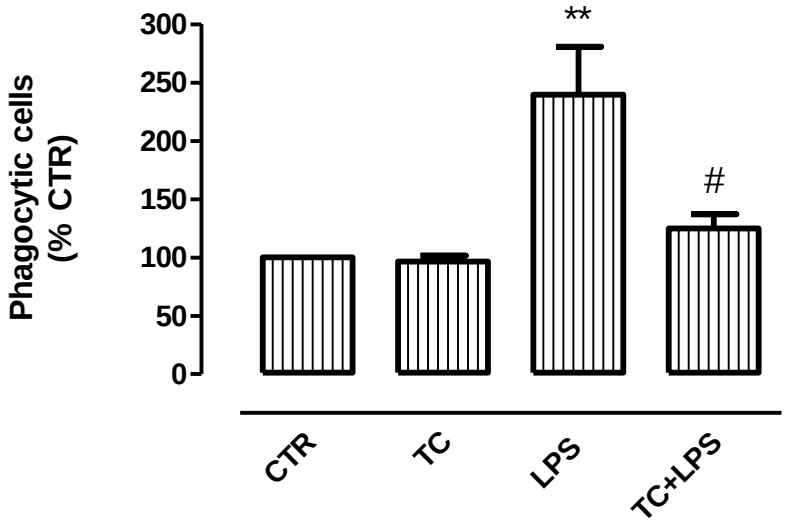

Figure 34. **STEP inibhitor in N9 microglial cell line reduces LPS-induced phagocytic activity.** Microglial cells was exposed to LPS (0.1 µg/ml) for twenty-four hours in the presence of TC-2153(1µM). Cells were incubated with fluorescent microspheres to evaluate phagocytic activity. Quantification of microglial cells that incorporated these microspheres (phagocytic cells). Data represent the mean ± SEM of three independent experiments performed in duplicate and expressed in percentage of CTR. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test ($^{**}p$ <0.01 compared to CTR and $^{\#}p$ <0.05 compared to LPS).

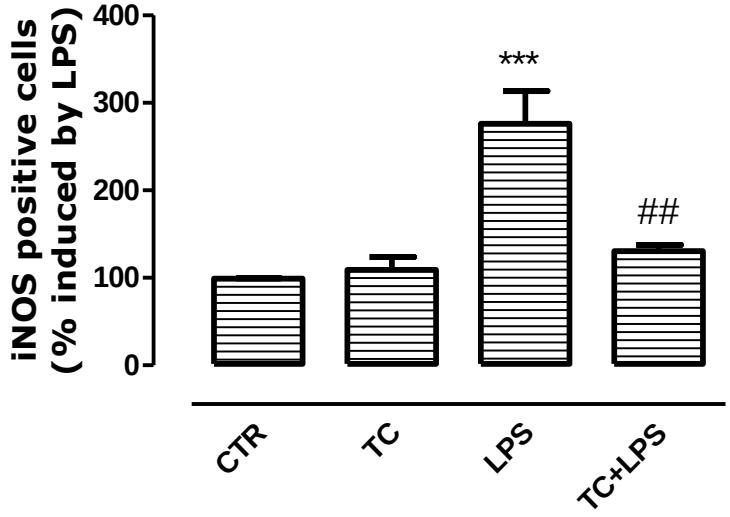

Figure 35. **STEP inibhitor in N9 microglial cell line reduces LPS-induced iNOS expression.** Microglial cells wer exposed to LPS (0.1 µg/ml) for 24h in the presence of TC-2153(1µM). iNOS expression was evaluated by immunocytochemistry. Quantification of iNOS-immunopositive cells for each condition. Data represent the mean ± SEM of three independent experiments performed in triplicate and expressed in percentage induced by LPS. Statistical analysis was performed using using one-way ANOVA followed by Bonferroni's Multiple Comparison Test (*** p <0.001 compared to CTR and $^{\#\#}$ p <0.01 compared to LPS).

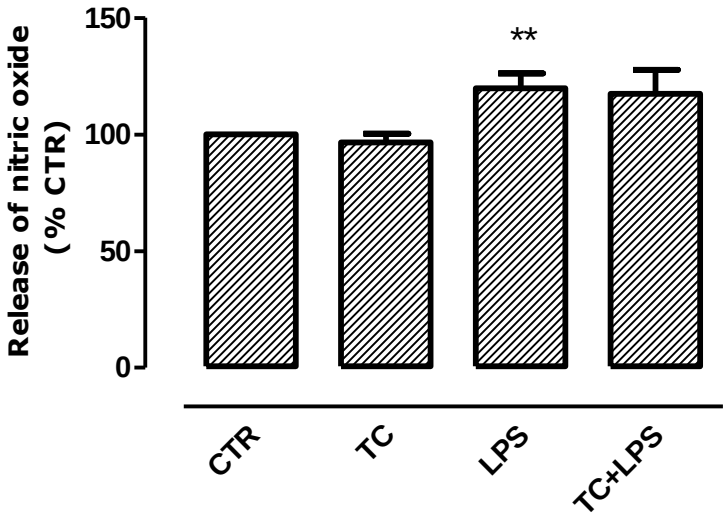

Figure 36. **NO release by LPS-stimulated microglia.** Microglial cells were exposed to LPS 0.1µg/ml for 24h in the presence of TC-2153 (1µM). Culture supernatants were collected and NO levels released by microglial cells quantified by Griess Method. Data represent the mean ± SEM of nine experiments performed in triplicate and expressed in percentage of CTR. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test ($^{**}p < 0.01$ compared to CTR).