A ingratidão dos agradecimentos (porque não quero arriscar esquecer alguém). Coragem. Trabalho. Dedicação. De todos involvidos de forma directa ou mais distante. De todos que me ensinaram, reindivicaram mais e melhor, riram ou choraram comigo. Agradeço-vos sem dizer os vossos nomes, que preencheriam um espaço maior do que aquele que poderia conceder-vos, nomes que não saberia ordenar, pois têm todos tanto valor para mim. Termino dizendo-vos que trarei todos os vossos nomes, profissionalismo, amizade e amor sempre comigo. Obrigada.

Raquel Ferreira

Neuropeptide Y Modulates Interleukin-1β-induced Microglia Activation

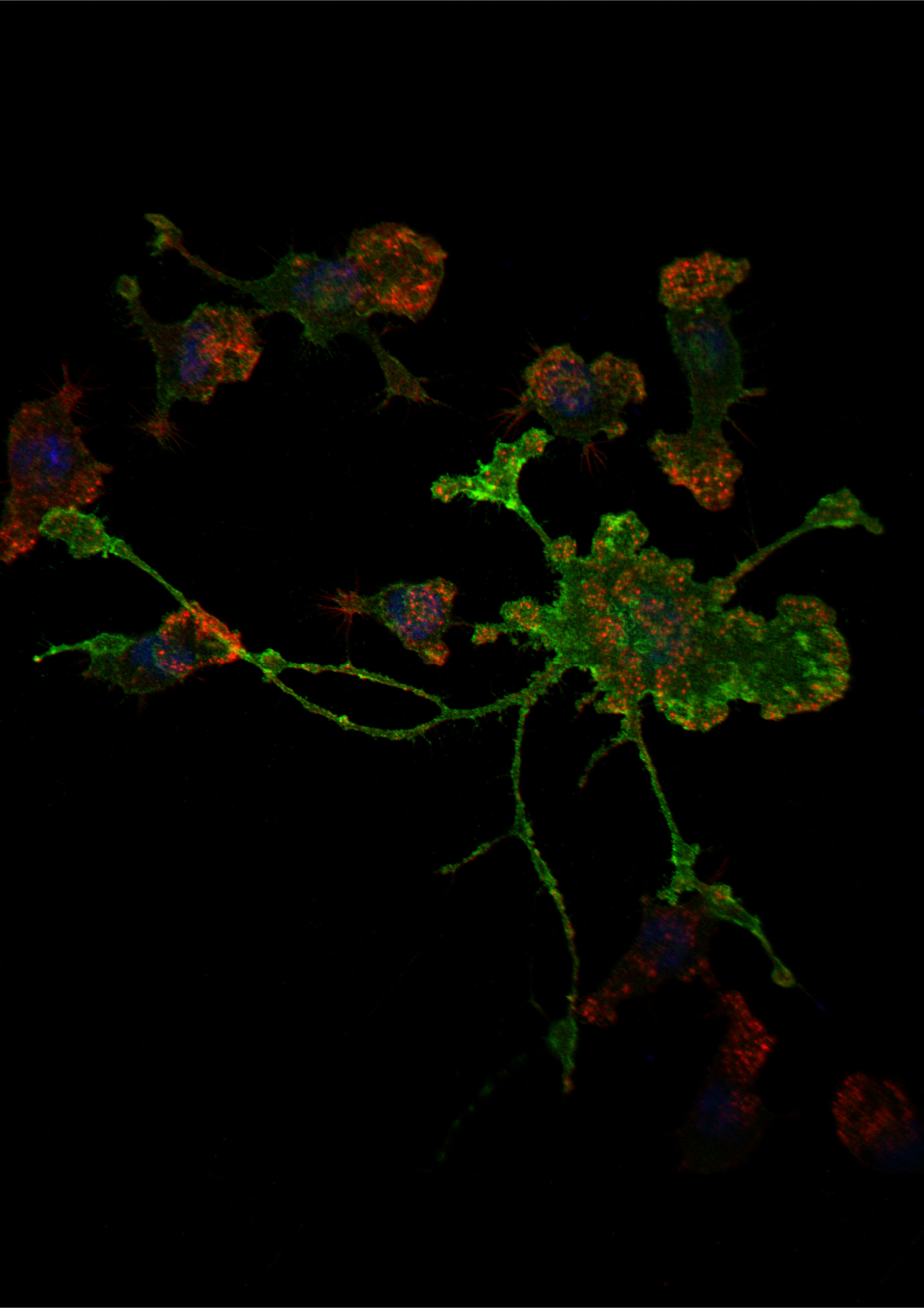
2010

Contribution of Microglia to Neural Inflammation

Neuropeptide Y Modulates Interleukin-1β-induced Microglia Activation

Raquel Margarida da Silva Ferreira

Universidade de Coimbra
2010



On the front page:

Microglial cells, treated with IL-1β and in the presence of IL-1ra, were modestly activated, preventing CD11b (in green) overexpression and actin cytoskeleton (in red) reorganization. In the center, an activated cell is interacting with resting-like microglia. Nuclear staining in blue (Hoechst 33342 staining).

Contribution of microglia to neural inflammation

Neuropeptide Y modulates interleukin-1β-induced microglia activation

by

Raquel Margarida da Silva Ferreira

Dissertation presented to the Faculty of Sciences and Technology of University of Coimbra in partial fulfillment of the requirements for the degree of Doctor of Philosophy (PhD).

University of Coimbra
2010

Work was accomplished at the Center for Neurosciences and Cell Biology of Coimbra (CNCB), Portugal, under the scientific supervision of João Oliveira Malva and Emília Conceição Pedrosa Duarte. Murine N9 microglial cell line was provided by Claudia Verderio, from National Research Council, Institute of Neuroscience and Department of Medical Pharmacology, Milan, Italy, and Paulo Santos from CNCB, Department of Life Sciences, University of Coimbra, Portugal. Monoclonal antibody NPY-05 was supplied by Eric Grouzmann from Division of Clinical Pharmacology and Toxicology, Centre Hospitalier Universitaire Vaudois, Switzerland, and Cláudia Cavadas from Faculty of Pharmacy, University of Coimbra, Portugal. Technical support was rendered by Luísa Cortes (confocal microscopy), Isabel Nunes and Isabel Dantas (cell culture), from CNCB, Department of Life Sciences, University of Coimbra, Portugal.
All collaborators, including Tiago Santos, Sara Xapelli, Ana Paula Silva, Armando Cristóvão, Stephànie Cochaud and Otília Vieira made important contributions for the published work.
Execution of this work was supported by Fundação para a Ciência e Tecnologia (FCT) and FEDER POCTI/SAU-FCF/58492/2004; POCTI/SAU-NEU/68465/2006. Grant reference: SFRH/BD/23595/2005.

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ABBREVIATIONS

$[Ca^{2+}]_i$	Intracellular calcium concentration
AC	Associational Comissural
AD	Alzheimer's disease
ADP	Adenosine diphosphate
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
APCs	Antigen-presenting cells
ASC	Apoptosis-associated speck-like protein containing a C-terminal
ATP	Adenosine triphosphate
Aβ	Amyloid beta
BAFF	B cell activating factor
BBB	Brain blood barrier
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine serum albumin
C/EBP	CCAAT-enhancer-binding proteins
CA	*Cornu ammonis*
CaMK	Calmodulin-regulated protein kinase
cAMP	Cyclic adenosine monophosphate
CARD	Caspase-activating recruiting domain
CCAAT	Cytidine-cytidine-adenosine-adenosine-thymidine
CD11b	Alpha chain of $\alpha_M\beta_2$-integrin or cluster of differentiation molecule 11b
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CPON	C-flanking peptide of NPY
CREB	cAMP response element-binding
CSF	Cerebral spinal fluid
DEPC	Diethylpyrocarbonate
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EC	Entorhinal cortex
ECM	Extracelular Matrix
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalogram
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FBS	Foetal bovine serum
GABA	γ-aminobutyric acid
GTP	Guanosine triphosphate
GTPase	GTP hydrolase
HSP	Heat shock protein
Hsp27	Heat shock protein 27
ICAM-1	Intercellular adhesion molecule
ICE	Interleukin converting enzyme or Caspase-1
IFN-γ	Interferon gamma
IGF-1	Insulin-like growth factor 1
IgG	Immunoglobulin
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
IL-1β	Interleukin-1 beta
iNOS	Inducible nitric oxide synthase
IP3	Ionositol triphosphate
IRAK	Interleukin-receptor associated kinase
IRF-3	Interferon regulatory factor-3
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KA	Kainic acid (or kainate)
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
LTβ	Lymphotoxin β
MAP	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MARCO	Macrophage receptor with collagenous structure
MCSF	Macrophage-colony stimulating factor
MF	Mossy fibres
MHC	Major histocompatibility complex
MK2	MAPK-activated protein kinase 2
mRNA	Messenger ribonucleic acid
MS	Multiple Sclerosis
MyD88	Myeloid differentiation primary response gene 88
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NALP3	Cryopyrin
NF-κB	Nuclear factor-κappa B
NGF	Neuronal growth factor
NMDA	N-methyl-D-aspartate
NMDAR	*N*-methyl-D-aspartate receptors
NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
nSMase	Neutral Sphingomyelinase
OD	Optical density
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PECAM-1	Platelet/endothelial cell adhesion molecule-1

PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PIL	Pilocarpine
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PNS	Peripheral nervous system
PP	Perforant path
PRR	Pattern recognition receptor
PtdSer	Phosphatidylserine
PTZ	Pentetrazol
RANKL	Receptor activator of NF-κB ligand
RASMC	Rat aortic smooth muscle cells
Rh1	Protopanaxatriol-type ginsenoside Rh1
Rho	Ras homolog gene
RNA	Ribonucleic acid
ROS	Reactive oxigen species
RT	Room temperature
RT-PCR	Reverse transcription PCR
Sb	Subiculum
SC	Schaffer collaterals
SDS	Sodium dodecyl sulfate
STAT	Signaling transducer and activator of transcription
TAB	TAK-1-binding proteins
TAK-1	TGF-β-activated kinase 1
TBS-T	Tris-buffered saline Tween-20®
TGF-β	Tumor growth factor-beta
Thr	Threonine
TIR	Toll IL-1 receptor
TLE	Temporal Lobe Epilepsy
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
Tollip	Toll-interacting protein
TRAF-6	TNF receptor associated factor 6
TREM2	Triggering receptor expressed on myeloid cells-2
Tyr	Tyrosine
UTP	Uridine triphosphate
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VX-765	ICE inhibitor
Y_1R	Y_1 receptor
Y_2R	Y_2 receptor
Y_5R	Y_5 receptor

PUBLICATIONS

Results presented in this dissertation have been published, or are in the process of submission, in international peer-reviewed journals:

Raquel Ferreira, Sara Xapelli, Tiago Santos, Ana Paula Silva, Armando Cristóvão, Luísa Cortes, João O. Malva (2010) Neuropeptide Y modulation of interleukin-1 beta (IL-1β)-induced nitric oxide production in microglia. Journal of Biological Chemistry.
DOI: 10.1074/jbc.M110.164020

Raquel Ferreira, Tiago Santos, Luísa Cortes, Stephànie Cochaud, Ana Paula Silva, Sara Xapelli, João O. Malva (2011) Neuropeptide Y inhibits interleukin-1 beta (IL-1β)-induced microglia motility. Submitted.

Raquel Ferreira, Tiago Santos, Luísa Cortes, Ana Paula Silva, Armando Cristóvão, Sara Xapelli, Otília Vieira, João O. Malva (2011) Neuropeptide Y inhibits interleukin-1 beta (IL-1β)-stimulated microglia phagocytosis. Submitted.

The awarded PhD grant also enabled the success of other paper submissions under co-authorship:

Silva A.P., Xapelli S., Pinheiro P.S., Ferreira R., Lourenço J., Cristóvão A., Grouzmann E., Cavadas C., Oliveira C.R., Malva J.O., Up-regulation of neuropeptide Y levels and modulation of glutamate release through neuropeptide Y receptors in the hippocampus of kainate-induced epileptic rats, *Journal of Neurochemistry* 2005; 93(1):163-70

Bernardino L., Ferreira R., Cristóvão A.J., Sales F., Malva J.O., Inflammation and neurogenesis in temporal lobe epilepsy, *Current Drug Targets - CNS & Neurological Disorders* 2005; 4(4):349-60

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Agasse F.[*], Bernardino L.[*], Ferreira R., Silva B.A., Grade S., Malva J.O., Response to histamine allows the functional identification of neuronal progenitors, neurons, astrocytes and immature cells in subventricular zone cell cultures, *Rejuvenation Research* 2008; 11(1):187-200. [*]both authors contributed equally

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Ferreira R., Xapelli S., Berdinadino L., Malva J.O., Epilepsia, In: Neurociências, Edições Lidel. In preparation.

SUMMARY

Neuropeptide Y (NPY) holds consistent neuroprotective and proneurogenic properties in the Central Nervous System (CNS). In light of growing evidence supporting a role for NPY in the regulation of the immune system, we sought to investigate the effect of this neuropeptide over several aspects of microglial response to inflammation, namely, the production of inflammatory mediators, cell motility and phagocytosis.

In chapter 1, we investigated the role of NPY in the modulation of LPS-induced release of inflammatory mediators, such as nitric oxide (NO) and interleukin-1β (IL-1β).
Upon lipopolysaccharide (LPS, 100 ng/ml) stimulation, we found that microglial cells increased the expression of inducible nitric oxide synthase (iNOS), as well as the production of NO, as quantified by Griess Assay. Moreover, microglial cells co-stimulated with LPS and adenosine triphosphate (ATP, 1 mM) responded with a massive release of IL-1β, as measured by ELISA. We observed that LPS (100 ng/ml) and IL-1β (1.5 ng/ml) stimulation induced NO production, a response prevented in the presence of a selective IL-1 receptor antagonist (IL-1ra, 150 ng/ml). Furthermore, LPS-induced NO production mediated by IL-1β occurred through a nuclear factor-kappaB (NF-κB)-dependent pathway. We observed that NPY inhibited IL-1β release and downstream nuclear translocation of NF-κB (determined by confocal microscopy and Western blotting), which is implicated in iNOS expression and NO synthesis. Pharmacological studies with a selective Y_1 receptor agonist ([Leu^{31},Pro^{34}]NPY, 1 μM) and selective antagonists for receptors Y_1 (BIBP3226, 1 μM), Y_2 (BIIE0246, 1 μM) and Y_5 (L-152,804, 1 μM) demonstrated that NPY inhibition was mediated exclusively through Y_1 receptor activation.

In chapter 2, we investigated the role of NPY in the modulation of IL-1β-induced microglial motility and the signaling pathway involved in this process.
Interestingly, co-stimulation of microglial cells with LPS (100 ng/ml) and ATP (1 mM) resulted in increased cell motility, an effect inhibited by IL-1ra (150 ng/ml), which strongly suggested the participation of IL-1β in this process. In our scratch wound assay, we also observed that IL-1β-induced motility was prevented by SB239063 (20 μM), a selective inhibitor of p38 mitogen-activated protein kinase (MAPK). IL-1β (1.5 ng/ml) induced p38 MAPK phosphorylation, followed by nuclear translocation, and this effect was inhibited by NPY *via* Y_1 receptor activation, as observed by confocal microscopy and Western blotting. Likewise, p38 MAPK inhibition decreased the extent of actin filament reorganization occurring during plasma membrane ruffling.

Given that both LPS and IL-1β induced significant alteration to the cell cytoskeleton, we proceeded to investigate the role of NPY in the regulation of LPS-induced microglial cell phagocytosis, in chapter 3.
Accordingly, we observed that LPS (100 ng/ml) increased latex bead phagocytosis by microglia. Consistently, co-administration of LPS (100 ng/ml) and ATP (1 mM) increased bead phagocytosis and this effect was blocked by IL-1ra (150 ng/ml), suggesting the involvement of

IL-1β. Moreover, direct application of IL-1β (1.5 ng/ml) augmented the number of phagocytosed beads, while NPY acting through Y_1 receptor activation inhibited this effect.

To conclude, we assigned a novel role for NPY in the regulation of important microglial responses to danger signals in the brain, involving the production and release of inflammatory mediators, cell motility and phagocytosis.

RESUMO

O neuropeptídeo Y (NPY) detém importantes propriedades neuroprotectoras e pró-neurogénicas no Sistema Nervoso Central (CNS). Dado o número crescente de evidências que sugerem um papel imuno-regulador para este neuropeptídeo, propusemo-nos estudar o efeito do NPY sobre vários aspectos da resposta da microglia à inflamação, nomeadamente, a produção de mediadores inflamatórios, motilidade celular e fagocitose.

No capítulo 3, investigámos o papel do NPY na modulação da libertação de mediadores inflamatórios, tais como óxido nítrico (NO) e interleucina-1β (IL-1β), após exposição a lipopolissacarídeo (LPS).
Após estimulação com LPS (100 ng/ml), as células da microglia aumentaram a expressão da sintetase induzível do óxido nítrico (iNOS), assim como a produção de NO, quantificado com recurso ao ensaio de Griess. Adicionalmente, as células da microglia estimuladas com LPS e adenosina trifosfato (ATP, 1 mM) responderam com uma libertação massiva de IL-1β, quantificado por ELISA. Observámos também que a exposição a IL-1β (1,5 ng/ml) induziu a produção de NO, uma resposta inibida na presença do antagonista selectivo para o receptor IL-1R (IL-1ra, 150 ng/ml). A produção de NO induzida por LPS é, assim, mediada por IL-1β. Observámos que NPY inibiu a libertação de IL-1β e a translocação nuclear de NF-κB (determinada por microscopia confocal e por Western blotting), um processo implicado na expressão de iNOS e na síntese de NO. Uma abordagem farmacológica, recorrendo à utilização de um agonista para o receptor Y_1 ([Leu^{31},Pro^{34}]NPY, 1 μM), e de antagonistas para os receptores Y_1 (BIBP3226, 1 μM), Y_2 (BIIE0246, 1 μM) e Y_5 (L-152,804, 1 μM) permitiu identificar o receptor Y_1 como o principal responsável pelo efeito inibitório do NPY.

No capítulo 4, investigámos o papel do NPY na modulação da motilidade da microglia induzida por IL-1β, e a via de sinalização envolvida neste processo.
A co-estimulação das células da microglia com LPS (100 ng/ml) e ATP (1 mM) resultou no aumento de motilidade celular, um processo inibido por IL-1ra (100 ng/ml), o que sugeriu o envolvimento de IL-1β neste processo. Com recurso a um ensaio de lesão, observámos que a motilidade induzida por IL-1β foi inibida por SB239063 (20 μM), um composto que inibe a activação da cinase p38. IL-1β (1,5 ng/ml) induziu a fosforilação da cinase p38, e a translocação da forma fosforilada para o núcleo. Este processo foi inibido por NPY através da activação do receptor Y_1 (observado por microscopia confocal e Western blotting). Da mesma forma, a inibição da p38 diminui a reorganização dos filamentos de actina que ocorre durante o "ruffling" membranar, um processo que reflecte a expansão da membrana, durante a migração celular.

No capítulo 5, investigámos o papel do NPY na regulação do processo fagocítico estimulado por LPS.
Assim, observámos que LPS (100 ng/ml) aumentou o número de micro-esferas de látex fagocitadas por microglia. A co-administração de LPS (100 ng/ml) e ATP (1 mM) aumentou a fagocitose de micro-esferas de látex e este efeito foi bloqueado por IL-1ra (150 ng/ml), sugerindo

o envolvimento de IL-1β. A aplicação directa de IL-1β aumentou o número de micro-esferas fagocitadas, enquanto o NPY inibiu este efeito através da activação do receptor Y_1.

Em suma, atribuímos um novo papel ao NPY na regulação de respostas da microglia a sinais inflamatórios no cérebro, envolvendo a produção e libertação de mediadores inflamatórios, motilidade celular e fagocitose.

CHAPTER 1. OVERVIEW

Within the nervous system, the brain has a privileged status. Until recently, the brain was portrayed as impenetrable to invading pathogens because a physical barrier between circulating blood and the brain parenchyma existes – the blood brain barrier (BBB) (Purves et al. 2009). However, the integrity of a healthy BBB can be compromised upon brain injury and disease. Hence, the brain parenchyma is continuously surveyed ensuring the protection of the central nervous system (CNS). For that purpose, there is a group of cells within the CNS which act as the primary immune effector cells of the brain. Microglial cells quickly respond to invading pathogens and injury through motility, phagocytosis and release of inflammatory mediators, that affect the final outcome of neuronal degeneration/repair. In that sense, disclosing the mechanisms underlying the role of microglia as immunocompetent cells could provide therapeutic targets for the prevention of neurological dysfunctions such as epilepsy, ischemia, stroke, Alzheimer's disease, or multiple sclerosis (Danton and Dietrich 2003; Garden and Möller 2006; Choi and Koh 2008; Amantea et al. 2009; Koning et al. 2009; Perry et al. 2010).

1.1. The blood-brain barrier

The BBB is composed by tightly juxtaposed endothelial cells sheathing the blood vessels in the brain. Surrounding the endothelial cells, a thin supporting basal lamina membrane made with laminin and fibronectin, among other proteins, contributes to insulation. Astrocytes and pericytes are the other cell components of the BBB. Under normal physiological conditions, this specialized continuous system of capillaries is only crossed by highly lipophilic small molecules such as oxygen (O_2) and carbon dioxide (CO_2). Larger molecules like insulin, leptin, and iron transferrin, cross the barrier via receptor-mediated endocytosis, or via selective membrane transporters involved in the transport of molecules like glucose or amino acids. However, in several neurological diseases, BBB dysfunction can occur through the passive diffusion of blood-borne substances across tight junctions (increased permeability) or through massive cellular infiltration. In hypoxia-ischemia, septic encephalopathy, HIV-induced dementia, multiple sclerosis, and Alzheimer's disease, among other pathologies, the blood-brain barrier is known to be compromised (Ballabh et al. 2004; Persidsky et al. 2006; Weiss et al. 2009).

Most importantly, in temporal lobe epilepsy (TLE), there is vascular remodeling and altered BBB permeability. Rigau and colleagues (2007) demonstrated for the first time the occurrence of aberrant angiogenesis specifically in TLE. Using TLE hippocampal samples and a rat model of limbic epilepsy, the group observed an increase in the density of the vascular network, which was significantly correlated with the frequency of seizures, transient up-regulation of vascular endothelial growth factor (VEGF) and extravascular immunoglobulin (IgG) staining (followed by neuronal uptake of IgG) (Rigau et al. 2007). Later in the same year, Van Vliet and colleagues also reported a positive correlation between BBB permeability and the occurrence of spontaneous seizures in chronic epileptic rats as well as serum albumin leakage (followed by albumin accumulation in neurons, astrocytes and microglia) (van Vliet et al. 2007).

Ultimately, the existence of a physical and selective blood-brain barrier protects the CNS from the potentially deleterious effect of a full immune response from the peripheral system. The delicate neuronal network would be unable to withstand long-lasting exposure to many of the molecules released by circulating leukocytes. Hence, within the CNS, immunosurveillance is mainly secured by microglia, a resident population of cells that act both as supportive glia and immunocompetent cells.

1.2. Microglia, the immunocompetent cells of the CNS

Microglial cells are critical in the protection of the brain parenchyma against brain injuries like infection, trauma, ischemia, brain tumors and neurodegenerative diseases. These cells are often regarded as the resident macrophages of the CNS since they have the ability to become phagocytes, when needed. However, in a healthy brain parenchyma environment, resting microglia does not engage phagocytic or pinocytic activity. When activated, microglial cells abandon their ramified resting-like morphology and become amoeboid, migrating to the site of injury and releasing several pro-inflammatory and trophic factors (Streit et al. 1999; Garden and Möller 2006; Block et al. 2007).

The involvement of microglia in inflammation and neurotoxicity has been demonstrated in models using lipopolysaccharide (LPS). This endotoxin is a complex macromolecule containing a polysaccharide covalently linked to lipid A and it is the main component of gram-negative bacteria outer cell membrane. LPS cannot mimic entirely the conditions under which microglia are activated in neurodegeneration but is one of the most used compounds to trigger inflammatory-like activity in microglial cells. Among other important properties, LPS is devoid of significant toxic effects directly on neurons but reveals a microglia-mediated neurotoxicity (Block et al. 2007). For the reasons stated above and because it is driven through specific recognition systems, microglial activation by LPS became the best described experimental model to study the development of an innate immune response (Rivest 2003), triggering microglial responses for the vast majority of parameters investigated, such as proliferation, migration and inflammatory mediators release.

Microglia are constantly prowling the CNS environment and have evolved to express multiple and diverse membrane receptors that identify a wide array of molecular determinants. Pattern recognition receptors (PRRs) are usually constitutively expressed by microglia to identify and bind pathogen-associated molecular patterns (PAMPs) in order to initiate the appropriate innate immune response. The Toll-like receptors (TLRs) are one of the most studied PRR families and as such, are activated by bacterial lipopolysaccharides, hypomethylated DNA, flagellin and double-stranded RNAs, among other molecules. Until now, thirteen TLRs have been identified in mice, although TLR10 is not functional, rendering them sensitive to almost every type of bacterial and viral challenge (Kawai and Akira 2010). Humans only present TLR1-TLR10. TLR4 is upregulated upon brain inflammation and, together with CD14, is considered the primary LPS receptor mediating LPS-induced neurodegeneration and oligodendrocyte damage (fig. 1.1) (Block et al. 2007).

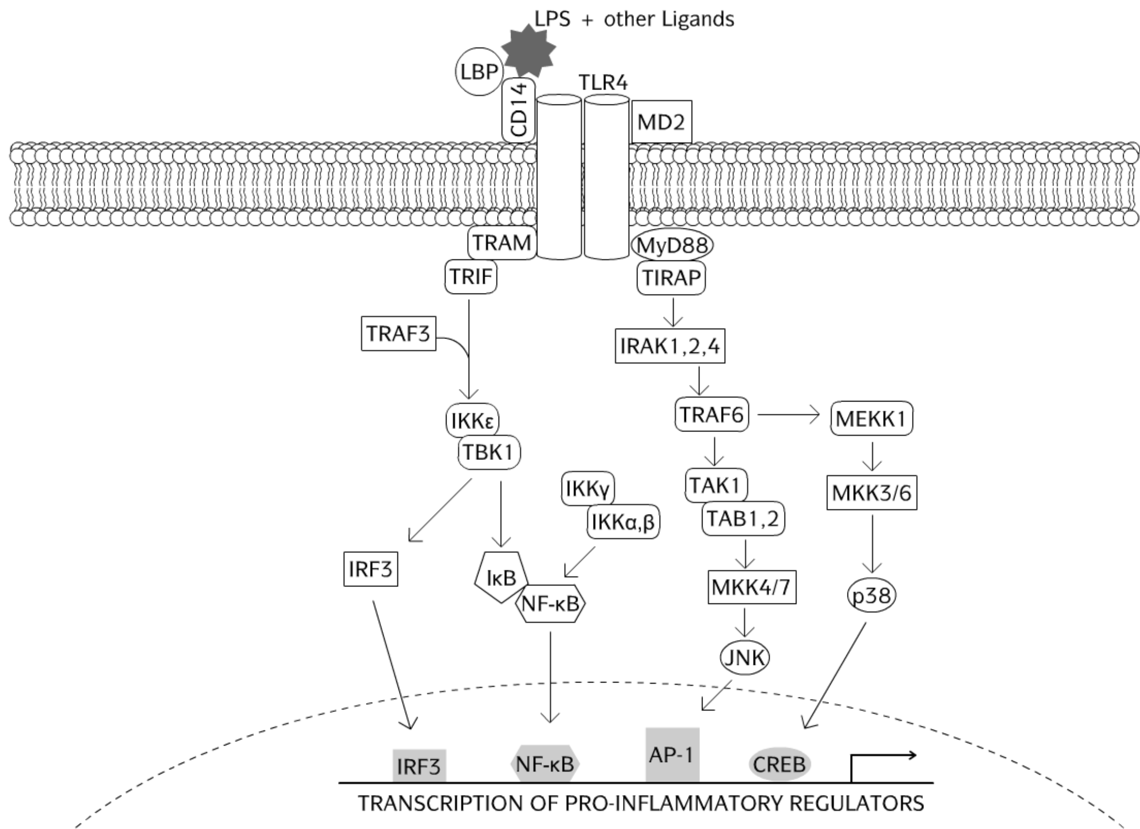


Fig. 1.1. Representative scheme of Toll-like receptor 4 (TLR4) signaling. LPS, a constituent of the cell wall of Gram-negative bacteria, is bound by the complex formed by pattern-recognition molecule Toll-like receptor 4 (TLR4), cell-surface receptor CD14 and MD2. The binding of LPS leads to recruitment of the adaptor proteins MyD88 and IRAK to the cytoplasmic domain of TLR4, leading to the phosphorylation of TRAF6 and to activation of JNK and p38 signaling pathways. Alternatively, LPS binding to TLR4 initiates a signaling cascade which recruits kinase IκK. IκK phosphorylates IκB, an inhibitor bound to the transcription factor NF-κB. Phosphorylated IκB is degraded, releasing NF-κB, which migrates to the nucleus where it activates the transcription of pro-inflammatory genes. Adapted from (Kawai and Akira 2010).

The interaction between TLRs and PAMPs results in the activation of intracellular signaling cascades that prompt the release of communicating signals to surrounding cells and invading leukocytes. Activated microglial cells release various signaling molecules, such as: cytokines and chemokines, like interleukin-1 (IL-1), IL-6, interferons (IFNs), tumor necrosis factor-α (TNF-α), tumor growth factor-beta (TGF-β), macrophage inflammatory protein-1 alpha (MIP-1α), MIP-1β, MIP-2, monocyte chemotactic protein-1 (MCP-1) and neurotrophins, like neuronal growth factor (NGF) and brain-derived growth factor (BDNF), just to mention a few (Garden and Möller 2006).

Some of these molecules, which are also produced by activated T cells with a T_H1 cytokine profile, alter the expression and function of tight junctions making the BBB more permissive to leukocyte migration. In fact, TNF-α, IFN-γ and IL-1 stimulation increases the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), platelet/endothelial cell adhesion molecule-1 (PECAM-1) and E-selectin by endothelial cells of

the BBB (Chavarria and Alcocer-Varela 2004). Leukocyte migration results from the interaction between a chemotactic signaling system composed by chemokines and chemokine receptors, the vascular system, through the expression of adhesion molecules and integrins, and lymphocytes (Persidsky et al. 2006).

Actually, in the normal brain, T-cells can migrate from the blood to the cerebrospinal fluid (CSF) or to the parenchyma where they are committed to die from apoptosis if no antigens are found (Bauer et al. 1998). If brain injury occurs, after the initial innate immune response, carried out by activated resident microglia, an adaptative response will follow. T-lymphocytes infiltrate the brain and accumulate at inflammatory sites. T-lymphocytes, together with infiltrating microglial cells, which act as better antigen-presenting cells (APCs) than their resident counterpart (since they express high levels of major histocompatibility complex (MHC) class II), reinforce a full adaptative immune response (Turrin and Rivest 2006).

Acting in concert, cellular interaction between endothelial cells and T-cells, and between T-cells and APCs leads to the recruitment of more microglia to the site of activation and to the production of more cytokines and chemokines resulting in an enhanced infiltration of immunomodulatory cells from the peripheral blood (Chavarria and Alcocer-Varela 2004; Garden and Möller 2006).

1.3. Interleukin-1β signaling

Interleukin (IL)-1 family has a leading role in the development of the innate immune response. A structural and functional basis for the central role of IL-1 related cytokines may reside on the similarity of the cytoplasmic domain of the IL-1 receptor type I (IL-1RI) and the cytoplasmic domains of all TLRs. IL-1 is implicated in fever, sickness behavior, metabolic and cardiovascular changes, brain trauma, stroke and epilepsy, as well as many forms of chronic neurodegenerative disorders like Alzheimer's and Parkinson's disease or multiple sclerosis (Dinarello 2009; Pinteaux et al. 2009). The most prominent member of this family is IL-1beta (IL-1β).

IL-1β is produced mainly by blood monocytes and tissue macrophages which produce specific inhibitors of caspase-1 in order to reduce the secretion of mature IL-1β, accumulating its precursor in the cytosol. Moreover, under basal conditions, IL-1β mRNA contains an instability element in the coding region that causes degradation of most of the mRNA, maintaining IL-1β levels low (Pinteaux et al. 2009). However, systemic/central infection or injury induces a rapid increase of IL-1β mRNA (around 15 minutes) and triggers a positive feedback loop leading to IL-1β stimulation of IL-1β synthesis over a period of 24 hours (Dinarello 2009). IL-1β is able to induce its own gene expression, amplifying the IL-1 response in an autocrine or paracrine manner (Weber et al. 2010). When IL-1β binds to its receptor the transcription of mRNA is induced and IL-1β precursor is translated into protein. Hence, disease conditions characterized by an increase of either basal procaspase-1 mRNA, IL-1β precursor levels or inflammasome components may develop into an autoimmune response.

Activated monocytes/macrophages release ATP to the extracellular space where it can activate $P2X_7$ receptors expressed on the membranes of other monocytes/macrophages. $P2X_7$ receptors

are ATP-gated ion channels with the ability to lose its ion selectivity and undergo dilation to form a non-selective pore during prolonged ATP exposure. For this reason, $P2X_7$ receptors cause massive calcium entry. These receptors, highly expressed in microglia, are also involved in apoptotic cell death and proliferation (Di Virgilio 1995; Ferrari et al. 1997a; Bianco et al. 2006; Franke et al. 2007). Along with $P2X_7$ receptor activation, potassium channels open. A rapid fall in intracellular potassium levels unfolds NALP3 molecule (or cryopyrin) enabling the assembly of an inflammasome complex. This structure is composed by NALP3, a caspase-activating recruiting domain (CARD), an adaptor protein termed apoptosis-associated speck-like protein containing a C-terminal CARD (ASC) and CARDINAL. The oligomerization of inflammasome complexes induces the processing of procaspase-1 to its active form (Dinarello 2009; Weber et al. 2010). The active caspase-1 cleaves the IL-1β precursor, in the cytosol or in secretory lysosomes, generating a carboxyl-terminal mature IL-1β (Fantuzzi and Dinarello 1999). A rise in intracellular calcium levels activates phosphatidylcholine-specific phospholipase C and calcium-dependent phospholipase A permitting the secretion of mature IL-1β with exocytosis of the lysosomal contents. Alternatively, IL-1β can exit the cell via shedding of plasma membrane, microvesicles, direct release via transporters, or multivesicular bodies containing exosomes (fig.1.2.).

IL-1β mature form can be obtained also in the absence of caspase-1. The inactive IL-1β precursor can be processed in the extracellular space by enzymes such as neutrophil proteinase 3, matrix metalloprotease 9, granzyme A and mast cell chymase (Fantuzzi and Dinarello 1999; Dinarello 2009).

Altogether, IL-1 receptor family includes ten members in which IL-1RI, IL-1RII and IL-1RIII are the receptors for both IL-1α and IL-1β. IL-RIII acts as a co-receptor and is also termed IL-1RAcP. When IL-1β binds to ubiquitously expressed IL-1RI, the receptor undergoes a conformational change and forms a heterodimer with IL-1RAcP. This event is facilitated by the approximation between the Toll IL-1 receptor (TIR) domains of both proteins and followed by the recruitment of myeloid differentiation primary response gene 88 (MyD88) and Toll-interacting protein (Tollip).

MyD88 binding to the heterodimer cytoplasmic domains triggers the phosphorylation of IL-1 receptor-associated kinases IRAK-1, IRAK-2 and IRAK-4. TNF receptor associated factor 6 (TRAF-6) joins the complex and after phosphorylation, it migrates to the membrane together with IRAK-2. This module associates with TGF-β-activated kinase-1 (TAK-1), TAK-1-binding protein (TAB)-1, and TAB-2. TAK-1, TAB-1, TAB-2 and TRAF-6 migrate to the cytosol, where TAK-1 is phosphorylated and TRAF-6 is ubiquitinated. TAK-1 phosphorylation activates the inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ) which in turn phosphorylates IκB. When IκB is degraded, p50 and p65 NF-κB subunits are released, translocating to the nucleus. TAK1 also induces p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) phosphorylation (Dinarello 2009; Weber et al. 2010).

In addition to IL-1RI, other receptors bind IL-1β, as previously mentioned. IL-1RII is a plasma membrane-anchored receptor which does not have a TIR domain thereby functioning as a

decoy receptor. IL-1β can bind to the soluble IL-1RII (sIL-1RII) and form a complex with soluble IL-1RAcP or cell-bound IL-1RAcP, neutralizing IL-1β activity (Weber et al. 2010).

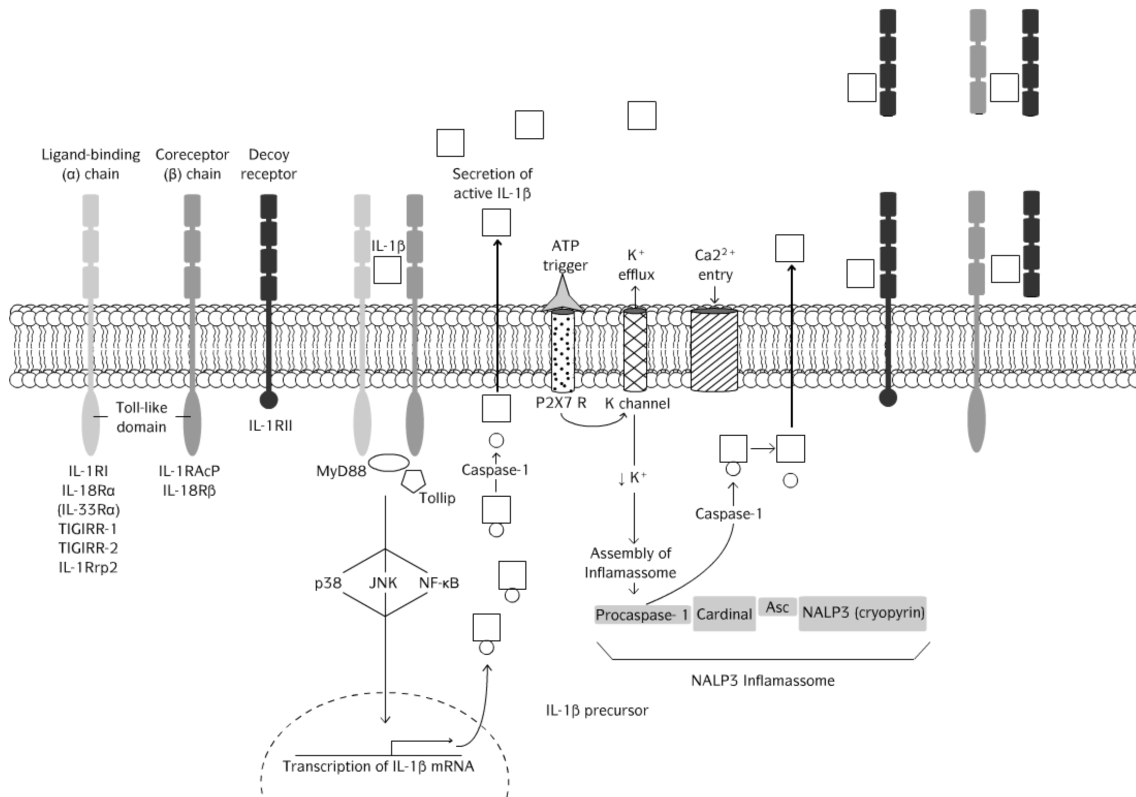


Fig. 1.2. Representative scheme of IL-1β signaling. Activation of IL-1 receptor complex triggers several signaling pathways that lead to the transcription of IL-1β mRNA. Transcription later takes place in the cytosol.
After activation of $P2X_7$ receptor by ATP, there is a rapid efflux of ions from the cell. Fall in intracellular potassium levels triggers the assembly of the components of the NALP3 inflammasome. Once assembled, processing of procaspase-1 into active caspase-1 beggins. Active caspase-1 processes the IL-1β precursor, resulting in the generation biologically active form IL-1β. Calcium influx increases intracellular calcium levels enabling the release of mature IL-1β. Cell membrane or soluble IL-1RII (sIL-1RII) may also bind IL-1β, neutralizing its activity. In addition, sIL-1RII can also form a complex with IL-1RAcP, impeding IL-1β binding to IL-1RI. (Adapted from (Dinarello 2009).

IL-1R antagonist (IL-1ra) is a specific inhibitor of the activity of both IL-1α and IL-1β. IL-1ra has two binding sites to IL-1RI, although high affinity binding to one site obstructs access to the other, necessary for the recruitment of IL-1RAcP. There are several autoinflammatory diseases that respond to IL-1 blockade ameliorating their symptoms, such as urate crystal arthritis (gout), type 2 diabetes, pericarditis, systemic-onset juvenile idiopathic arthritis and neonatal-onset multi-inflammatory disease, to name a few (Dinarello 2009).
To conclude, IL-1ra and IL-1RII serve as negative regulators of IL-1 signaling in the extracellular space and therefore can limit or terminate IL-1 effects (Weber et al. 2010).

IL-1 exerts cell-specific and concentration-dependent actions in the CNS. In astrocytes, IL-1 acts as a mitogenic factor and as a strong stimulant for the production and release of several secondary inflammatory mediators: cytokines, adhesion molecules, prostaglandins, neurotoxic factors and matrix metalloproteinases, as well as growth factors. In these cells, IL-1 acts through the canonical pathway (activating NF-κB and MAPKs) (Crespel et al. 2002). Alternatively, IL-1 can activate interferon regulatory factor-3 (IRF-3), signal transducers and activators of transcription factor-1 (STAT-1), Ras homolog gene family member A (RhoA) and protein kinase C (PKC) signaling pathways (Pinteaux et al. 2009). In neuronal cells, IL-1 also induces chemokine and growth factor production involving the NF-κB pathway (Pinteaux et al. 2009).
IL-1β can play an important role in excitotoxicity by elevating extracellular glutamate levels, either by inhibiting glial re-uptake of glutamate (Hu et al. 2000) or by increasing glial glutamate release via TNF-α production (Bezzi et al. 2001). IL-1β and IL-1RI are optimal inflammatory markers in epilepsy since they are rapidly up-regulated in the brain after seizures (Vezzani and Granata 2005). Moreover, IL-1β alters BBB permeability via tight-junction disruption resulting in increased neuronal excitability (Ferrari et al. 2004; Allan et al. 2005b; Ravizza et al. 2006). In pyramidal neurons of the hippocampus, IL-1RI co-localizes with NMDA receptors. IL-1β activation induces tyrosine phosphorylation of the NR2B subunit leading to a transient calcium influx and to the activation of the Ca^{2+}/calmodulin-dependent protein kinase II (CamKII) and cAMP response element-binding protein (CREB). Cell depolarization can be further caused by IL-1 activation of neutral sphingomyelinase (nSMase) and src kinase (Viviani et al. 2003; Pinteaux et al. 2009).
Conversely, IL-1 can inhibit neuronal activity by decreasing AMPA receptor and N-type Ca^{2+} channel expression and by enhancing GABAergig inhibition (Pinteaux et al. 2009). In conclusion, IL-1 activation has relevant functional consequences on neuronal excitability and cell survival.

1.4. p38 mitogen-activated protein kinase signaling pathway

Extracellular stimuli trigger cellular responses mostly through the activation of kinase and phosphatase cascades. A kinase family that clearly stands out is mitogen-activated protein kinases (MAPKs). In mammalian cells, the most relevant kinases belonging to this family are extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-jun N-terminal kinases (JNKs) and p38 MAPKs (Koistinaho and Koistinaho 2002).
JNKs are important regulators of physiological and pathological processes in the central and peripheral nervous system. These kinases are involved in cell mitosis and differentiation, neurite formation and outgrowth, but also in neuronal degeneration in response to stress and injury. ERK1/2 is also involved in cell cycle, cell growth and differentiation becoming activated in response to stress stimuli, including oxidative stress, glutamate receptor stimulation, or increased intracellular calcium levels (Koistinaho and Koistinaho 2002; Waetzig et al. 2006).
p38 MAPKs are serine threonine kinases present in the cytoplasm until activated by dual phosphorylation on tyrosine (Tyr) and threonine (Thr) residues. LPS stimulation of

macrophages results is phosphorylation of only p38α isoform suggesting that the α-isoform plays a central role in the inflammatory response of p38 MAPK pathway in microglia as well (Koistinaho and Koistinaho 2002). p38 MAPKs includes four isoforms differentially synthesized and regulated in a tissue-specific manner. p38α is abundant in monocytes and macrophages, p38β isoform is prevalent in endothelial cells, p38γ is particularly enriched in skeletal muscle, whereas p38δ predominates in endocrine glands (Cuadrado and Nebreda 2010).The α-isoform is implicated in cancer, heart and neurodegenerative diseases while the other isoforms have not been attributed to any obvious health condition (although p38γ and p38δ may be involved in metabolic diseases, cancer and tissue regeneration). In ischemic brain injury, the BBB becomes leaky and thrombin, among other molecules, reaches microglia. *In vitro* studies have demonstrated that thrombin activates p38 MAPK in microglia, resulting in nitric oxide (NO) release and expression of CD40 receptor. In cultured microglia, LPS-induced NO and TNF-release appears to require both p38 and p44/42 MAPK activity (Koistinaho and Koistinaho 2002).

Given its role in the immune system and in the regulation of cell survival and differentiation, p38 has become an interesting pharmaceutical target. However, MAPK inhibitors have repeatedly failed in clinical trials, mainly because liver and neural problems occur as side effects (Cuadrado and Nebreda 2010).

1.5. Nuclear factor-kappa B signaling pathway

Nuclear factor-kappa B (NF-κB) represents a family of transcription factors kept inactive in the cytoplasm, under normal physiological conditions, through the interaction with inhibitory molecules of the IκB family. The NF-κB/Rel family includes NF-κB1 (p50/p105), NF-κB2 (p52/p100), p65 (RelA), RelB, and c-Rel. The most common activated form of NF-κB is a heterodimer consisting of a p50/p65 or p52/p65 subunits, in which p65 contains a trans-activation domain necessary for gene induction (Tak and Firestein 2001; Israel 2010) .

NF-κB activation is involved in human inflammatory diseases such as rheumatoid arthritis, atherosclerosis, asthma and multiple sclerosis, among others (Tak and Firestein 2001). Once NF-κB heterodimer is freed from the IKK subunit it enters the nucleus and activates the expression of a variety of genes coding for cytokines (e. g. TNF-α, IL-1β, IL-6, IL-8), chemokines and adhesion molecules (e. g. E-selectin, VCAM-1, ICAM-1).

There is a canonical and an alternative pathway for NF-κB activation (fig.1.3.). The first pathway is triggered by microbial products and pro-inflammatory cytokines, usually leading to activation of RelA- or cRel-containing complexes that regulate pro-inflammatory and cell survival genes. The latter is activated by TNF-family cytokines—lymphotoxin β (LTβ), CD40 ligand (CD40L), B cell activating factor (BAFF) and receptor activator of NF-κB ligand (RANKL), but not TNF-α. The alternative pathway results in the activation of RelB/p52 complexes and regulates genes required for lymph-organogenesis and B-cell activation. These pathways can be distinguished by the different requirements they have for IKK subunits. The IKK complex consists of two kinase subunits, IKKα (IKK1) and IKKβ (IKK2), and a regulatory subunit IKKγ (NEMO). IKKα is

required for activation of the alternative pathway through the phosphorylation and processing of p100, the precursor for p52, and this is independent of both IKKβ and IKKγ. IKKβ regulates activation of the canonical pathway through phosphorylation of IkBs and requires the IKKγ subunit but not IKKα (Lawrence 2009).

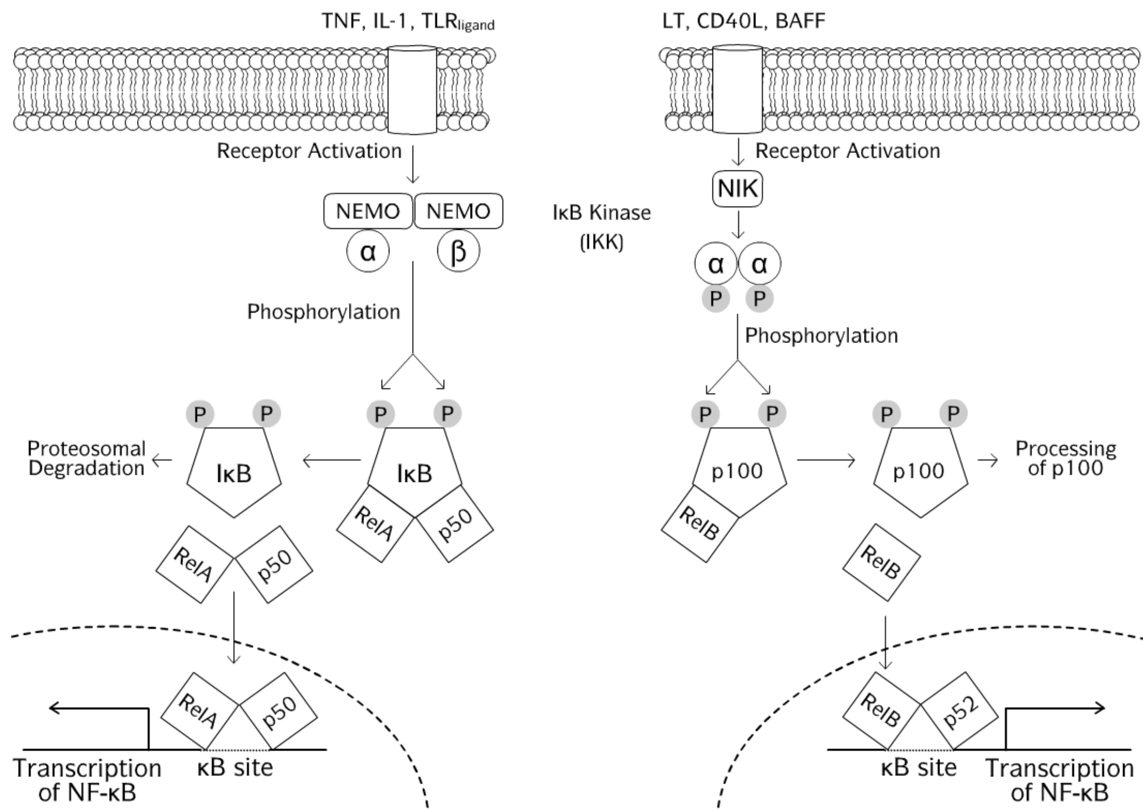


Fig. 1.3. Representative scheme of canonical and alternative NF-kB pathways. In the canonical pathway, TLRs activation by pro-inflammatory cytokines leads to the activation of RelA, through phosphorylation of IκB. RelA and p50 subunits translocate to the nucleus and regulate the expression of pro-inflammatory and cell survival genes. In the alternative pathway, LT, CD40L, BAFF, and RANKL, but not TNFα, results in the activation of RelB/p52 complexes, after phosphorylation and processing of p100, the precursor for p52. Activation of the alternative pathway regulates genes required for lymph-organogenesis and B-cell activation. (Adapted from (Lawrence 2009).

Regarding the role NF-κB plays in inflammation, therapeutic strategies have been developed to block NF-κB activity. For instance, the use of IKK-β-dominant-negative gene therapy, NF-κB decoy oligonucleotides or T-cell specific NF-κB inhibitors have proven to ameliorate adjuvant-induced arthritis, streptococcal cell wall–induced arthritis and collagen-induced arthritis, and inflammatory bowel disease symptoms (Tak and Firestein 2001).

1.6. Crosstalk between inflammation and excitotoxicity

The hippocampus is part of the limbic system which also includes the hypothalamus, thalamus, fornix, cingulate gyrus of the cerebral cortex and amygdala. This brain structure is an important center for memory regulation and processing, learning and emotional behavior.

The hippocampal formation is formed by several well defined cellular layers: the dentate gyrus (DG) and the *cornu ammonis* (CA), the subiculum (Sb) and fimbria. The CA is structurally divided in three main subfields – CA1, CA2 and CA3, while the DG contains the fascia dentate and the hilus (Purves et al. 2009). The DG receives input from the entorhinal cortex (EC) via the perforant path (PP), and then the DG granule cells project to the CA3 pyramidal neurons via mossy fibres axons (MF). CA3 pyramidal cells communicate ipsilaterally with CA1 pyramidal cells through the Schaffer Collateral Pathway (SC) and contralaterally via the Associational Commissural pathway (AC). CA1 neurons also receive input from the PP and complete the circuit sending axons to the subiculum which in turn projects to the EC. Hence, synaptic transmission in the hippocampus involves three main cellular groups: granule cells from the DG, pyramidal cells from the CA3 and from CA1 and, for that reason, is termed trisynaptic circuit (Amaral and Witter 1989; Purves et al. 2009).

Epileptogenesis can unfold in several brain regions. Nonetheless, most experimental models privilege the hippocampus because it has such well defined circuits, afferent and efferent pathways. Epileptogenesis designates a process by which the neural network develops recurring seizures, ultimately culminating in chronic epilepsy. During the epileptogenic process, alterations in the neuronal network at a physiological and structural level occur including cell death, increased excitation, altered inhibition, neuronal circuit reorganization, neurogenesis and aberrant mossy fiber formation (Morimoto et al. 2004; Bausch 2005).
In normal physiological conditions, hippocampal excitability is mediated by glutamatergic synapses. On the contrary, exacerbated glutamate release, the major excitatory neurotransmitter in the mammalian CNS, may induce neuronal damage/death in a process designated excitotoxicity. Excitotoxicity is associated to stroke, brain trauma, spinal cord injury, and neurodegenerative diseases such as multiple sclerosis (MS), Alzheimer's disease, Parkinson's disease, Huntington's disease and epilepsy. In response to damage, similarly to what occurs under epileptogenic treatments, collateral axon sprouting may develop. Mossy fiber sprouting is the aberrant projection of granule cell axons into supragranular layers of DG and within the hilus (de Lanerolle et al. 1989; Wenzel et al. 2000). The occurrence of mossy fiber sprouting is closely associated to the occurrence of spontaneous seizures supporting the idea that new mossy fiber synapses are mainly excitatory, thereby contributing to hippocampal excitability (Gorter et al. 2001; Zhang et al. 2002).
The reorganization of the hippocampal neuronal circuitry can be deeply altered by either enhancing existing excitatory connections or by forming new connections. These can create recurrent excitatory loops or amplify the response of the affected region contributing to the generation of epileptiform discharges.

Epilepsy combines a group of neurological disorders characterized by spontaneous and recurring seizures caused by neuronal hyperactivity. Epilepsy affects approximately 3% of the world population without distinguishing age, social status or gender (Morimoto et al. 2004).

One of the most common forms is temporal lobe epilepsy (TLE), characterized by complex partial seizures and for developing into a drug-resistant condition (Engel 1996).
To disclose the molecular and cellular alterations subjacent to the development of TLE, various animal models have been implemented. In experimental models of epilepsy, seizure activity triggers different pathways with opposite outcomes over neuronal replacement. Seizure activity can either stimulate neurogenesis, through injury-associated mechanisms, and axonal sprouting, or otherwise, cause neuronal cell death and the activation and proliferation of astrocytes and resident microglia (Morimoto et al. 2004). Hence, increasing the number of newborn neurons after seizures may be a compensatory mechanism to deal with the effects of injury (Parent 2002).

Most importantly, experimentally induced seizures in rodents trigger a massive inflammatory response in the brain. Activated microglia release pro-inflammatory cytokines (e. g. IL-1β, TNF-α, IL-6) creating an inhibitory environment for neurogenesis (Vezzani et al. 1999a; Jankowsky and Patterson 2001). The few neurons that survive this inflammatory process are particularly vulnerable to recurrent seizures (Ekdahl et al. 2003). However, differentiation of new neurons is stimulated by seizure activity itself (Parent et al. 1997).
Seizures also induce TLR expression in microglia which prompt the transcriptional activation of cytokines, chemokines, MHC class I and II and co-stimulatory molecules. This process could facilitate the presentation of antigenic peptides to infiltrating T lymphocytes. The inflammatory response is modulated by the production and release of pro-inflammatory molecules, anti-inflammatory mediators and binding proteins (Vezzani and Granata 2005).
The occurrence of generalized seizures is also believed to contribute to the increase of pro-inflammatory cytokine levels in the plasma of epileptic patients (Peltola et al. 2002; Lehtimäki et al. 2003; Hulkkonen et al. 2004; Bernardino et al. 2005). During epileptic seizures, the BBB becomes transiently disrupted near the epileptogenic focus enabling a bidirectional movement of monocytes. Permeability of the BBB to monocytes enables their activation in the brain and the initiation of a series of cytokine releasing cascades in the blood.

Insufficient clinical data has been gathered to establish a direct correlation between intracellular cytokine and chemokine levels in peripheral blood from TLE patients and seizure activity. Interestingly, some immunomodulatory drugs have anticonvulsant effects (e. g. adrenocorticotropic hormone) while some anti-epileptic drugs (e. g. valproate) have anti-inflammatory actions. Whether the immune/inflammatory challenge is initiated within the CNS (and the infiltration of blood-borne immune cells or circulating inflammatory mediators a consequent response to this intrinsic event) or whether the CNS is the target of an immune/inflammatory response that originates within peripheral lymphoid tissues remains unknown. Epileptic seizures alter significantly the expression pattern of several neurotransmitters, particularly in the hippocampus. Under pathological hyperactivity, neuropeptide Y acts as a fierce regulator of neuronal activity acting as pro- or anticonvulsant, depending on the receptor subtype activated (Vezzani et al. 1999b).

1.7. Neuropeptide Y – physiological role in the CNS

Many of the peptides known to be widely distributed in the CNS were actually discovered in non-neural tissues. Raising considerable attention is neuropeptide Y (NPY), a 36 amino-acid residue polypeptide involved in regulation of blood pressure, circadian rhythms, feeding behavior, anxiety, epilepsy, memory processing and cognition, pain and drug addiction (Silva et al. 2002). NPY is part of a larger family of peripheral and central peptides with a high degree of conservation which also includes peptide YY and pancreatic polypeptides. While NPY acts as a neurotransmitter and is mainly synthesized and released by neurons, peptide YY is found in intestinal endocrine cells and pancreatic polypeptide in pancreatic cells, acting as hormones (Lundberg et al. 1984; Larhammar 1996; Michel et al. 1998). These peptides share considerable amino-acid homology, have amidated C-terminal ends and a large number of tyrosine residues in their chemical structure. Structurally, the peptide family adopts a U-shape extended polyproline helix with an alpha helix connected by a type II beta turn, termed PP-fold (Larhammar 1996; Michel et al. 1998).

NPY synthesis involves a series of proteolytic alterations to the initial precursor peptide, termed pre-pro-NPY, which includes a hydrophobic signal peptide, the mature peptide, the amidation-proteolytic site and the C-flanking peptide of NPY (CPON). This large peptide is directed into the endoplasmic reticulum where the signal peptide is removed, generating pro-NPY by a signal peptidase. Cleavage of the precursor pro-NPY at the dibasic site by prohormone convertases further generates NPY(1–39) and CPON. Truncation at the C-terminal end by a carboxypeptidase forms NPY(1-37), a substrate for peptidylglycine α-amidating monooxygenase, which then results in biologically active amidated NPY(1-36). The amidated C-terminal of NPY prevents degradation by carboxypeptidases. However, mature NPY can be further processed by dipeptidyl peptidase IV and aminopeptidase P to generate NPY(3–36) and NPY(2–36), respectively (Cerda-Reverter and Larhammar 2000; Silva et al. 2002).

NPY exerts its functions through a group of receptor subtypes belonging to the G-protein-coupled receptor superfamily. From all known receptors, 5 have been cloned: Y_1, Y_2, Y_4, and Y_5, which represent fully-defined subtypes, as long as y_6, a truncated non-functional receptor (Y abbreviates for tyrosine). Since there is no physiological correlate for y_6, the International Union of Pharmacology recommends that capital Y should not be used to address this receptor subtype (Larhammar 1996; Michel et al. 1998). Y_1, Y_2, and Y_5 preferentially bind NPY and peptide YY, whereas Y_4 preferentially binds pancreatic polypeptides (Michel et al. 1998). Additionally, other receptor subtypes have been reported raising the question on whether these sequences represent species homologues or gene duplicates of the mammalian subtypes. The Y_3 subtype was postulated from pharmacological studies on mammalian tissues but does not seem to exist as a separate gene (Larhammar 1996). Overall, three subfamilies named after their first members, Y_1, Y_2 and Y_5 have been assembled. The Y_1 subfamily includes the mammalian subtypes Y_1, Y_4 and

y_6 receptors. Y_2 subfamily includes Y_7, found in zebrafish and in frogs (Fredriksson et al. 2004), whereas Y_5 stands alone.
Mammalian Y_1, Y_2 and Y_5 receptors are coupled to G proteins, specifically to $G_{i/o}$ protein subunits which inhibit cAMP formation, mobilize intracellular Ca^{2+}, and modulate K^+ channels (Michel 1991). Mobilization of intracellular calcium stores occurs *via* inositol phosphate dependent and independent pathways. Moreover, NPY acts through p44/42 MAPK signaling pathway in human erythroleukemia cells (Keffel et al. 1999). In addition, nitric oxide may be involved in the intracellular events activated by NPY (Fetissov et al. 2003).

The complex architecture of neural connections in the hippocampus can be challenging for the characterization of the existing cellular subpopulations. Due to the absence of more selective ligands and subtype-specific antibodies for NPY receptors it becomes difficult to investigate the amount and contribution of each specific Y-receptor subtype to hippocampal activity. Parker and Herzog were the first to compare mRNA expression of NPY receptors Y_1, Y_2, Y_4 and Y_5 in different regions of the rat hippocampus. For that purpose they analyzed consecutive sections - CA1, CA2, CA3 and DG, using highly sensitive *in situ* hybridization technique. CA3 pyramidal neurons exhibit the highest proportion of all receptor mRNAs, while Y_2 receptor subtypes are the predominant known Y-receptor subtype in the rat hippocampus. Y_4 receptor was found in several discrete areas of the rat hippocampus at low levels (Parker and Herzog 1999). In primary cultures of hippocampal neurons, the Y_1 receptor subtype was preferentially expressed over Y_2 receptor, determined by reverse transcriptase polymerase chain reaction (RT-PCR), binding assays and emulsion receptor autoradiography (St-Pierre et al. 1998).

Growing evidence suggest that NPY plays an important role in the immune system, particularly in the regulation of T cell responses in autoimmunity, where NPY appears as a putative therapeutic target. Although a role for the other receptors remains elusive, Y_1 receptor activation seems to suppress T cell activation (Wheway et al. 2007a). Macrophages release NPY, upon TLR activation, which can act in an autocrine fashion or by stimulating surrounding cells. Noteworthy, Y_1 receptor is expressed by B cells, T cells, dendritic dells (DCs) and macrophages. Y_1 receptor activation leads to the reduction of IFN-γ production ultimately inhibiting Th1 responses (Bedoui et al. 2003b; Prod'homme et al. 2006; Wheway et al. 2007a). Moreover, in the CNS, Y_1 receptors have been appointed as pro-convulsant receptors in experimental models of epilepsy (Vezzani et al. 1999b). Y_2 and Y_5 receptors are also involved in neuronal excitability and epilepsy (Woldbye et al. 1997; Baraban 2002; Guo et al. 2002; Richichi et al. 2004; Woldbye et al. 2005; Lin et al. 2006). Additionally, Y_2 receptor is involved in several physiological functions such as feeding behavior (Sainsbury et al. 2006), cardiovascular regulation (Malmstrom 2001), bone formation (Allison et al. 2007) and gastrointestinal motility (Fujimiya et al. 2000), to name a few. Y_5 receptors are implicated in food intake (Mashiko et al. 2007; Elbers et al. 2009), as well as in the regulation of circadian rhythms (Gamble et al. 2005).

In present day, NPY is a well-recognized neuropeptide of considerable importance to the regulation of CNS neurophysiology. In fact, our group has developed pioneering work uncovering the neuroprotective, antiepileptic and proneurogenic role of NPY (fig. 1.4.). In 2001, Silva and colleagues were the first to characterize, through single cell calcium imaging, the role of Y_1 and Y_2 receptors on $[Ca^{2+}]_i$ in cultured rat hippocampal neurons (Silva et al. 2001). Since then, authors have demonstrated that NPY is a key modulator of glutamate release and is neuroprotective against excitotoxicity in organotypic hippocampal slice cultures (Silva et al. 2003a; Silva et al. 2003b; Silva et al. 2005; Silva et al. 2007; Xapelli et al. 2007). Opening new perspectives for the development of cell-based brain therapy, NPY was shown to promote neurogenesis in the subventricular zone acting through Y_1 receptor (Agasse et al. 2008).

Since the initial work of Dureus and colleagues, suggesting a role for NPY in the regulation of human granulocyte and monocyte activation and chemotaxis (Dureus et al. 1993), increasing evidence have supported the relevance of this peptide in the immune system (Bedoui et al. 2003a; Bedoui et al. 2003b; Bedoui et al. 2004; Nave et al. 2004; Wheway et al. 2005; Prod'homme et al. 2006; Bedoui et al. 2007; Wheway et al. 2007a; Wheway et al. 2007b; Bedoui et al. 2008). Therefore, to fully comprehend the role of NPY in CNS/immune system communication, it is necessary to further characterize and dissect the anti-inflammatory properties of NPY.

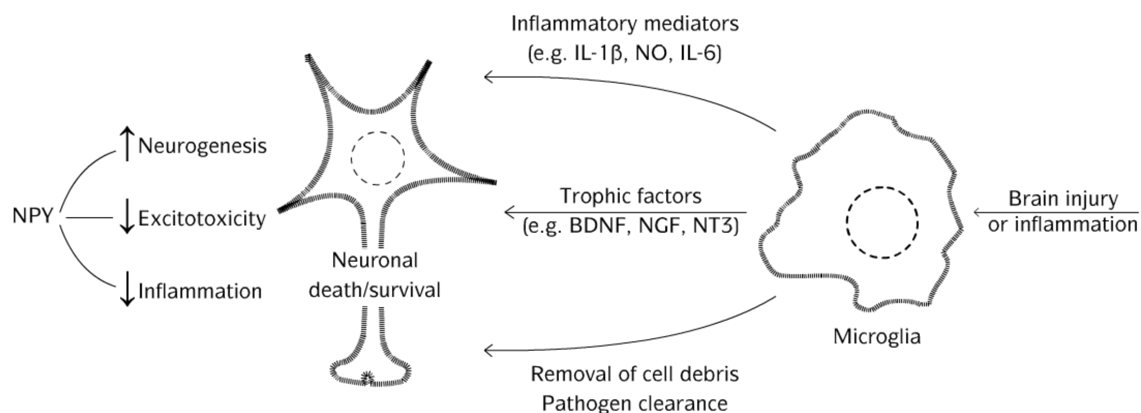


Fig. 1.4. Representative scheme illustrating the role of NPY in the CNS regarding the crosstalk between neurons and microglia. Upon brain injury or inflammation, microglia can clear cell debris, along with extracellular aggregates, and invading pathogens. Also, in an inflammatory context, microglia release both neurotoxic and trophic factors. Playing an important part on the final outcome on neuronal death/survival, NPY is able to promote neurogenesis and decrease the effects of excitotoxic and inflammatory insults. (Adapted from (Xapelli et al. 2006; Block et al. 2007).

1.8. OBJECTIVES

Our main objective was to disclose the mechanisms subjacent to the development of microglial cell responses to inflammation, using murine N9 microglial cell line as a biological model to study endotoxin-induced inflammation.

NPY plays a seminal role in the regulation and neuroprotection of brain cells. Given the mounting evidence that further support a modulatory role for NPY in the immune system, our objectives were set to investigate the effect of this neuropeptide in:

i) Production and subsequent release of inflammatory mediators such of NO and IL-1β by microglia, upon LPS challenge;
ii) Regulation of microglial cell motility;
iii) Modulation of cell phagocytosis.

Regarding each objective, we sought to disclose which receptors were involved in NPY actions, as well as which signaling pathways were implicated in the process.

CHAPTER 2. METHODOLOGY

2.1. Cell line culture

Murine N9 microglia cell line (kind gift from Prof. Claudia Verderio, CNR Institute of Neuroscience, Cellular and Molecular Pharmacology, Milan, Italy) was grown in RPMI medium supplemented with 30 mM glucose (Sigma, St. Louis, MO, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO, Invitrogen, Barcelona, Spain). Cells were kept at 37ºC in a 95% atmospheric air and 5% CO_2 humidified atmosphere. Number of viable cells was evaluated counting trypan blue-excluding cells. For immunocytochemistry studies, cells were plated at a density of $2x10^4$ cells *per* well in 24 well trays or were plated at a density of $5x10^5$ cells *per* well in 6 well trays (for remaining experiments).

Cell treatment included the following incubation setup: NPY (human, rat/amidated sequence) (1 µM) (Bachem, Bubendorf, Switzerland) for 6 or 24 hrs, LPS (from Escherichia coli 055:B5) (100 ng/ml) (Sigma) for 6 or 24 hrs, IL-1β (Escherichia coli-derived) (1.5 ng/ml) (R&D System, Minneapolis, MN, USA) for 15 min or 6 hrs, ATP (from bacterial source) (1 mM) (Sigma) for 30 min, Y_1 receptor agonist [Leu^{31},Pro^{34}]NPY (porcine, amidated sequence) (1 µM) (Bachem) for 6 or 24 hrs, Y_1 receptor antagonist BIBP3226 (1 µM, in water) (Bachem), Y_2 receptor antagonist BIIE0246 (1 µM, in 30% DMSO) (Tocris, Bristol, UK) and Y_5 receptor antagonist L152-804 (1 µM, in 100% DMSO) (Tocris), IL-1ra (150 ng/ml) (R&D Systems). For motility studies, cell treatments also included p38 MAPK inhibitor SB239063 (chemically synthesized) (20 µM) (Tocris, Bristol, UK). ATP, SB239063 and all receptor antagonists were added 30 min prior to cell treatment and maintained during the course of experiments.

2.2. RNA isolation from N9 microglial cells

The mRNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, cells were first lysed in a highly denaturing buffer containing guanidine thiocyanate which ensured the inactivation of RNases. Samples were applied to spin columns where total RNA bound to the membrane. Exclusion of contaminants and small size RNA allowed the purification of a high-quality mRNA enriched solution. RNA samples were stored in DEPC-treated water (Sigma) at -80ºC prior to quantification by optical density (OD) measurement at 260 nm (RNA/DNA calculator GeneQuant II, Amersham Pharmacia Biotech, Uppsala, Sweden). Purity and integrity of the samples were determined using the ratio OD_{260}/OD_{280} (only samples whose ratios were between 1.7 and 2.2 were transcribed), and by visual confirmation on the agarose gel.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of NPY and NPY receptors expression in N9 microglial cells

A total of 2 μg of mRNA extracted was transcribed using 10 U/μl Reverase reverse transcriptase (Bioron GmbH, Ludwigshafen, Germany) and 0.05 μg/μl Oligo-p(dT)15 primers (Bioron GmbH). Amplification of NPY, NPY receptors and β-actin was performed in a 50 μl reaction system (Bioron GmbH) containing 5 μl of template cDNA, 5 μl of 10X PCR reaction buffer, 10 mM deoxynucleotide mix, 0.2 μM of upstream and downstream primers, 5000 U/mL of Taq DNA polymerase (Amersham Biosciences, Buckinghamshire, UK) and RNase-free water.
Primer sequences were as follows: NPY forward 5'-AGA GAT CCA GCC CTG AGA CA-3'; NPY reverse 5'-AAC GAC AAC AAG GGA AAT GG-3'; Y_1 receptor forward 5'-AAC CTC TCC TTC TCA GAC TTG C-3'; Y_1 receptor reverse 5'-CAC AGT GTT GAA GAT GGT AAG G-3'; Y_2 receptor forward 5'-CTC CAA GCA AAT CAG CTT CC-3'; Y_2 receptor reverse 5'-GTT TTG TGC CTT CGC TGA TGG-3'; Y_5 receptor forward 5'-GTG TTC CCG AGG TGC TTC TA-3'; Y_5 receptor reverse 5'-ATT CCG AGC AGC AGC TGT AT-3'. Amplicons for NPY (236 bp), for Y_1 receptor (615 bp), for Y_2 receptor (318 bp), for Y_5 receptor (524 bp) and for β-actin (428 bp) were run in a 1.5% agarose gel stained with ethidium bromide for visual confirmation. Densitometrical analysis for the evaluation of mRNA expression of NPY and NPY receptors was performed on Versa-Doc Imaging System (Model 3000, BioRad Laboratories, CA, USA).

2.4. Griess Assay

Production of NO was determined through the formation and accumulation of its stable metabolite product, nitrite (NO_2^-). Cells were incubated with lysis cocktail solution (137 mM NaCl, 20 mM Tris-HCl, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM sodium vanadate (all from Sigma), pH 8.0). After gentle homogenisation, total amount of protein was quantified using the BioRad Method. Standard solution of 10 mM $NaNO_2$ (Sigma) was diluted to concentrations ranging from 1 μM to 100 μM and applied in duplicates to 96 multiwell EIA/RIA plates (Costar, Corning Incorporated, NY, USA). Griess reagents were added (1:1) to each well: 0.1% N-1-naphthylenediamine dihydrochloride, and 1% sulphanilamide in 5% phosphoric acid (all from Sigma). Under acidic conditions, in the presence of nitrite, a pink chromophoric azo compound is produced (protocol adapted from Huygen (Huygen 1970), originally reported by Griess, 1879). Optical density was recorded at 540 nm in an ELISA plate reader (SPECTRA max 384 Plus, Molecular Devices, CA, USA).

2.5. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (PFA) (Sigma) and then placed for 20 min in permeabilizing solution (0.3% bovine serum albumin (BSA) and 3% Triton X-100 (all from Sigma)). Unspecific binding was prevented by incubating cells in a 3% BSA and 0.3% Triton X-

100 solution for 30 min, at RT. Cells were kept overnight at 4°C in a primary antibody solution, then washed with PBS, and incubated for 1 hr at RT with the corresponding secondary antibody. Antibodies were used as listed: rabbit polyclonal anti-NPY (1:1000) (Sigma); sheep polyclonal anti-Y_1R (1:1000) (AbD Serotec, Oxfordshire, UK); rabbit monoclonal anti-phosphorylated p38 (1:1000) (Cell Signaling, Tech, Beverly, MA, USA); rabbit monoclonal anti-iNOS (1:250) (Millipore Corporation Bedford, MA, USA); rat monoclonal anti-CD11b (1:1000) (AbD Serotec); rabbit monoclonal anti-NF-κB p65 (1:100) (Santa Cruz Biotechnology, Inc., California, USA) in 0.1% Triton X-100, 0.3% BSA solution; Alexa Fluor 594 goat anti-rabbit; Alexa Fluor 594 donkey anti-sheep, Alexa Fluor 594 goat anti-rat; Alexa Fluor 488 donkey anti-rabbit; Alexa Fluor 488 goat anti-rat (all 1:200 in PBS, from Molecular Probes).
Membrane ruffling was observed using a marker for filamentous actin, phalloidin. Cells were incubated for 2 hrs in phalloidin-Alexa Fluor 594 conjugate (1 U/ml) (Molecular Probes) in PBS, at RT.
For nuclear labelling, cell preparations were stained with Hoechst 33342 (2 μg/ml) (Molecular Probes, Eugene, Oregon, USA) in 0.3% BSA, for 5 min at RT and mounted in Dakocytomation fluorescent medium (Dakocytomation Inc., California, USA). Fluorescent images were acquired using a confocal microscope (LSM 510 Meta, Carl Zeiss, Göttingen, Germany).

2.6. Nuclear and cytosolic extracts

After cell treatment with 1.5 ng/ml IL-1β and/or 1 μM NPY, cells were lysed and collected in 500 μl of buffer 1 (10 mM HEPES, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% Triton X-100, 1 mM EGTA, 0.1% chymostatin, 0.1% leupeptin, 0.1% antipain, and 0.1% pepstatin (all from Sigma), pH 7.5). Samples were centrifuged for 12 min at 2300 g at 4ºC, and the supernatant corresponding to the cytosolic extract collected. Pellets were resuspended in 30 μl of buffer 2 (25 mM HEPES, 300 mM NaCl, 5 mM $MgCl_2$, 20% glycerol, 1 mM EGTA, 0.1% chymostatin, 0.1% leupeptin, 0.1% antipain, and 0.1% pepstatin (all from Sigma), pH 7.5), centrifuged at 10,600 g for 20 min, at 4ºC, and the supernatant corresponding to the nuclear extract collected. Protocol was adapted from Santos and colleagues (Santos et al. 2001). Total protein from each sample was quantified using the BioRad method.

2.7. Western Blotting

Total protein from cell lysates (prepared as described in section Griess Assay) was quantified using the BioRad Method. Afterwards, samples were loaded onto 10% acrylamide/bisacrilamide gels (BioRad, Hercules, CA, USA) (for NPY and NF-κB p65 detection the percentage of acrylamide/bisacrilamide used was 15%). Proteins were separated by SDS-PAGE using a bicine/SDS (Sigma) electrophoresis buffer (pH 8.3) and then transferred to PVDF membranes (Millipore) with 0.2 μm pore size for NPY and 0.45 μm pore size for remaining proteins under the following conditions: 300 mA, 90 min at 4ºC in a solution containing 10 mM CAPS (Sigma) and 10% methanol (VWR International S.A.S. France), pH 11.0) (protocol adapted from

Pinheiro and collaborators (Pinheiro et al. 2005)). Membranes were blocked in Tris-buffer saline containing 5% low-fat milk and 0.1% Tween® 20 (Sigma) for 1 hr, at RT, and then incubated overnight at 4ºC with the primary antibody solution diluted in 1% TBS-Tween, 0.5% low fat milk. For the detection of phosphorylated proteins, membranes were blocked in Tris-buffer saline containing 5% BSA 0.1% Tween® 20 (Sigma) for 1 hr, at RT, and then incubated overnight at 4ºC with the primary antibody solution diluted in 1% TBS-Tween, 5% BSA.

The following primary antibodies were used: rabbit monoclonal anti-iNOS (1:1,000) (BD Transduction); rabbit monoclonal anti-NF-κB p65 (1:100) (Santa Cruz Biotechnology, Inc); rabbit polyclonal anti-NPY (1:100) (Sigma); sheep polyclonal anti-Y_1R (1:10,000) (AbD Serotec); rabbit monoclonal anti-phosphorylated p38 (1:1000) and rabbit polyclonal anti-total p38 (1:1000) (both from Cell Signaling). After rinsing three times with TBS-T, membranes were incubated for 1 h at RT with an alkaline phosphatase-linked secondary antibody anti-rabbit IgG (1: 20,000), and anti-sheep IgG (1: 1,000), in 1% TBS-T 0.5% low-fat milk (GE Healthcare UK Limited, Buckinghamshire, UK). Regarding the identification of phosphorylated proteins, after rinsing three times with TBS-T, membranes were incubated for 1 hr at RT with an alkaline phosphatase-linked secondary antibody anti-rabbit IgG 1: 20,000, in 5% BSA 0.1% Tween® 20 (Sigma) 1% TBS-T (GE Healthcare UK Limited, Buckinghamshire, UK).

For endogenous control immnunolabelling, primary antibody solutions consisted of mouse monoclonal anti-α-tubulin (1:10,000) and rabbit monoclonal anti-histone (1:10,000) (Millipore, MA, USA). Protein immunoreactive bands were visualized in a Versa-Doc Imaging System (Model 3000, BioRad Laboratories, CA, USA), after incubation of the membrane with ECF reagent (GE Healthcare UK Limited) for 5 min.

2.8. Enzyme-linked immunosorbent assay (ELISA) for IL-1β

Cells were plated and treated with NPY as previously described (see cell line culture section). For the quantification of IL-1β protein levels a mouse IL-1β ELISA Kit was used following the manufacturer's instructions (eBioscience, San Diego, CA, USA). Cells were left at RT for 5 min in lysis buffer (137 mM NaCl, 20 mM Tris-HCl, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 1 µg/ml leupeptin, 0.5 mM sodium orthovanadate (all from Sigma), pH 8.0). Total protein concentration was determined by the Bicinchoninic acid method (BCA) and samples were stored at -80ºC.

Microtiter plates (MaxiSorp, Nunc A/S, Roskilde, Denmark) were coated with 100 µl/well of capture antibody in coating buffer. Plates were sealed and left overnight at 4ºC. Wells were washed, blocked with 1X Assay Diluent and left at RT for 1 hr. After washing, 100 µl of each sample was added, as well as standard solutions, after performing 2-fold serial dilutions of the top standard. The plate was sealed and left incubating for 2 hrs at RT. Afterwards, 100 µl/well of detection antibody diluted in 1X Assay Diluent was added, the plate was sealed and incubated at RT for 1 hr. Washes were repeated and 100 µl/well of Avidin-HRP diluted in 1X Assay Diluent was added. Then the plate was sealed and kept at RT for 30 min. Wells were soaked in washing buffer for 5 min prior to aspiration and 100 µl/well of Substrate Solution was added to each well

and incubated at RT for 15 min. Afterwards, 50 µl/well of Stop Solution was added. Optical density was recorded at 450 nm and at 570 nm (values later subtracted to those obtained with 450 nm) in an ELISA plate (SPECTRA max 384 Plus, Molecular Devices, CA, USA).

2.9. Motility assay

Before cell seeding, two parallel lines were carved on the underside of each well with a scalpel. These lines served as a guidance axis together with the line given by the scratch wound. Cell monolayer was approximately 95% confluent before the motility assay took place. One hour before performing the wound, the medium was replaced by serum-free medium to ensure no proliferation occurred in order to compromise final results. Wound was made by a perpendicular scratch made with a P10 pipette tip (Gilson S.A.S., Villiers-le-Bel, France). After cell treatment, images were taken with an inverted Axiovert 200 microscope (Carl Zeiss), with a 5x objective and a CoolSNAP digital camera (Roper Scientific, Tucson, AZ, USA). Differential interference contrast (DIC) images were acquired using MetaFluor Software (Universal Imaging, Downingtown, PA, USA) and analyzed with NIH ImageJ Software. Cell migration was determined counting the number of cells that migrated towards the middle of the wound within a 12 hr period of treatment. Protocol adapted from Valster and colleagues (2005) (Valster et al. 2005).

2.10. Bead phagocytosis assay

Beads were opsonised with goat serum (1 µg/ml) (Vector Laboratories Inc., Burlingame, CA, USA), under constant agitation at 8 rpm, overnight at 4ºC. Beads were then resuspended in previously heated growth medium and distributed by each well at a density of $5x10^5$ beads *per* well. After 30 min of incubation, cells were washed with PBS and fixated with 4% paraformaldehyde (PFA). Beads were labelled with secondary antibody Alexa Fluor 594 donkey anti-goat (Molecular Probes, Oregon, USA), 1: 200, in PBS. Only negative-labelled beads were considered as internalised. For nuclear labelling, cell preparations were stained with Hoechst 33342 (2 µg/ml) (Molecular Probes) in 0.3% BSA, 0.1% Triton-X100 solution, for 5 min at room temperature (RT) and mounted in Dakocytomation fluorescent medium (Dakocytomation Inc., California, USA). Fluorescent images were acquired using a confocal microscope (LSM 510 Meta, Carl Zeiss, Göttingen, Germany).

2.11. Data analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Statistical significance was considered relevant for p values < 0.05 using one-way analysis of variance followed by Bonferroni *post hoc* test for comparison among experimental settings and Dunnett *post hoc* test for comparison with control condition. In western blotting studies for NPY and Y_1R, statistical significance was determined using unpaired one-tailed Student's t test.

Data were presented as means ± standard error of mean (SEM). For every immunocytochemistry analysis, 5 independent microscopy fields were acquired, *per* coverslip, with a 40x objective (about 40 cells *per* field). For the migration assay, 4 images *per* experimental condition were acquired. Every experimental condition was tested in three sets of independent experiments, unless stated otherwise, and performed in duplicates.

CHAPTER 3. Neuropeptide Y modulation of interleukin-1β (IL-1β)-induced nitric oxide production in microglia

3.1. Introduction

Brain inflammation is characterised primarily by microglia activation (Streit et al. 1999). This process, which is accompanied by significant morphological changes (Garden and Möller 2006), is triggered by several stimuli such as adenosine triphosphate (ATP) (Inoue 1998), blood-derived factors or microbial signals (e.g. lipopolysaccharide (LPS)). One of the outcomes of microglia activation is the production of nitric oxide (NO) from the conversion of L-arginine to L-citrulline by Ca^{2+}-independent inducible nitric oxide synthase (iNOS) (fig. 3.1.) (Palmer et al. 1988; Knowles and Moncada 1994; Moncada and Bolaños 2006).

NO, which is mainly known as a vasodilator, is of particular importance for blood flow regulation, sleep-wake cycle, food intake and thermal regulation, immune system function and neuronal transmission (Calabrese et al. 2007). Particularly, in the Central Nervous System (CNS), NO regulation presents itself as an opportunity to intervene therapeutically in human health.

3.1.1. Nitric oxide biosynthesis

NO diffuses through the cell membrane of presynaptic axons to neighboring cells where it stimulates the production of cyclic guanosine monophosphate (cGMP), a secondary messenger. NO, and L-citrulline, is generated from L-arginine in a chemical reaction catalysed by nitric oxide synthase (NOS). Hence, NO synthesis is dependent on the availability and metabolism of L-arginine. Dioxygen and nicotinamide-adenine dinucleotide phosphate (NADPH) function as co-substrates and tetrahydropteridin, flavin adenine dinucleotide, flavin mononucleotide, thiol and heme are used as co-factors (fig. 3.1.) (Purves et al. 2009).

NO synthase is present in three isoforms: NOS1 (or neuronal NOS), NOS2 (also known as inducible NOS or iNOS) and NOS3 (endothelial NOS) (Knowles and Moncada 1994; Moncada and Bolaños 2006; Calabrese et al. 2007). NOS1 and NOS3 are expressed constitutively under normal conditions and their activation is triggered by a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). When $[Ca^{2+}]_i$ is elevated (e.g. by acetylcholine or bradykinin), these isoforms bind to calmodulin forming tetramers, and can bind to other co-substrates in the N-terminal domain of NOS. Interestingly, NO has the ability to regulate its own synthesis by forming a stable ferrous-nitrosyl complex with NOS, preventing synthesis *de novo*. The activity and subcellular distribution of NOS is also affected by the degree of phosphorylation achieved through specific kinases. Nonetheless, there is a catalytically active NOS, which is non-phosphorylated, bound to plasma membrane, responsible for the release of NO to the extracellular space (Bredt and Snyder 1994). Contrary to other constitutive isoforms, iNOS can act independently of $[Ca^{2+}]_i$ because it already binds tightly to calmodulin. Hence, in the presence of L-arginine and cofactors, it can produce a continuous flow of NO (Cho et al. 1992).

The presence of interferon-γ (IFN-γ), interleukin-1β (IL-1β), tumour necrosis factor- α (TNF-α) and lipopolysaccharide (LPS) increases mRNA transcription coding for iNOS (Murphy 2000). This process can be conducted through the activation of several signaling cascades involving protein kinases and phosphatases.

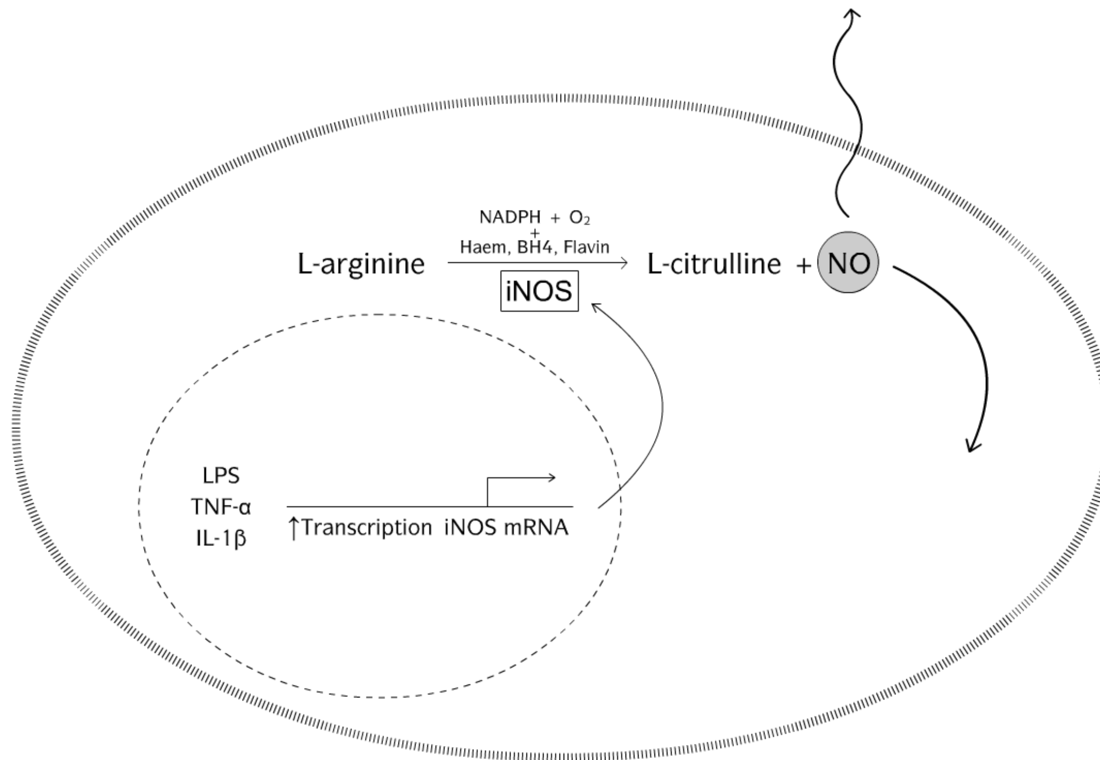


Figure 3.1. Scheme illustrating NO synthesis. Upon inflammatory challenge, mRNA transcription for inducible nitric oxide synthase (iNOS) is up-regulated. Nitric oxide and L-citrulline are generated from L-arginine, in the presence of oxygen (O_2) and nicotinamide adenine dinucleotide phosphatase oxidase (NADPH), as well as co-factors like flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), haem and tetrahydrobiopterin (BH4). NO diffuses within the cell or to the extracellular space, where it can act in an autocrine or paracrine manner. Adapted from (Calabrese et al. 2007).

Although NO is produced by several cells of CNS, most of the work dissecting the intracellular signaling pathways that lead to the transcriptional activation of NOS has been developed in astrocytes. Both p38 and extracellular regulated protein regulated kinase (ERK) from the mitogen-activated protein kinase (MAPK) family are involved in the regulation of iNOS activation in rat astrocytes and microglia (Bhat et al. 1998; Bhat et al. 2002). On the other hand, inhibition of p38 and c-Jun N-terminal kinase (JNK) prevented IL-1β induction of iNOS in human fetal astrocytes (Hua et al. 2002).

Moreover, the inhibition of LPS-and cytokine-mediated expression of iNOS by tyrosine kinase inhibitors has been long ago described (Feinstein et al. 1994; Kong et al. 1996). The tyrosine kinase family includes Janus kinase (JAK) family, src family, mitogen-activated protein kinase kinase (MAPKK) family, and receptor-linked tyrosine kinase family. Actually, IFN-γ induces tyrosine phosphorylation of signaling transducer and activator of transcription-1α (STAT1α)

but not STAT1β via JAK2 and that tyrosine phosphorylation of STAT1α seems to be essential for IFN-γ-induced expression of iNOS in glial cells (Dell'Albani et al. 2001). Also, inhibiting IFN-γ-induced JAK/STAT1 signaling, the administration of vasoactive intestinal peptide and the pituitary adenylate cyclase activating polypeptide compromised iNOS expression (Delgado 2003). More recently, protopanaxatriol-type ginsenoside Rh1 (Rh1) was shown to inhibit NO, reactive oxygen species (ROS), and TNF-α production in IFN-γ-stimulated microglia. Rh1 inhibited DNA binding of several transcription factors, such as NF-κB, IRF-1, and STAT1, as well as inhibited the phosphorylation of JAK1, STAT1, STAT3, and ERK, thereby inhibiting iNOS gene expression (Jung et al. 2010).

Protein kinase A (PKA) is part of a protein kinase cascade that couples a number of extracellular signals to variety of cellular functions. Along with pro-inflammatory cytokines such as TNF-α, prostaglandin E2 (PGE2) may activate the cyclic adenosine monophosphate (cAMP)/PKA cascade, triggering p38MAPK and inositol triphosphate (IP3) IP3/Ca^{2+} signaling. The latter activates cAMP response element-binding (CREB) directly or *via* protein kinase C (PKC) activation resulting in increased expression of iNOS in astrocytes (Hsiao et al. 2007). Chio and colleagues have also demonstrated that PKA activation in macrophages stimulates PKC and p38 MAPK, inducing NF-κB activation and, consequently, iNOS and IL-6 genes (Chio et al. 2004).

3.1.2. Nitric oxide in health and disease

Nitric oxide (NO) is now considered a neurotransmitter to the mammalian brain. NO stimulates the release of acetylcholine through the stimulation of neighboring glutamatergic neurons (Prast et al. 1998; Lydic et al. 2006). In addition, NO can modulate the release of γ-aminobutyric acid (GABA) (Getting et al. 1996; Ohkuma et al. 1996; Saransaari and Oja 2006), noradrenaline (Feleder et al. 2007), glutamate (Lonart et al. 1992; Bal-Price and Brown 2001), dopamine (Kaehler et al. 1999; Hull and Dominguez 2006; Di Matteo et al. 2010) and serotonin (Kaehler et al. 1999; Iuras et al. 2005). NO has been implicated also in the regulation of synaptic plasticity, in cognitive processes such as memory. NO can modulate long-term depression or long-term potentiation depending on whether it acts post- or presynaptically, respectively (Bon and Garthwaite 2003).

Moreover, NO can grant neuroprotection through the following mechanisms: reduction of Ca^{2+} influx, due to *S*-nitrosylation of caspase 3 and NR1 and NR2 subunits of the *N*-methyl-D-aspartate receptors (NMDAR), which leads to a decrease of cell death; activation of cyclic-AMP-responsive-element-binding protein and Akt *via* stimulation of soluble guanylate cyclase–cyclic GMP–protein kinase G pathway; generation of biliverdin, a precursor of bilirubin, which acts as an antioxidant and anti-nitrosive molecule, through the induction of the activity of haem oxygenase 1 (Calabrese et al. 2007).

However, NO can act as a pathophysiological agent since it is associated to hypertension, diabetes, heart failure, among other pathologies (Moncada and Bolaños 2006). In the CNS, high amounts of NO inhibit mitochondrial cytochrome oxidase in neurons, causing them to depolarize and to release glutamate, and ultimately to die by excitotoxicity *via* NMDAR (Bal-

Price and Brown 2001; Brown 2007). NO can also react with superoxide anions and form peroxynitrite, which detains strong oxidant properties, and can damage cellular components when protein nitration takes place (Calabrese et al. 2007).

In the CNS, NO production is associated to neurotransmission, regulation of food intake, sleep-wake cycle, body temperature, neuroprotection/neurotoxicity, spanning its role to the periphery where NO regulates smooth-muscle relaxation (Calabrese et al. 2007). Given the ample involvement of neuropeptide Y (NPY) in the regulation and neuroprotection of brain cells (Silva et al. 2003a; Agasse et al. 2008; Xapelli et al. 2008), we sought to disclose, in the present chapter, the anti-inflammatory properties of NPY in order to provide therapeutic targets for the prevention of neurological dysfunctions in several CNS injuries and chronic diseases, such as epilepsy, ischemia, stroke, Alzheimer's disease, or multiple sclerosis (Danton and Dietrich 2003; Garden and Möller 2006; Choi and Koh 2008; Amantea et al. 2009; Koning et al. 2009; Perry et al. 2010).

3.2. RESULTS

3.2.1. Expression of NPY and Y_1 receptor increase in murine N9 microglia cell line upon LPS-induced inflammation

Murine N9 microglia cell line was used as a biological model to study endotoxin-induced inflammation. Firstly, we performed conventional polymerase chain reaction (PCR) as a qualitative approach to identify the expression of NPY and NPY receptors. We amplified cDNA coding for NPY, Y_1 receptor (Y_1R), Y_2 receptor (Y_2R) and Y_5 receptor (Y_5R). In order to have a semi-quantitative analysis, β-actin was used as an endogenous control given its stable expression in every experimental condition (Bustin 2000). Mouse hippocampal samples were used as a positive control, since the hippocampus is a brain region known to highly express NPY and its receptors Y_1, Y_2 and Y_5 (de Quidt and Emson 1986a; de Quidt and Emson 1986b; Naveilhan et al. 1998). As a negative control, we used samples from negative transcription reactions (no template controls). Before RNA extraction, cells were treated with 1 μM NPY and challenged with 100 ng/ml LPS for 24 hrs. LPS is a key element of the outer membrane of Gram-negative bacteria which binds to CD14/TLR4/MD2 receptor complex, promoting the secretion of pro-inflammatory cytokines and the activation of several signalling cascades (Cohen 2002). N9 microglial cell line did not abundantly express NPY in control conditions, although there was a significant increase of NPY cDNA when cells were treated with 100 ng/ml of LPS ($p < 0.001$, n = 3) (fig. 3.2. A). Moreover, NPY treatment inhibited the described LPS effect ($p < 0.01$, n = 3) (fig. 3.2. A). Y_1R, Y_2R and Y_5R were detected in N9 microglia cell line, and a significant increase in Y_1R expression was observed upon LPS challenge ($p < 0.05$, n = 3) (fig. 3.2. B), while no significant differences were obtained for Y_2 R (fig. 3.2. C) or for Y_5 R (fig. 3.2. D). Furthermore, NPY treatment caused a significant decrease in Y_1R cDNA copies, when challenged with LPS, as compared to LPS alone ($p < 0.05$) (fig. 3.2. B). This decrease did not differ significantly from control levels.

To determine if the differences observed in cDNA levels translated into significant alterations of the pattern of protein expression, we performed immunocytochemistry for NPY and Y_1R. To visualise microglia morphology we labelled the alpha chain of $\alpha_M\beta_2$-integrin, CD11b, a well known surface marker, closely associated to microglial activation, and a mediator of the diapedesis process of leukocytes through the endothelium (Vetvicka et al. 1999). As expected, LPS treatment led to an altered cell morphology shown by an increase of CD11b expression and a bloated cell body (fig. 3.3. A, LPS). Furthermore, we observed an increase in NPY labelling (fig. 2 A, top panel) and in both Y_1R signal and distribution (fig. 3.3. A, bottom panel) induced by LPS. In addition, by western blotting analysis we could observe a significant increase in NPY (4 kDa) and Y_1R (44 kDa) protein levels ($mean_{NPY} = 135.5 \pm 12.25\%$; $p < 0.05$, n = 6; $mean_{Y1R} = 150.20 \pm 18.74\%$; n = 4) after LPS challenge (fig. 3.3. B).

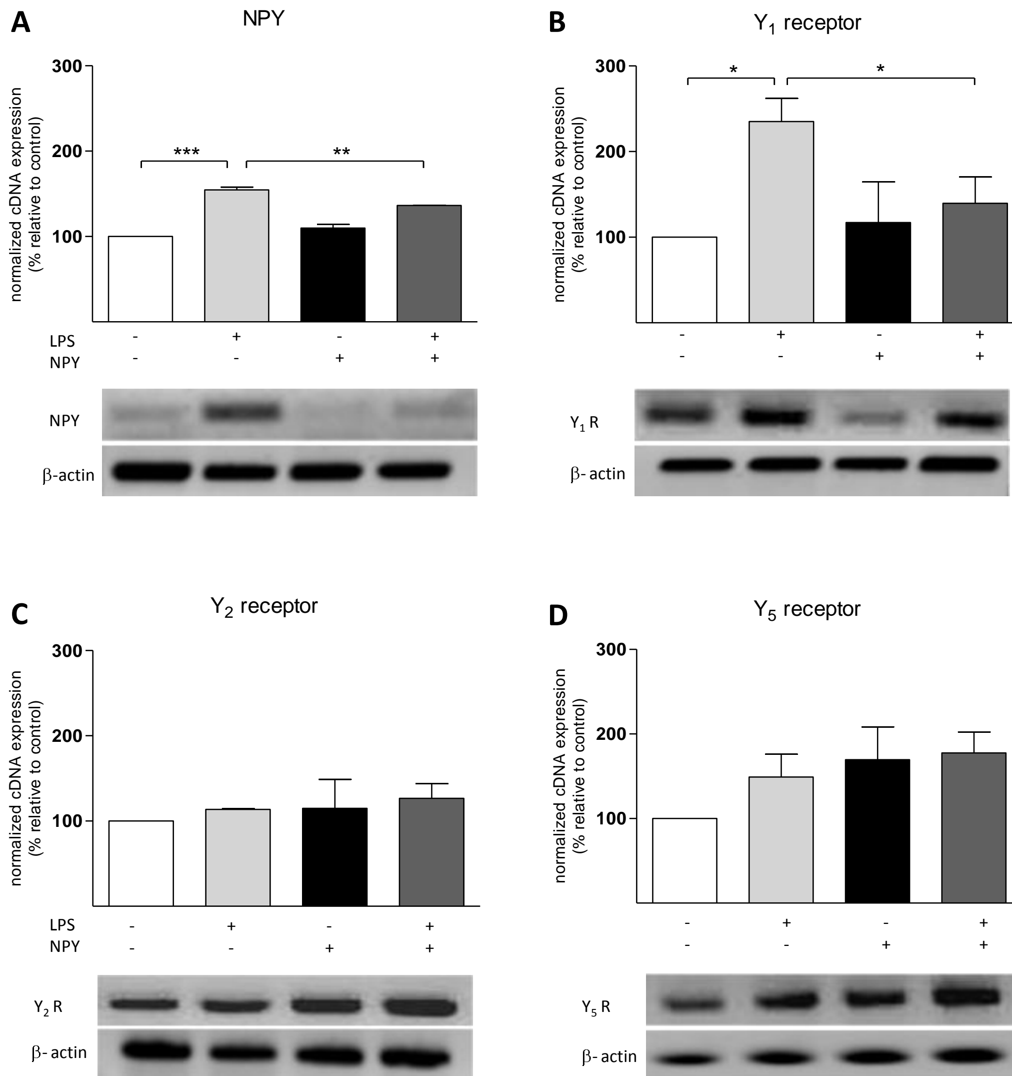


Fig. 3.2. Murine N9 microglial cell line expresses NPY and receptors Y1, Y2 and Y5. RT-PCR detection of amplified products for: (A) NPY (236 bp), (B) Y1R (616 bp), (C) Y2R (318 bp) and (D) Y5R (524 bp). Cells were treated with 1 μM NPY and challenged with 100 ng/ml LPS for 24 hrs. For semi-quantitative analysis, results were normalized to β-actin (428 bp), an endogenous control. LPS-stimulated microglia significantly expressed higher levels of Y1R and NPY cDNA copies. Representative agarose gels for each amplified PCR product are depicted below the respective graph. Data are expressed as mean ± SEM (n=3) and as a percentage of control (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, using Bonferroni's Multiple Comparison Test).

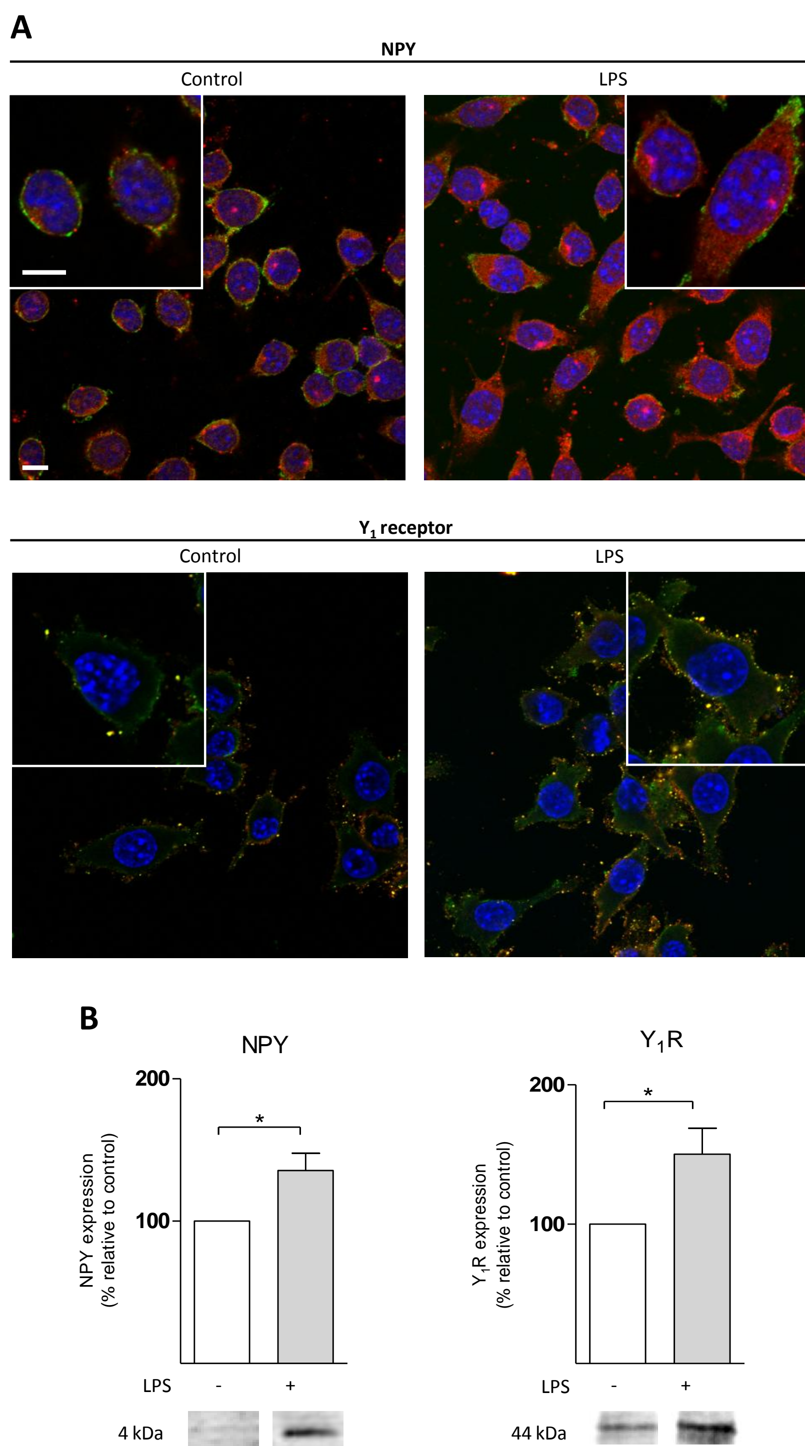


Fig. 3.3. LPS induces NPY and Y1 receptor expression. (A) Confocal microscopy photomicrographs show NPY and Y1R localisation (in red) on microglial cells (green) under basal conditions (control) and after 100 ng/ml LPS challenge for 24 hrs (LPS). Microglial cells stimulated with LPS exhibited an activated phenotype and expressed higher levels of both NPY and Y1R. Cell morphology was visualised with CD11b labelling (in green) and nuclear morphology is shown with Hoechst 33342 staining (in blue). Scale bar 10 µm. (B) Western blotting analysis of LPS stimulatory effect on NPY (4 kDa) and Y1R (44 kDa). After LPS challenge, an increase in NPY and Y1R protein levels was observed. A representative blot is shown below each graph. Data are expressed as mean ± SEM (n=6 for NPY and n=4 for Y1R) and as a percentage of control (*$p < 0.05$; using Student's t test for comparison with control).

3.2.2. NPY prevents the production of NO and decreases iNOS expression after LPS stimulation

Activation of microglia by inflammatory stimuli such as pathogens, adhesion molecules and cytokines, leads to the expression of high levels of nitric oxide synthase (NOS), with consequent increasing levels of NO (Moss and Bates 2001; Liu et al. 2002). Using the Griess assay, we quantified NO production by microglial cells after 100 ng/ml LPS incubation for 24 hrs (fig. 3.4.). LPS-stimulated cells produced significantly more NO than control cells ($mean_{CTR}$ = 100%; $mean_{LPS}$ = 280.65±50.38%) and this effect was reverted in the presence of NPY (1 μM) ($mean_{NPY}$ = 98.88±7.41%; $mean_{LPS+NPY}$ = 101.85±6.59%; $p < 0.01$, n = 3) (fig. 3.4. A). These results suggested that NO production stimulated by LPS was inhibited by NPY treatment. To discard any contribution from endogenous NPY, we treated cells with the monoclonal antibody NPY-05 (6 μg/ml) which acts as a NPY scavenger by binding to the carboxyl terminal of this peptide (Brakch et al. 2002). As expected, nitrite levels after NPY-05 treatment were similar to control ($mean_{NPY05}$ =109.89%, n = 3) and significantly different from those obtained with LPS challenge alone ($p < 0.001$). In the presence of NPY-05 and LPS, nitrite production was similar to LPS alone ($mean_{LPS}$ = 264.52±7.27%; $mean_{LPS+NPY05}$ = 256.14±10.13%; n = 3), indicating that, in our experimental conditions, endogenous NPY did not play any role in NO inhibition (fig. 3.4. B). The efficacy of the neutralizing antibody was determined by performing a concentration-response curve with increasing concentrations of NPY-05 (ranging from 60 ng/ml to 6 μg/ml) in the presence of LPS and NPY.

To determine how NPY exposure was involved in the inhibition of NO production, we tested whether NPY was affecting the synthesis of the converting enzyme iNOS, the isoform present in microglia. By western blotting, we observed that LPS significantly induced an increase in iNOS protein levels ($mean_{LPS}$ = 167.86±10.43%; n = 3, $p < 0.001$) and that this effect was abolished by NPY ($mean_{LPS+NPY}$ = 116.47±10.13%; $p < 0.01$, n = 3) (fig. 3.5. A). Moreover, we also performed immunocytochemistry in the same experimental conditions (fig. 3.5. B), and we observed that cells treated with NPY alone (fig. 3.5., i-l) displayed a weak labelling signal for iNOS and CD11b that was similar to control conditions (fig. 3.5., a-d). The strongest fluorescent signal was observed when cells were challenged with LPS (fig. 3.5., e-h), furthermore a moderate effect was visualised when cells were treated with both NPY and LPS (fig. 3.5., m-p).

3.2.3. Y_1 receptor activation mimics the effect of NPY on NO production

To further characterize the action of NPY over the inhibition of NO production, we aimed at determining which NPY receptor(s) could be involved. For that purpose we started by incubating cells with a selective agonist for Y_1 receptor, $[Leu^{31},Pro^{34}]$NPY (1μM) for 24 hrs. Microglial cells treated with $[Leu^{31},Pro^{34}]$NPY and LPS ($mean_{LPS+Leu31,\ Pro34}$ = 109.14±9.36%), produced NO levels similar to control ($mean_{CTR}$ = 99.65%), to NPY-treated cells ($mean_{NPY}$ = 98.88±7.41%) (fig. 3.4. A) and to cells exposed to LPS plus NPY ($mean_{NPY+LPS}$ = 116.30±1.29%, n = 3) (fig. 3.6. A). Additionally, we used a selective antagonist for Y_1R, BIBP3226 (1μM), to

further confirm that NPY-mediated inhibition of NO production was exclusively *via* Y_1R. In fact, when Y_1R was blocked, microglia stimulated with LPS, or with LPS plus NPY, significantly increase NO levels ($mean_{LPS+BIBP3226}$ = 236.07±5.32%; $p < 0.001$; $mean_{LPS+NPY+BIBP3226}$ = 211.8±26.70%; $p < 0.001$, n = 3) when comparing with cells treated with LPS and NPY ($mean_{NPY+LPS}$ = 116.30±1.29%) (fig. 3.6. A). To further exclude any contribution by other NPY receptors, cells were co-incubated with BIIE0246 and L152-804 (selective antagonists for Y_2R and Y_5R, respectively), and then treated with NPY and challenged with LPS. Blocking Y_2R and Y_5R did not affect the ability of NPY to inhibit NO production, even after LPS challenge ($mean_{LPS+NPY+BIIE0246+L152-804}$ = 105.98±5.19%; $p < 0.001$, n = 4) (fig. 3.6. B).

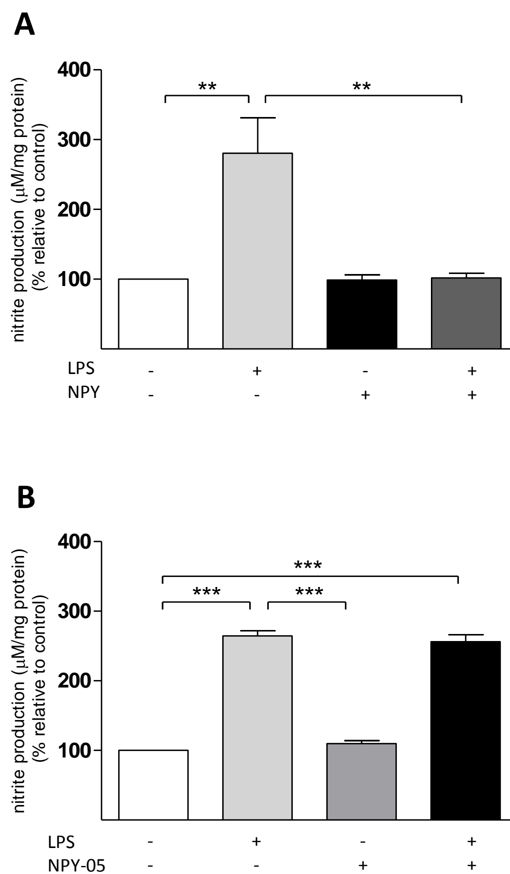


Fig. 3.4. NPY inhibits the production of nitric oxide. (A) LPS (100 ng/ml) significantly induced nitrite production, an indirect measure of the amount of NO, while NPY (1 μM) inhibited nitrite production upon LPS stimulation. (B) Pre-incubation with NPY-05 (6 μg/ml), a NPY scavenger, did not change the amount of NO when compared to control, indicating that, in our experimental conditions, endogenous NPY does not contribute to the inhibition of LPS-induced nitrite production. Data are expressed as mean ± SEM (n=3) and as a percentage of control (**p < 0.01; ***p < 0.001, using Bonferroni's Multiple Comparison Test).

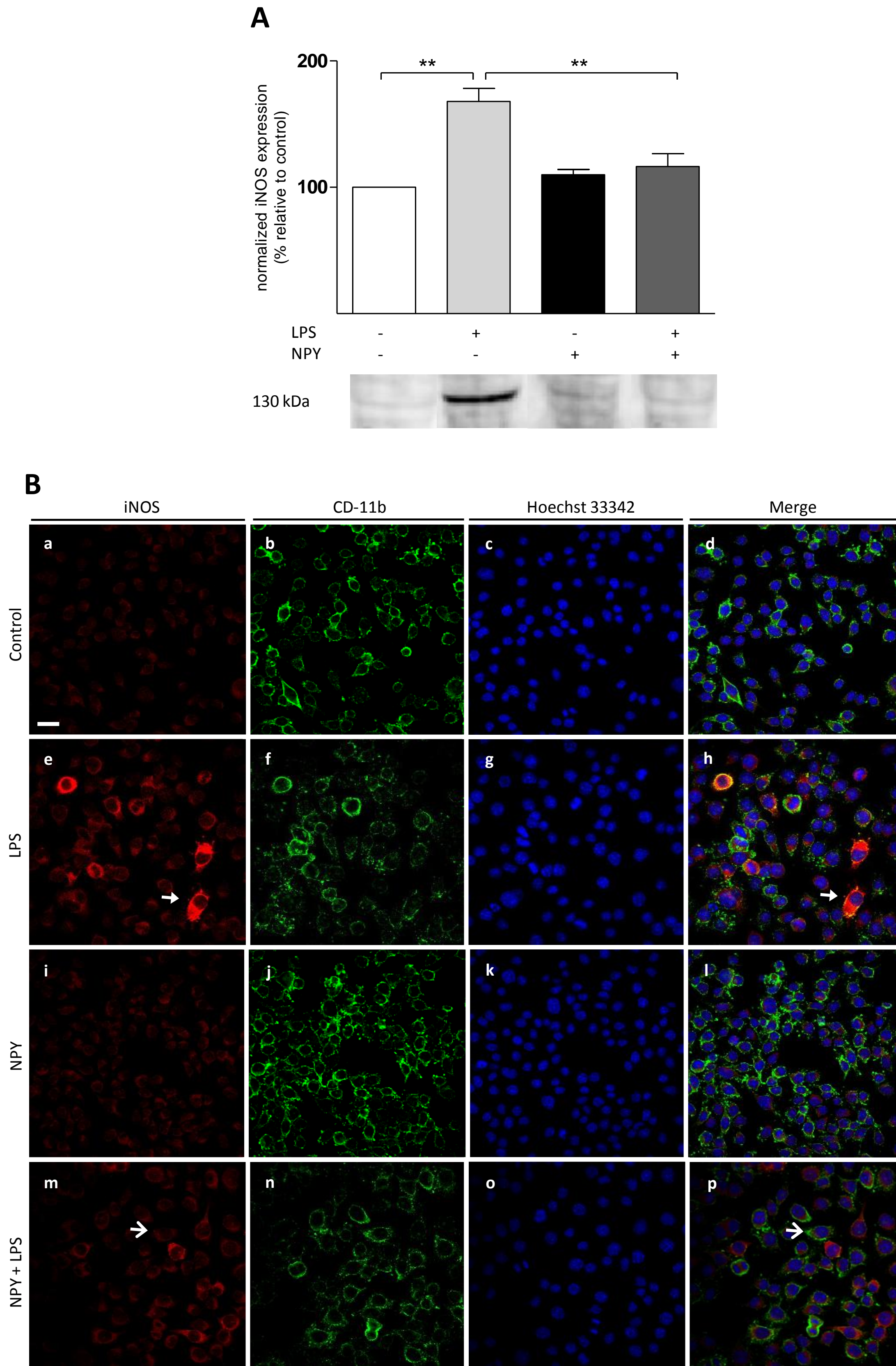


Fig. 3.5. NPY inhibits inducible nitric oxide synthase expression. Microglial cells were treated with 1 µM NPY and challenged with 100 ng/ml LPS for 6 hrs to assess the effect of NPY over iNOS (130 kDa) protein levels. (A) NPY significantly inhibited LPS-stimulated iNOS protein levels. Bellow the graph, a representative blot illustrates this effect. Data are expressed as mean ± SEM (n=3) and as a percentage of control (**$p < 0.01$, using Bonferroni's

Multiple Comparison Test). (B) Immunolabelling against iNOS (in red) and CD11b (in green) shows a weaker fluorescent signal when cells were treated with NPY alone (i-l), similar to control conditions (a-d). The strongest fluorescent signal was observed when cells were challenged with LPS (e-h). A moderate effect was visualised when cells were treated with both NPY and LPS (m-p). Closed arrowheads point to iNOS-positive cells, whereas open arrowheads show negative labelling. Nuclear morphology is shown with Hoechst 33342 staining (in blue). Scale bar 20 μm.

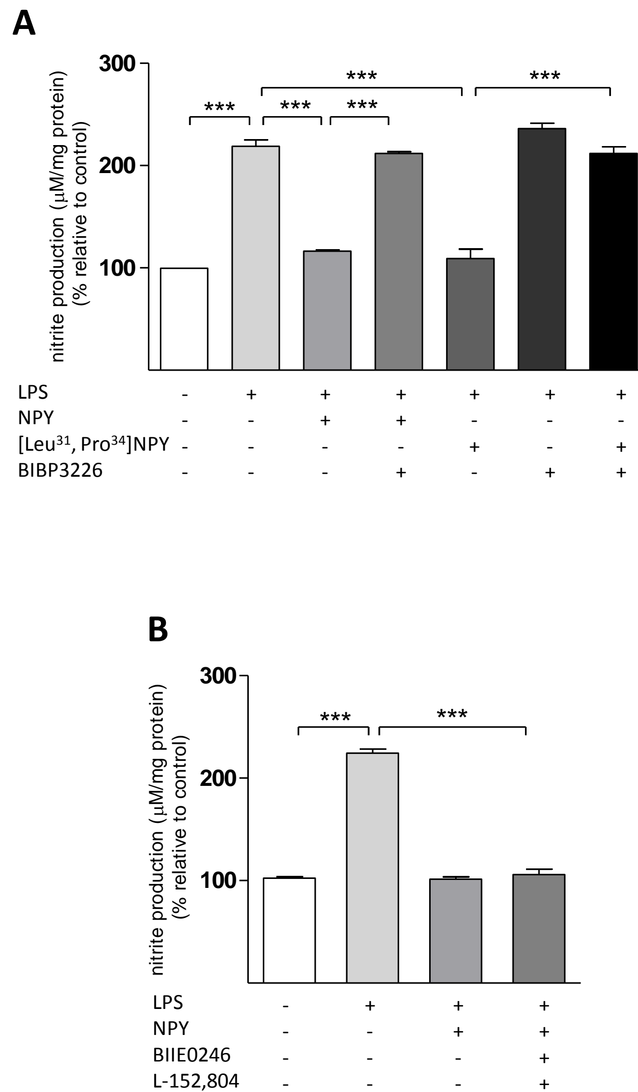


Fig. 3.6. NPY inhibits nitric oxide production *via* Y1 receptor activation. Microglia cells were treated with a selective Y1 receptor agonist [Leu31, Pro34]NPY (1 μM) and a selective Y1 receptor antagonist BIBP3226 (1 μM) to determine the effect of Y1R activation in LPS-induced nitrite production.

(A) Cells challenged with LPS and treated with [Leu31, Pro34]NPY display nitrite production similar to control levels. Accordingly, when cells were pre-treated with BIBP3226, no NPY inhibitory effect was observed. (B) The involvement of other receptors was excluded with the use of selective antagonists for Y2 receptor (BIIE0246, 1 μM) and for Y5 receptor (L152-804, 1 μM). When Y2R and Y5R were blocked, NPY inhibited NO production stimulated by LPS. Data are expressed as mean ± SEM (n=3 for fig. A and n=4 for fig. B) and as a percentage of control (***p < 0.001, using Bonferroni's Multiple Comparison Test).

3.2.4. NPY modulates the release of IL-1β

Another key feature of inflammation is the release of IL-1β by microglial cells. Using a quantitative method such as ELISA, we observed that in the presence of LPS there was a significant release of biological active IL-1β (mature form) to the media (439.13±58.90 pg; $p < 0.001$, n = 5) (fig. 3.7. A). When cells were simultaneously treated with NPY and LPS, the release of IL-1β was similar to control (control = 28.34±9.06 pg; LPS+NPY = 36.94±5.09 pg; $p < 0.001$, n = 5). To perceive the strength of this effect, cells were treated with ATP (1 mM). This nucleotide activates interleukin converting enzyme (ICE) in an inflammatory context (Abreu and Arditi 2004) and, when co-administered with LPS, triggers a massive release of IL-1β (Griffiths et al. 1995; Ferrari et al. 1997b; Grahames et al. 1999; Bernardino et al. 2008). In the presence of these stimuli, we observed a highly significant release of IL-1β to the media (1412.69±82.02 pg; $p < 0.001$, n = 5). Nevertheless, in microglia treated with NPY, this effect greatly diminished (41.21±2.81 pg; $p < 0.001$, n = 5) (fig. 3.7. A). Moreover, since IL-1β has been described as a stimulator of NO production, a concentration-response curve was performed to determine which concentration of IL-1β would lead to a significant production of NO. We observed that 1.5 ng/ml IL-1β (201.67±29.06%; $p < 0.001$, n = 3) (fig. 3.7. B) was the only concentration able to significantly increase NO production. Then, we challenged cells with the selected concentration of IL-1β for 6 h, 12 h and 24 h and we found that 1.5 ng/ml IL-1β treatment for 6 h lead to a significant production of NO (218.20±32.85%; $p < 0.05$, n = 3) (fig. 3.7. C).

3.2.5. NPY blocks IL-1β-induced production of NO through Y_1 receptor activation

We sought to determine whether NPY could prevent the effect mediated by IL-1β on NO production. As previously shown, cells challenged with 1.5 ng/ml IL-1β for 6 hrs showed significant levels of NO ($mean_{IL\text{-}1\beta}$ = 209.57±6.42%; $p < 0.001$, n =3), while NPY co-exposure prevented this effect ($mean_{NPY}$ = 126.10±1.77%; $mean_{IL\text{-}1\beta+NPY}$ = 111.28±6.81%; $p < 0.001$, n = 3) (fig. 3.8. A). To assess how selective and robust was IL-1β-induced production of NO, we co-incubated microglial cells with a selective antagonist of IL-1β receptor (IL-1ra). To block the functional effects of IL-1β a 10^2-10^3-fold higher dose of IL-1ra is needed (Arend et al. 1990); therefore we used 150 ng/ml. We observed that microglial cells stimulated with IL-1β showed an increase of NO production ($mean_{IL\text{-}1\beta}$ = 204.66±4.27%; $p < 0.001$, n = 3) and that IL-1ra inhibited this effect ($mean_{IL\text{-}1\beta+IL\text{-}1ra}$ = 112.96±5.32%; $p < 0.001$, n = 3). Upon LPS challenge, and in the presence of IL-1ra, NO production was inhibited ($mean_{LPS}$ = 236.07±5.32%; $mean_{IL\text{-}1ra+LPS}$ = 114.94±4.29%; $p < 0.001$, n = 3) (fig. 3.8. B). Moreover, we investigated if Y_1R could be involved in this effect. In fact, when cells were exposed to IL-1β, and treated with NPY and Y_1R selective antagonist, BIBP3226, the inhibitory effect of NPY was not observed ($mean_{IL\text{-}1\beta+NPY}$ = 112.98±4.29%; $mean_{IL\text{-}1\beta+NPY+BIBP3226}$ = 209.14±9.36%; $p < 0.001$, n = 3). In contrast, in the presence of Y_1R selective agonist, [Leu^{31},Pro^{34}]NPY, cells challenged with IL-1β produced NO

levels similar to control ($mean_{CTR}$ = 99.65%; $mean_{IL\text{-}1\beta+Leu31,\ Pro34}$ = 99.66±0.67%; $p < 0.001$, n = 3) (fig. 3.8. C).

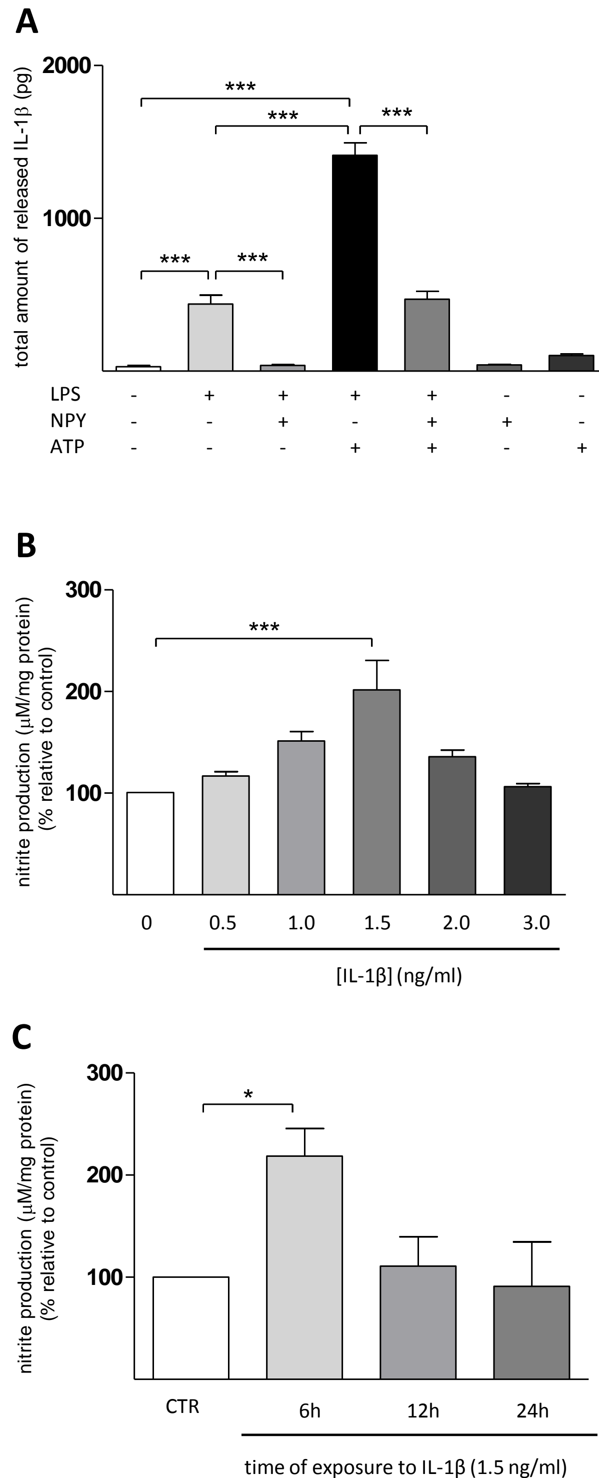


Fig. 3.7. NPY inhibits the release of interleukin-1β. (A) Microglia were stimulated with LPS (100 ng/ml) for 24 hrs to determine the effect of this endotoxin on IL-1β release. LPS induced the release of IL-1β, an effect inhibited by NPY treatment. Additionally, cells were challenged with both LPS and ATP (1 mM) for 30 minutes, which induced a massive release of IL-1β. NPY was also able to significantly reduce the amount of IL-1β released by microglial cells. (B) A concentration-response curve was performed for IL-1β to observe which concentration induced a significant

nitrite production. (C) The selected concentration (1.5 ng/ml) was used to study which time of incubation is necessary to obtain a significant increase in NO production. Data are expressed as mean ± SEM (n=5 for fig. A; n=3 for fig. B and C) and as a percentage of control (fig. 6 A, ***p < 0.001, using Bonferroni's Multiple Comparison Test; fig. 6 B and 6 C, *p < 0.05, *** p < 0.001, using Dunnett's Multiple Comparison Test;).

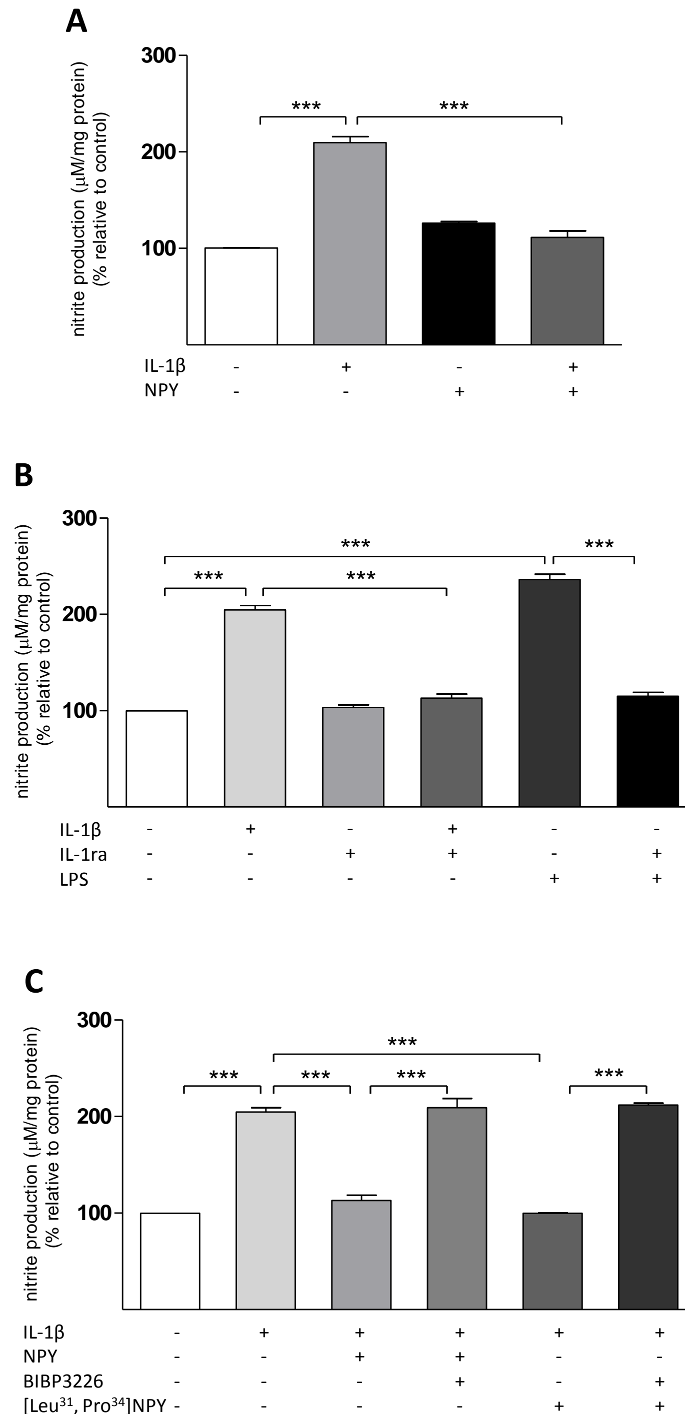


Fig. 3.8. NPY inhibits IL-1β-induced nitric oxide production through Y1 receptor activation. (A) Cells treated with 1.5 ng/ml IL-1β for 6 hrs produced significantly higher levels of NO; in the presence of NPY, IL-1β-induced NO production was prevented. (B) Selective IL-1β receptor antagonist IL-1ra (150 ng/ml) completely blocked the action of IL-1β over NO production. LPS-induced nitrite production is mediated by IL-1β since IL-1ra blocked this effect

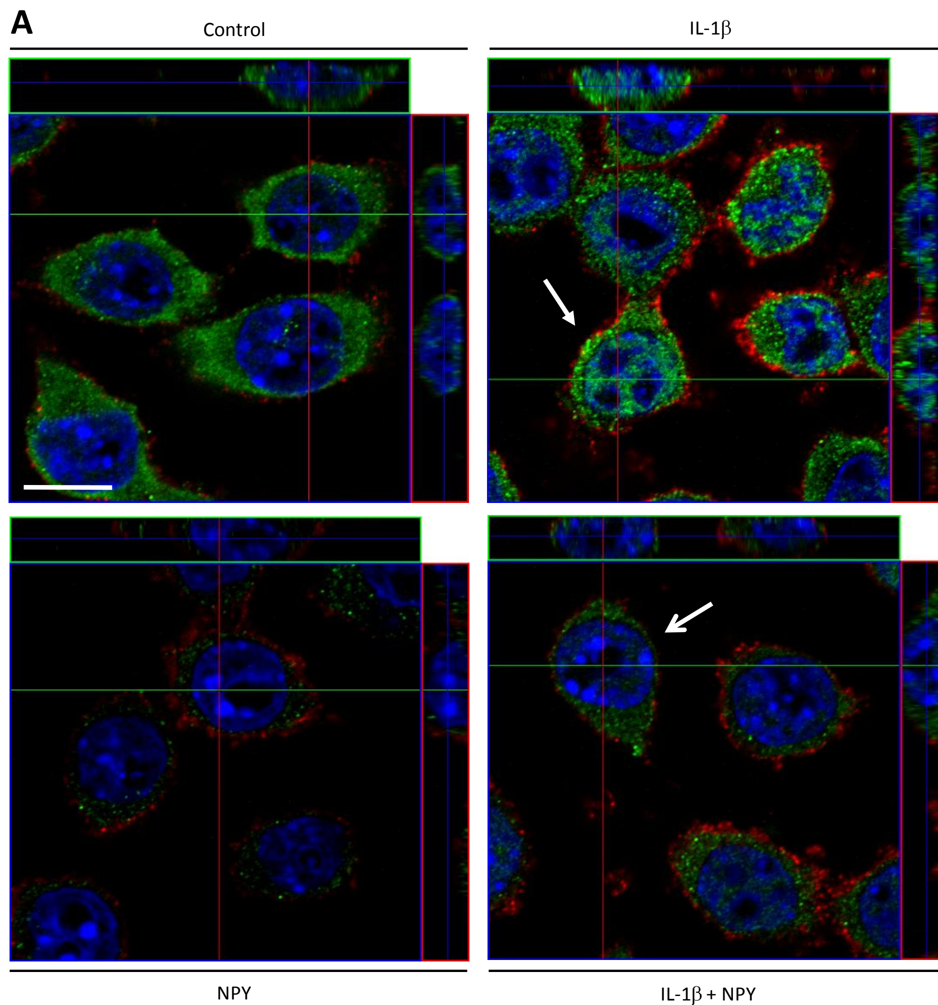
upon LPS challenge. (C) Cells were treated with a selective Y1 receptor agonist [Leu31, Pro34]NPY (1 μM) or with a selective Y1 receptor antagonist BIBP3226 (1 μM) to determine the effect of Y1 receptor activation on IL-1β-induced nitrite production. Activation of Y1R prevented IL-1β-induced NO production while pre-incubation with BIBP3226 abolished this effect. Data are expressed as mean ± SEM (n=3) and as a percentage of control (***$p < 0.001$, using Bonferroni's Multiple Comparison Test).

3.2.6. NPY inhibits nuclear translocation of NF-kB p65 induced by IL-1β challenge

NF-κB is a well known transcription factor, which upon microglia activation, is able to induce iNOS synthesis as well as pro-inflammatory cytokines, such as IL-1β (Mukaida et al. 1996). In the presence of IL-1β we observed an increase in the signal of NF-κB p65 positive cells (fig. 3.9. A, top panel, right pictogram). When cells were treated with 1 μM NPY, or NPY plus IL-1β, few nuclear translocation of NF-kB p65 was detected (fig. 3.9. A, bottom panel), as it occurred in control cells (fig. 8 A, top panel, left pictogram). Nuclear fraction extracts showed significantly increased NF-κB protein levels for cells challenged with IL-1β ($mean_{IL\text{-}1\beta} = 148.72 \pm 8.37\%$; $p < 0.05$, n = 3) opposite to cells treated with NPY plus IL-1β ($mean_{NPY+IL\text{-}1\beta} = 107.31 \pm 14.85\%$; $p < 0.05$, n = 3) (fig. 3.9. B).

3.2.7. NPY blocks IL-1β-induced iNOS expression

IL-1β induces the activation of NF-κB pathway, ultimately leading to the synthesis of iNOS mRNA and NO production (Murphy and Grzybicki 1996; Saha and Pahan 2006; Weber et al. 2010). Therefore, we aimed at uncovering if NPY would inhibit IL-1β-induced iNOS expression. Cells that were stimulated with 1.5 ng/ml IL-1β showed an increased expression of iNOS when compared to control cells or to cells treated with NPY, as observed by immunocytochemistry (fig. 3.10. A). To quantify this effect we performed a western blotting analysis of iNOS (130 kDa) protein levels under the same experimental conditions (fig. 3.10. B). We observed that IL-1β significantly induced an increase in iNOS protein levels ($mean_{IL\text{-}1\beta} = 129.52 \pm 5.05\%$; $p < 0.01$, n = 4) and that this effect was abolished by NPY ($mean_{IL\text{-}1\beta+NPY} = 107.76 \pm 2.44\%$; $p < 0.01$, n = 4) (fig. 3.10. B).



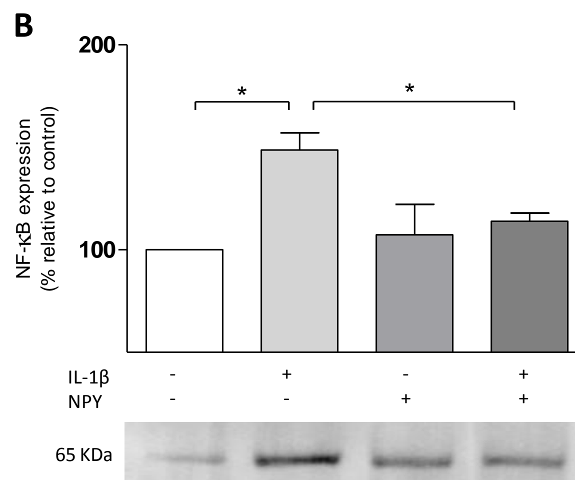


Fig. 3.9. NPY inhibits nuclear translocation of Nf-κB after IL-1β challenge. (A) Confocal microscopy photomicrographs of microglial cells treated with 1 μM NPY and 1.5 ng/ml IL-1β for 15 min were taken to assess the role of NPY and IL-1β over NFκB signalling pathway. Cells were stained for NFκB (in green), for CD11b (in red) and Hoechst 33342 (nuclei in blue). Nuclear translocation of NF-κB was promoted by IL-1β and inhibited when cells were treated with NPY. Orthogonal sections show nuclear localisation of NF-κB (in green). Scale bar 10 μm. (B) Western blotting analysis was performed to study the inhibitory effect of NPY over NF-κB (65 kDa) nuclear translocation

upon IL-1β stimulation. After IL-1β challenge, a significant increase in NF-κB protein levels was observed. When cells were treated with NPY, the amount NF-κB was reduced to values comparable to control. A representative blot is shown below the graph. Data are expressed as mean ± SEM (n=3) and as a percentage of control (*$p < 0.05$, using Bonferroni's Multiple Comparison Test).

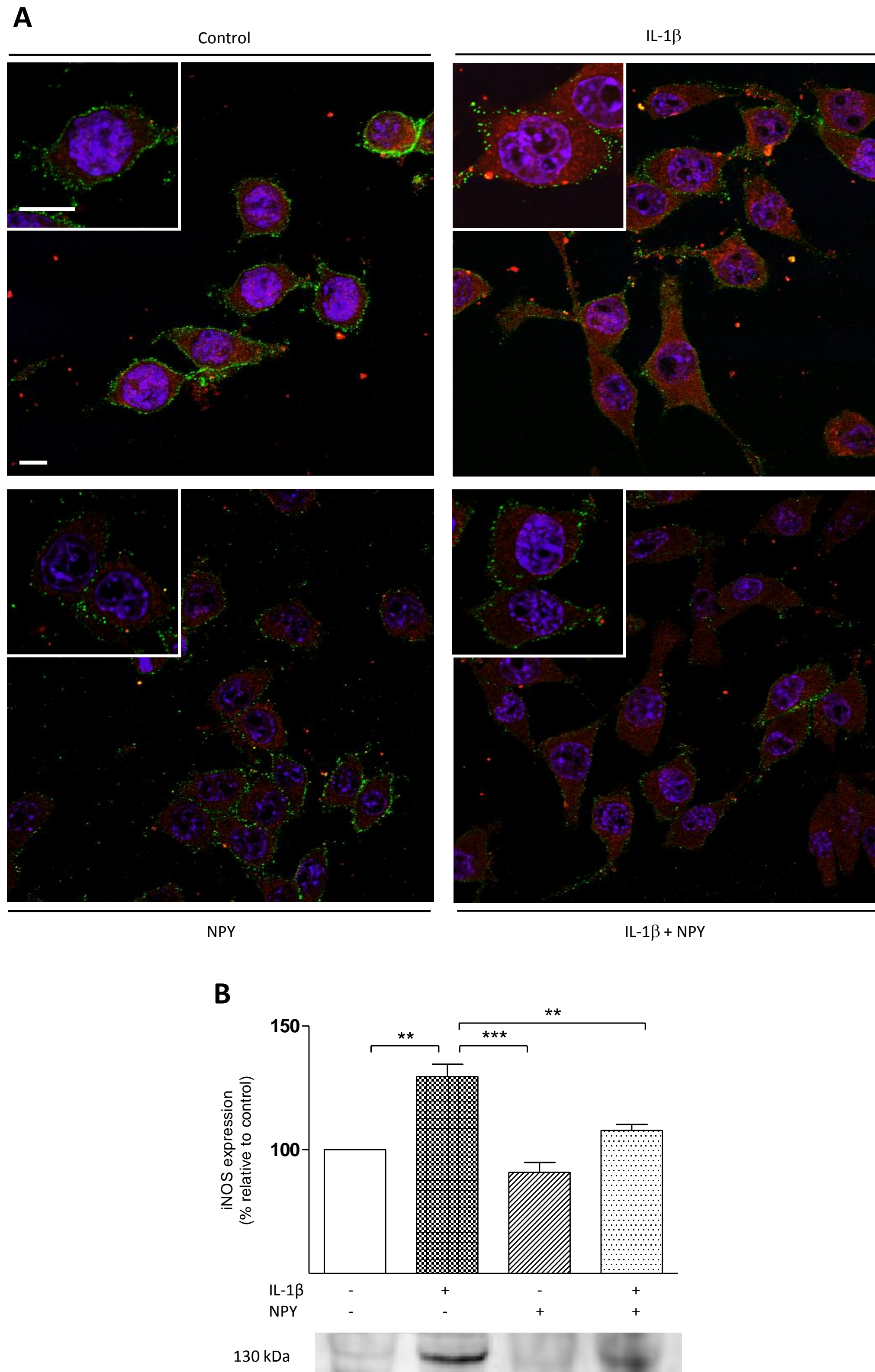


Fig. 3.10. (legend in the next page)

Fig. 3.10. NPY inhibits IL-1β-induced iNOS protein levels. (A) Confocal microscopy photomicrographs illustrate microglial cells treated with 1μM NPY and 1.5 ng/ml IL-1β for 6 hrs to assess the role of NPY and IL-1β over iNOS synthesis. To determine whether NPY was blocking the synthesis of NO induced by IL-1β treatment, cells were stained for NFκB (in green), for CD11b (in red) and Hoechst 33342 (nuclei in blue). An increase of iNOS labelling was induced by IL-1β administration and inhibited to comparable intensity of fluorescence control values when treated with NPY. Scale bar 10 μm. (B) To provide a quantitative analysis, iNOS protein values were measured by western blot. NPY inhibited IL-1β-induced iNOS levels. A representative blot is shown below the graph. Data are expressed as mean ± SEM (n=4) and as a percentage of control (**$p < 0.01$; ***$p < 0.001$ using Bonferroni's Multiple Comparison Test).

3.3. DISCUSSION

Activated microglia respond to brain injury or infection, acting as immunocompetent cells capable of phagocytosis and able to release a diversity of chemical mediators of inflammation, including chemokines, cytokines, reactive oxygen and nitrogen intermediates (Turrin and Rivest 2006; Bernardino and Malva 2007). In the immune system, increasing evidences have appointed NPY as a key modulator (De la Fuente et al. 2001; Bedoui et al. 2003a; Bedoui et al. 2004; Nave et al. 2004; Bedoui et al. 2007). In fact, NPY has been shown to play a major role in important functional properties of the Central Nervous System, such as neural stem cell proliferation and differentiation, modulation of neurotransmission, neuroprotection, response to brain injury and epilepsy (Howell et al. 2003; Silva et al. 2003a; Agasse et al. 2008; Xapelli et al. 2008). These findings suggest that NPY could work as a modulator of the inflammatory reaction of the brain immune system, eventually acting as microglial activation repressor.

In order to address this hypothesis, we used an endotoxin-mediated model of inflammation to unravel the role of NPY over inflammatory mediators such as IL-1β and NO, produced by a microglial cell line. Our results showed that NPY was able to prevent NO production by microglia, after LPS challenge. Additionally, NPY inhibited the release of IL-1β and also prevented IL-1β-induced production of NO, *via* activation of Y_1 receptor. This effect was mediated through NF-κB p65 signalling pathway, since NPY was able to block nuclear translocation of this transcriptional factor and associated synthesis of iNOS.

Using conventional reverse transcription PCR, immunocytochemistry analysis and Western blotting, we characterised murine N9 microglial cell line regarding the expression of NPY and its receptors Y_1, Y_2 and Y_5. Our results clearly showed an increase of Y_1R and NPY labelling, and protein levels, across cell membrane and cytoplasm, respectively, when microglia were challenged with LPS. Additionally, NPY treatment inhibited this effect suggesting it could act as a negative regulator of Y_1 receptor expression. In accordance to this observation, Teixeira and collaborators (Teixeira et al. 2009) also demonstrated that treatment with NPY resulted in a significant decrease of Y_1 receptor transcript in differentiating osteoblasts.

In the present work we identified an inhibitory role for NPY in LPS-induced NO production. It has been previously reported that N9 murine microglial cells produce and release NO following exposure to LPS (Dimayuga et al. 2007). Activation of macrophages by bacterial cell-wall components can lead to the expression of high levels of NOS with the most expressed isoform being iNOS, which oxidizes L-arginine to yield L-citrulline and NO. For that reason, we performed Western blotting and immunocytochemistry to determine possible differences in iNOS expression levels attributable to NPY. Our results showed that iNOS expression was significantly reduced when NPY was present, implying that NPY could be preventing *de novo* synthesis of this enzyme. To unveil through which receptor NPY was acting upon, we treated cells with selective Y_1R agonist [Leu^{31},Pro^{34}]NPY and selective antagonists for Y_1R, Y_2R and Y_5R: BIBP3226, BIIE0246 and L152-804, respectively, and as previously reported in the olfactory mucosa (Cervin et al. 1999), the inhibitory effect of NPY on NO production involved the activation of Y_1 receptor. In a study conducted in healthy human volunteers to determine dose-

dependent effects of NPY on nasal mucosal blood flow, NPY was able to inhibit intranasal NO production (Cervin et al. 1999). Moreover, RT-PCR analysis performed on nasal mucosa biopsies revealed only Y_1 receptor mRNA detection, leading to the suggestion that NPY-evoked vasoconstriction was mediated *via* Y_1 receptors.

Upon brain insult, IL-1α/β is synthesised and, proteolytically processed to mature IL-1β by caspase-1 (Dinarello 2009). As part of the repertoire of inflammation, excessive IL-1β synthesis and release from microglia can be detrimental to the injured brain. Accordingly, Bernardino and colleagues (Bernardino et al. 2008) showed that 100 μM VX-765, a selective ICE/caspase-1 inhibitor, or 1 μM IL-1ra (IL-1 receptor antagonist) blocked exacerbation of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA)-induced neuronal damage, during transient exposure to LPS and ATP. It has been described that LPS activates Toll-like receptor 4 (TLR4) and under co-activation of $P2X_7$ receptors by ATP cause the release of IL-1β from microglial cells (Bianco et al. 2006; Bernardino et al. 2008). Moreover, Ohtani and colleagues (Ohtani et al. 2000) had shown that, in rat cultured microglia, ATP induced iNOS expression and NO production, presumably in cooperation with macrophage-colony stimulating factor (MCSF) present in the culture media. Also, Schroeder and colleagues (Schroeder et al. 1999) had shown that inhibition of NO synthesis leads to an increase of IL-1β protein expression in ANA-1 murine macrophages. The authors suggested a negative feedback mechanism through which NO production inhibited the synthesis of IL-1β by S-nitrosation of NF-κB, a transcription factor implicated in immune and inflammatory reactions. Our findings can further provide an insightful understanding of the liaison between IL-1β and NO, suggesting NPY as a key modulator of their interplay. Our results showed that microglia significantly released IL-1β (the biologically active form) to the medium when cells were treated with LPS and that this effect was potentiated when cells were treated simultaneously with LPS and ATP. In the presence of NPY the release of IL-1β was significantly reduced and this effect was mimicked using a selective Y_1R agonist, implying that NPY acted *via* Y_1 receptor. Moreover, cells treated with IL-1β significantly increased NO production: an effect abolished in the presence of NPY or Y_1R agonist. Furthermore, LPS challenge, together with IL-1ra treatment, led to the inhibition of NO production. Hence, blockage of IL-1β receptor with IL-1ra inhibited NO production, suggesting that LPS action on NO production is mediated through this cytokine. In fact, some reports have also shown that IL-1ra inhibited iNOS in astrocytes (Mollace et al. 1997; Hu et al. 1999; Akama and Van Eldik 2000).

In activated microglia, induction of iNOS, and consequently NO production is likely to involve NF-κB (Mukaida et al. 1996). In broad terms, Toll-like receptors are activated by pathogen-associated molecular patterns and trigger a cascade of cellular signals leading to the activation of NF-κB. The Toll-like receptor superfamily includes IL-R1, through which IL-1β leads to NF-κB activation *via* a serine/threonine kinase called interleukin-receptor associated kinase (IRAK) (Doyle and O'Neill 2006). In relation to this, competitive inhibitors of serine/threonine protein kinases, such as calmodulin regulated protein kinases (CaMKs), can modulate iNOS expression. Watterson and colleagues (Watterson et al. 2001) screened the action of low molecular weight cell permeable compounds described as CaMK inhibitors and found them to block the

induction of both iNOS and IL-1β in primary cortical glial cultures and microglial BV-2 cell line. Also, in rat aortic smooth muscle cells (RASMC), NF-κB and CCAAT-enhancer-binding proteins (C/EBP) mediated IL-1β-induced iNOS expression (Teng et al. 2002). Hitherto, our data support a role for NF-κB signalling pathway in the inflammation model used, since this transcriptional factor was not able to translocate to the nucleus upon NPY treatment, even after IL-1β challenge. Interestingly, early in 1995, Ball and colleagues (Ball et al. 1995) reported the existence of a binding site for NF-κB in a promoter region of the human and murine Y_1 receptor gene. Later, Musso and colleagues (Musso et al. 1997) showed that the murine Y_1 receptor promoter region contained consensus sites for members of the kB-Rel family of transcription factors, which were able to bind kB-related nuclear complexes in a specific manner. Authors speculated on whether Y_1 receptor could represent one of the kB site-containing genes regulated by kB-related factors responding to inflammatory stimuli.

In summary, our work revealed a novel role for NPY in the regulation of key events occurring during inflammation converging relevant evidence from the literature. Upon an endotoxin challenge, microglia respond with increased IL-1β and NO production, an effect inhibited by NPY *via* Y_1 receptor activation, showing the involvement of NF-κB signalling pathway in this process. Microglia are the smallest member of the glia family but greatly responsible for vital physiological responses to brain injury. Taken together, our data indicate a new integrated functional response of microglia cells and a key modulatory role for NPY. These findings may be valuable to reveal new drug targets to modulate the inflammatory reaction upon brain injury.

CHAPTER 4. Neuropeptide Y inhibits interleukin-1 beta (IL-1β)-induced microglia motility

4.1. Introduction

Cell migration is a complex process mediated by dynamic changes in the actin–myosin cytoskeleton and occurs during important phases of development (e. g. gastrulation and nervous system formation) and adulthood (e. g. wound healing and immune system function). This process is modulated by the microtubule system, and associated motors, or by the action of intermediate filament systems (Ridley et al. 2003). Migration is a multistep event that involves a network of internal and external signals, complex signal transduction cascades, highly dynamic cytoskeleton and intricate cellular interactions (fig. 4.1.). It is worth mentioning that during cell movement, the intracellular machinery (e. g. nucleus, mitochondria, endoplasmatic reticulum, Golgi apparatus) needs to be transported, but the underlying mechanisms remain unknown.

4.1.1. Cell polarization

Overall, cells respond to a migration-promoting agent by polarizing and extending membrane protrusions in the sought direction. These protrusions, usually driven by actin polymerization, adhere to the extracellular matrix (ECM) or to adjacent cells which serve as traction sites to propel the cell forward (Raven et al. 2004). To sense the surrounding environment, the cell develops large broad lamellipodia or forms spike-like filopodia, which become orientated in the direction of movement. This change in cell morphology is often referred to as cell polarization, a process involving Rho family of guanosine triphosphate (GTP)-binding proteins (GTPases) (e.g. Cdc42), phosphoinositide 3-kinases (PI3Ks), integrins, microtubules and vesicular transport (Ridley et al. 2003).

Lamellipodia are flattened, sheet-like structures, composed by a cross-linked F-actin tangle that projects from the cell surface (Frame et al. 2002). Actin polymerization in lamellipodia is mediated by the Arp2/3 complex whose activation is regulated by WASP/WAVE family members. Other actin-binding proteins regulate actin polymerization: profilin (prevents self-nucleation), ADF/cofilin family (promotes actin dissociation), cortactin (stabilizes branches) and filamin-A and α-actinin (promote the cross-linking of filaments) (Welch and Mullins 2002). Most importantly, controlled lamellipodia growth provides the basis for directional migration.

Filopodia are long, thin protrusions at the periphery of cells, and growth cones, organized into long parallel F-actin bundles (Frame et al. 2002). Filament bundling is promoted by fascin which provides the structural rigidity needed to drive the cell membrane. Filopodia assembly occurs by filament elongation, endorsed by Ena/VASP proteins, instead of branched nucleation. Filopodia have a structural design that enables local environment exploration. The formation of lamellipodia and filopodia is regulated, particularly, by Rac, Cdc42, and RhoG proteins, also members of the Rho family.

4.1.2. Cell migration

A cell will move in a given direction by conciliating two actions: the formation of focal adhesions near the leading edge and cell-substrate detachment at the cell rear. Focal adhesions are multimeric protein complexes that physically connect the ECM to the intracellular actin cytoskeleton providing the necessary traction required to move the cell body forward (Frame et al. 2002). If the ECM integrity is affected, cell migration may be compromised. For instance, changes in this structure, or in the matrix proteins such as β2-integrin CD11a and tenascin-R, impairs microglia migration (Angelov et al. 1998; Ullrich et al. 2001). The formation of adhesion sites requires integrin clustering and the recruitment of structural and signaling components. This process is activated by talin and mediated by PKC, Rap1 and PI3K signaling pathways. Simultaneously, at the rear of the cell, focal-adhesion disassembles and cell detaches from the substrate allowing tail retraction. Focal adhesion disassembly is promoted by calpains through proteolytic cleavage of protein components of the focal-adhesion complex (Frame et al. 2002). This process involves other signaling pathways and molecules such as Src/FAK/ERK, Rho, myosin II, calcium, calcineurin and the delivery of components by microtubules (Schaller 2001; Ridley et al. 2003)

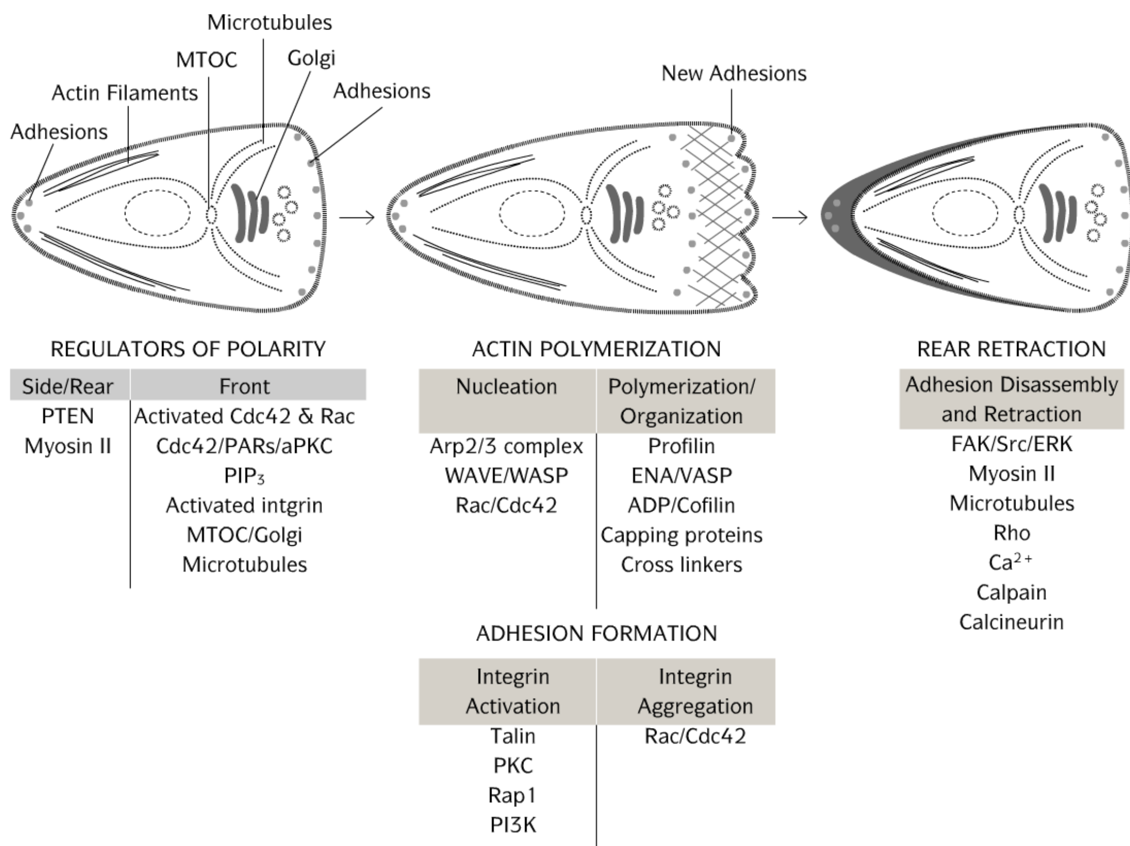


Fig. 4.1. Representative scheme regarding proteins and structures mobilized for in cell migration. Several proteins are implicated in polarity, which prompts directed vesicle trafficking toward the leading edge, organization of microtubules (in some cells), and the localization of the microtubule organizing center (MTOC, in some cells) and Golgi apparatus in front of the nucleus. Migration requires the formation of a cell protrusion enabled by the

reorganization of actin cytoskeleton. Protrusions are stabilized by the formation of adhesions, which requires integrin activation, clustering, and the recruitment of structural and signaling components to nascent adhesions. Finally, at the cell rear, adhesions disassemble as the rear retracts pushing the cell forward. Adapted from (Ridley et al. 2003).

4.1.3. Cell chemotaxis

In response to the activating stimulus, microglial cells migrate to the site of injury or inflammation, where they release inflammatory mediators that promote more cell recruitment (including peripheral blood cells). Microglia appear to be stimulated to move into the developing CNS *via* the same chemotactic gradients observed in experimental models (Cartier et al. 2005). Chemotaxis is a process that designates cell movement in response to a chemical gradient, involving direction sensing followed by directed migration (Affolter and Weijer 2005). This course of action is mediated by membrane receptors that detect the extracellular signals and activate signal transduction pathways that locally mould the cytoskeleton to enable movement. Microglia respond to various stimuli such as blood-derived factors caused by damage to the blood vessels, extracellular ATP and ADP released from ischemic and traumatic CNS injuries, microbial signals, adhesion molecules, cytokines, complement molecules, among others (Honda et al. 2001; Forstreuter et al. 2002; Allan and Rothwell 2003; Minghetti et al. 2005; Nimmerjahn et al. 2005; Block et al. 2007).

Given the association between cell migration and pathologies like vascular disease, osteoporosis, rheumatoid arthritis, multiple sclerosis, cancer and mental retardation (Ridley et al. 2003), it is important to understand the underlying mechanisms of this cellular process in order to develop effective therapeutic approaches. In the present chapter, we support a role for NPY in the regulation of an important microglial feature – motility, and the molecular pathways involved.

4.2. RESULTS

4.2.1. NPY prevents microglial cell motility trough Y_1 receptor activation.

Murine N9 microglia cell line was used to study the role of NPY in endotoxin-induced motility. LPS is part of the outer membrane of Gram-negative bacteria and binds to CD14/TLR4/MD2 receptor complex present at the cell membrane, triggering several signaling cascades (Cohen 2002). We have previously used this cell line as a biological model to dissect the effects of LPS over microglial physiological responses, such as production of inflammatory mediators (e. g. NO and IL-1β), and we observed that NPY-mediated inhibition of microglia activation occurred via Y_1 receptor (Y_1R) (Ferreira et al. 2010).

To evaluate the effect of NPY on LPS-induced cell motility, we determined the number of cells that migrated *in vitro* across scratch wounds (fig. 4.2. A). LPS significantly increased cell motility, determined after 12 h, compared to control ($mean_{CTR}$ = 100.0±8.8%, n=11; $mean_{LPS}$ = 182.7±11.8%; n = 10, $p < 0.001$). NPY inhibited the stimulatory effect of LPS on cell motility, while NPY treatment alone was similar to control ($mean_{NPY}$ = 98.1±12.8%, n=4; $mean_{LPS+NPY}$ = 93.1±13.1%, n=6, $p < 0.001$) (fig. 4.2. A, B).

To disclose a possible contribution from endogenous NPY, we treated microglial cells with monoclonal antibody NPY-05 (6 μg/ml) which acts as a NPY scavenger by binding to the carboxyl terminal of this peptide (Brakch et al. 2002). The number of migrating cells after NPY-05 treatment was similar to controls, but significantly different from LPS challenge ($mean_{CTR}$ = 100±8.8%, n=11; $mean_{LPS}$ = 182.7±11.8%; n = 10; $mean_{NPY05}$ =102.5±11.9%, n=3, $p < 0.001$). In the presence of NPY-05 and LPS, the motility rate was similar to LPS alone ($mean_{LPS+NPY05}$ = 172.3±5.4%), indicating that in our experimental conditions endogenous NPY did not significantly contribute to cell motility. The efficacy of the neutralizing antibody was determined by performing a concentration-response curve with increasing concentrations of NPY-05 (ranging from 60 ng/ml to 6 μg/ml) in the presence of LPS and NPY (fig. 4.2. D).

In order to characterize the subset of receptors involved in the inhibitory role of NPY, and in accordance with preliminary results, we treated cells with a selective agonist for Y_1R, $[Leu^{31},Pro^{34}]$NPY (1μM). We observed that LPS-increased cell motility across the scratch was completely inhibited by $[Leu^{31},Pro^{34}]$NPY ($mean_{CTR}$ = 100±8.8%, n=11; $mean_{LPS+[Leu31,\ Pro34]NPY}$ = 114.6±5.6%, n=4). Additionally, we used a selective antagonist for the Y_1R, BIBP3226 (1μM) to further confirm that NPY-mediated inhibition of LPS-induced cell motility was *via* Y_1R activation. When Y_1R was blocked, the number of microglial cells stimulated with LPS and NPY, found in the lesion area, was similar to LPS alone and significantly different from controls ($mean_{LPS+NPY+BIBP3226}$ = 181.5±7.8%, n=4; $mean_{LPS}$ = 182.7±11.8%, n=10; $mean_{CTR}$ = 100.0±8.8%, n=11, $p < 0.001$). To discard any contribution from other NPY receptors, cells were co-incubated with BIIE0246 and L-152,804 (selective antagonists for Y_2R and Y_5R, respectively), and then treated with NPY and challenged with LPS. The blockage of Y_2R and Y_5R did not affect the ability of NPY to inhibit microglia motility into the scratch ($mean_{LPS+NPY+BIIE0246+L152\text{-}804}$ =

123.6±6.9% n = 4, $p < 0.001$) (figs. 4.2. A, C) which demonstrates that NPY-mediated inhibition of LPS-induced cell motility is exclusively *via* Y_1R activation.

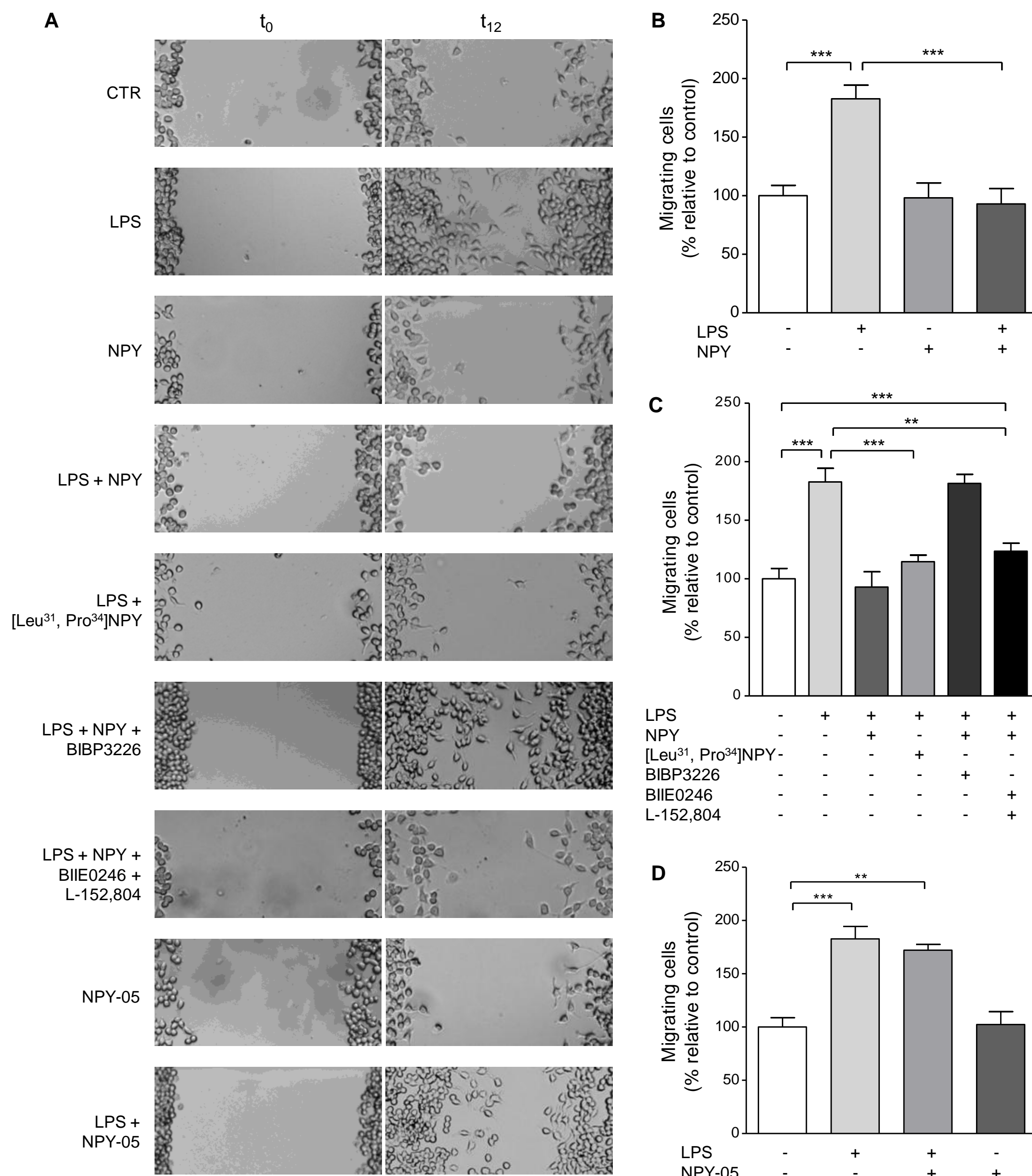


Fig. 4.2. NPY inhibits LPS-stimulated motility of murine N9 microglial cell line through Y1 receptor activation. (A) Representative photomicrographs illustrate the inhibitory effect of NPY *via* Y1 receptor on LPS-induced cell motility. (B) LPS (100 ng/ml) significantly induced cell motility, while NPY (1 μM) inhibited this effect upon LPS stimulation. (C) To determine the effect of Y1 receptor in LPS-induced cell motility, microglial cells were treated with a selective Y1 receptor agonist [Leu31, Pro34]NPY (1 μM) and antagonist BIBP3226 (1 μM) to determine the effect of Y1R activation in LPS-induced cell motility. The involvement of other receptors was determined with the use of selective antagonists for Y2 receptor (BIIE0246, 1 μM) and for Y5 receptor (L-152,804, 1 μM). (D) Pre-incubation with NPY-05 (6 μg/ml), a NPY scavenger, demonstrated that, in our experimental conditions, endogenous NPY does not contribute to the inhibition of LPS-induced cell motility. Data are expressed as mean ± SEM (n=4-11) and as a percentage of control (**p < 0.01, ***p < 0.001, using Bonferroni's Multiple Comparison Test).

4.2.2. LPS-induced motility is mediated by IL-1β

We have previously shown that murine N9 microglial cells release the biologically active form of IL-1β upon LPS challenge, particularly when co-stimulated with ATP (Ferreira et al. 2010). This nucleotide activates interleukin converting enzyme (ICE) in an inflammatory context (Abreu and Arditi 2004) and, when co-administered with LPS, triggers a massive release of IL-1β (Griffiths et al. 1995; Ferrari et al. 1997b; Grahames et al. 1999; Bernardino et al. 2008). Accordingly, we tested if cells stimulated with LPS and ATP (1 mM) increased cell motility (fig. 2A). In these experimental conditions, cells migrated in a very similar fashion to LPS alone ($mean_{LPS+ATP}$ = 191.5±16.6%, n=4; $mean_{LPS}$ = 182.7±11.8%, n=10). Moreover, this effect was abolished in the presence of selective receptor antagonist for IL-1β (IL-1ra) ($mean_{LPS+ATP+IL\text{-}1ra}$ = 119.4±9.4%, n=3), known to block the functional effects of IL-1β at 10^2-10^3-fold higher doses (150 ng/ml) (Arend et al. 1990) Accordingly, the effect induced by IL-1β was abolished by IL-1ra ($mean_{IL\text{-}1\beta+IL\text{-}1ra}$ = 107.2±3.5%, n=3, $p < 0.001$). In addition, we treated cells with NPY that were previously challenged with LPS and ATP. As previously observed in N9 microglial cells treated only with LPS (fig. 1D), NPY also inhibited the increased of migrating microglia induced by both LPS and ATP ($mean_{LPS+ATP+NPY}$ = 122.9±13.0%, n=11, $mean_{CTR}$ = 100±8.8%, n=11, $mean_{LPS+ATP}$ = 191.5±16.6%, n=4, $p < 0.01$) (fig. 4.3. B).

In light of the last result, we stimulated microglial cells directly with IL-1β (1.5 ng/ml) and observed that the percentage of migrating cells significantly increased compared to control and, strikingly, this effect was prevented by NPY treatment ($mean_{CTR}$= 100.0±8.8%, n=11; $mean_{IL\text{-}1\beta}$ = 190.0±11.8%, n=6; $mean_{IL\text{-}1\beta+NPY}$ = 107.9±18.7%, n=5, $p < 0.01$) (Fig. 4.3. C). In agreement with previous experiments, we incubated cells with the selective agonist for Y_1R, [Leu^{31},Pro^{34}]NPY (1 μM) and with IL-1β. As expected, the blockade of Y_1R significantly inhibited microglial motility ($mean_{LPS+[Leu31,\ Pro34]NPY}$ = 117.8±910.8%, n=4; $mean_{IL\text{-}1\beta}$ = 190.0±11.8%, n=6, $p < 0.001$). The inhibitory effect on motility/migration mediated by Y_1R activation was not observed following exposure to a selective antagonist for Y_1R, BIBP3226 (1μM) ($mean_{IL\text{-}1\beta+NPY+BIBP3226}$= 189.4±14.3%, n=3; $mean_{IL\text{-}1\beta+NPY}$= 107.9±18.7%, n=5, $p < 0.001$) (fig. 4.3. C).

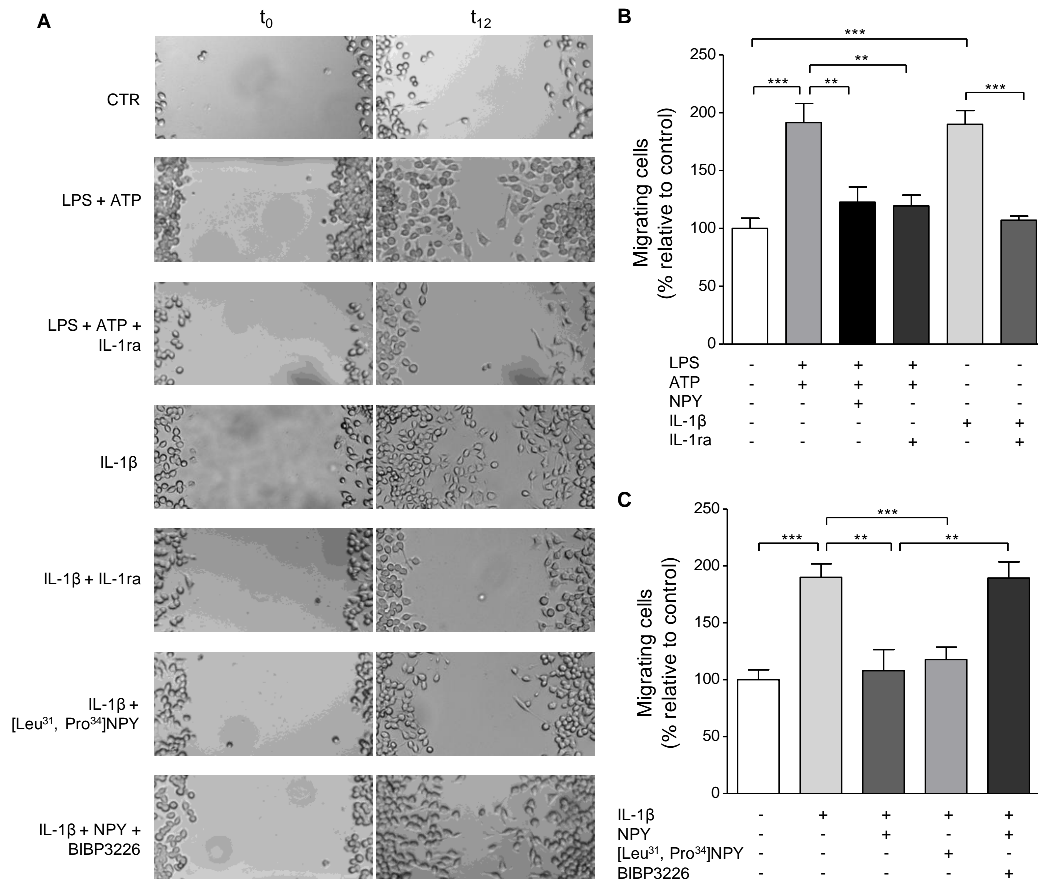


Fig. 4.3. NPY inhibits IL-1β-induced motility *via* Y1 receptor activation. (A) Representative photomicrographs illustrate the inhibitory effect of NPY *via* Y1 receptor on IL-1β-induced cell motility. (B) LPS (100 ng/ml) plus ATP (1 mM) significantly induced cell motility, while NPY (1 μM) inhibited this effect. LPS-induced motility was prevented by IL-1ra application (150 ng/ml) suggesting the involvement of IL-1β. IL-1β-stimulated (1.5 ng/ml) motility was completely inhibited by IL-1ra. (C) Cells were treated with a selective Y1 receptor agonist [Leu31, Pro34]NPY (1 μM) or with a Y1 receptor-selective antagonist BIBP3226 (1 μM) to determine the effect of Y1 receptor activation on IL-1β-induced microglia motility. Data are expressed as mean ± SEM (n=3-11) and as a percentage of control (**p < 0.01, ***p < 0.001, using Bonferroni's Multiple Comparison Test).

4.2.3. IL-1β-induced motility is p38-dependent

Several cell types such as smooth muscle cells, endothelial cells, neutrophils, mast cells, epithelial cells recruit p38 signaling activation for migration/motility (Huang et al. 2004). We tested the effect of the selective p38 inhibitor SB239063 (20 μM) in microglial cell motility induced by either LPS or IL-1β (fig. 4.4. A), and we concluded that SB239063 significantly inhibited motility in microglia in both conditions ($mean_{LPS+SB236063}$ = 110.1±4.8%, n=10; $mean_{LPS}$ = 182.7±11.8%, n=10, $p < 0.001$; $mean_{IL\text{-}1\beta+SB239063}$ = 110.1±4.8%, n=3; $mean_{IL\text{-}1\beta}$ = 190.0±11.8%, n=6, $p < 0.01$) (figs. 4.4. B).

To further assess the involvement of p38, we performed a western blotting analysis of p38 protein levels (38 kDa), in total extracts, under the same experimental conditions. As expected, LPS and IL-1β induced p38 phosphorylation ($mean_{LPS}$ = 162.7±11.0%, n=4; $mean_{IL\text{-}1\beta}$

=161.6±8.2%, n=4; $mean_{CTR}$ = 100%, n=4, $p < 0.01$), whereas NPY abolished this effect ($mean_{LPS+NPY}$ = 113.1±13.8%, n=4, $p < 0.05$; $mean_{IL\text{-}1\beta+NPY}$ = 95.6±3.7%, n=4, $p < 0.01$; $mean_{NPY}$ = 104.0±4.1%, n=4) (Fig. 4.5. A). Incubation with SB239063 prevented LPS- and IL-1β-induced motility and concomitant phosphorylation of nuclear p38 protein levels ($mean_{LPS+SB239063}$ = 102.4±15.24%, n=4, $p < 0.01$; $mean_{IL\text{-}1\beta+SB239063}$ = 102.20±5.43%, n=4, $p < 0.05$) (fig. 4.5. A). These effects were further illustrated by immunocytochemistry studies in the same experimental conditions (fig. 4B). We could only observe a distinct nuclear labeling of phosphorylated p38 (in red) when cells were stimulated with LPS or with IL-1β. Upon activation, p38 is phosphorylated and translocates to the nucleus (Ben-Levy et al. 1995). Otherwise, if this pathway is not activated p38 labeling in the nuclear fraction is almost absent. To visualize microglia morphology we labeled the alpha chain of $\alpha_M\beta_2$-integrin, CD11b, a well known surface marker, whose overexpression is associated to microglial activation, and a mediator of the diapedesis process of leukocytes through the endothelium (Vetvicka et al. 1999).

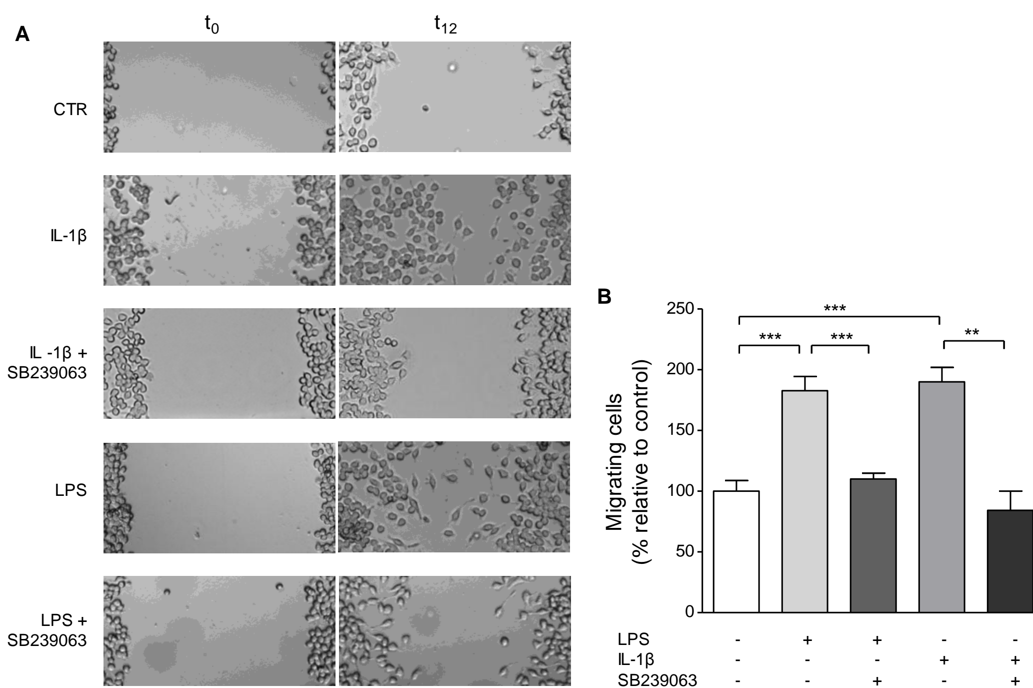


Fig. 4.4. LPS and IL-1β-induced cell motility depends on the activation of p38-MAPK pathway. (A) Representative photomicrographs illustrate the inhibitory effect of SB239063, a selective p38 inhibitor, on LPS and IL-1β-induced cell motility. (B) Both LPS and IL-1β-induced cell motility were inhibited by treatment with SB239063 (20 μM). Data are expressed as mean ± SEM (n=3-11) and as a percentage of control (**$p < 0.01$, ***$p < 0.001$, using Bonferroni's Multiple Comparison Test).

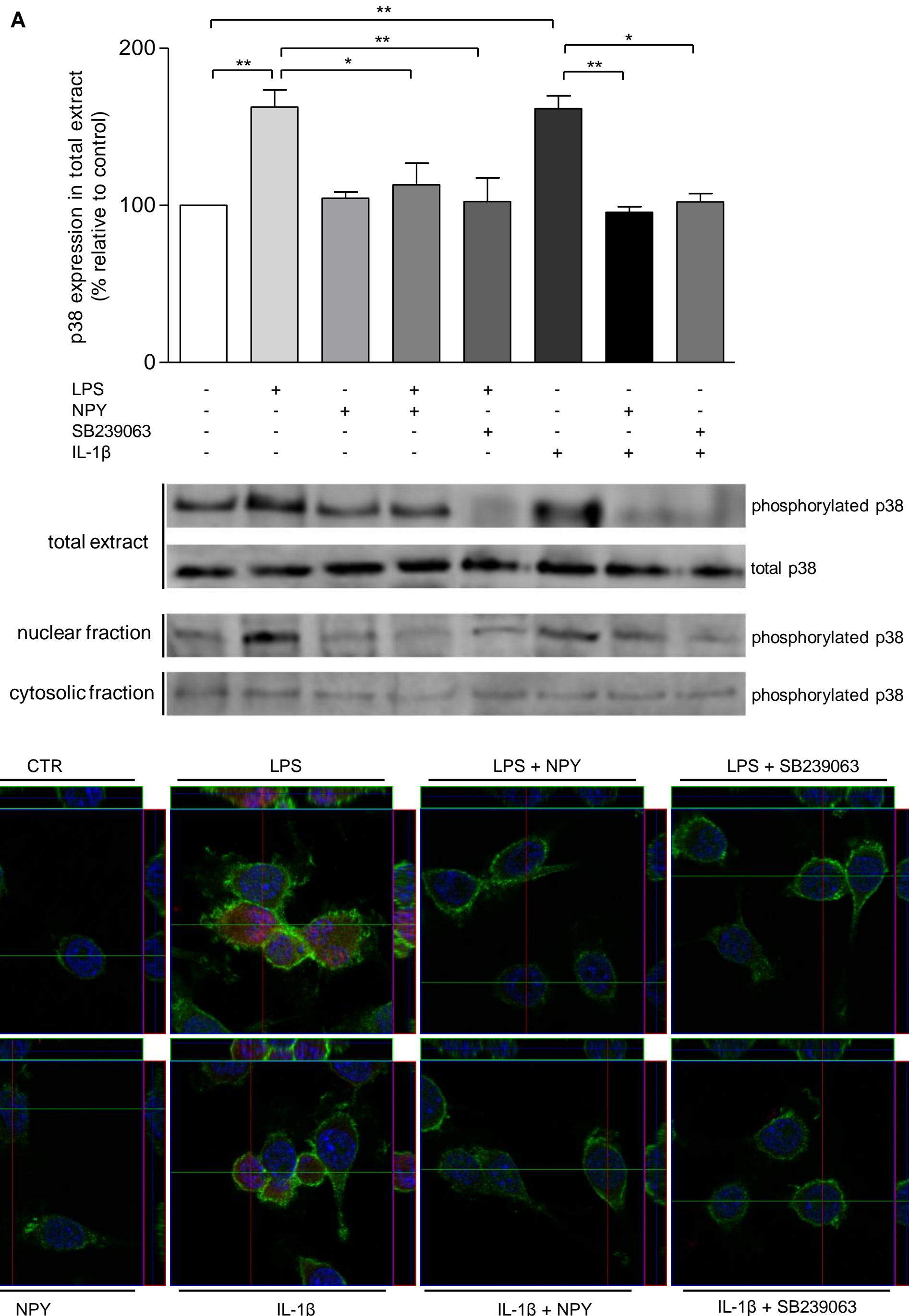


Fig. 4.5. NPY decreases the levels of activated p38 in the nucleus. (A) To provide a quantitative analysis of the effect of NPY on p38 activation, protein levels were quantified by western blot. Microglial cells were treated with 1 μM NPY and challenged with 100 ng/ml LPS for 1 hr. NPY inhibited both LPS and IL-1β-induced p38 phosphorylation (38 kDa) in the total extract. The inhibitory effect of NPY was more obvious in the nuclear fraction. SB239063, a selective p38 inhibitor, blocked p38 phosphorylation. A representative blot is shown below the graph. (B) Confocal microscopy photomicrographs illustrate microglial cells treated with 1μM NPY and 1.5 ng/ml IL-1β or 100 ng/ml LPS for 1 hr to assess the role of NPY over p38 phosphorylation (in red). SB239063 (20 μM), a selective p38 inhibitor, blocked p38 phosphorylation. Cells were stained for CD11b (in green) and Hoechst 33342 (nuclei in blue). Scale bar 10 μm. Data are expressed as mean ± SEM (n=4) and as a percentage of control (*p < 0.05, **p < 0.01, using Bonferroni's Multiple Comparison Test).

4.2.4. Interleukin-1β and lipopolysaccharide induce significant cytoskeleton reorganization of microglial cells.

Migration mainly depends on cytoskeleton actin filament rearrangement, allowing the cell to sense the chemical and physical properties of the surrounding environment and to respond by moving the membrane and cell body in the direction of the migration-inducing stimulus (Ridley et al. 2003). Cells were stimulated with 100 ng/ml LPS, 1.5 ng/ml IL-1β, and treated with 1 μM NPY and 1μM [Leu^{31},Pro^{34}]NPY, for 6 hrs. In order to determine changes in cytoskeleton reorganization, namely in the arrangement of actin filaments, we stained cells with phalloidin. This toxin binds specifically to actin filaments and prevents their depolymerization (Small et al. 1999). LPS treatment, as well as IL-1β, led to the formation of fine protrusions and membrane ruffling (phalloidin staining) accompanied by altered cell morphology and a bloated cell body, as shown by CD11b expression. NPY treatment prevented this effect, through Y_1R activation, and cells showed a control resting-like morphology. Moreover, according to the previous data, SB239063 (20 μM) also inhibited actin reorganization, highlighting the involvement of p38 in crucial steps required for microglial migration (fig. 4.6.).

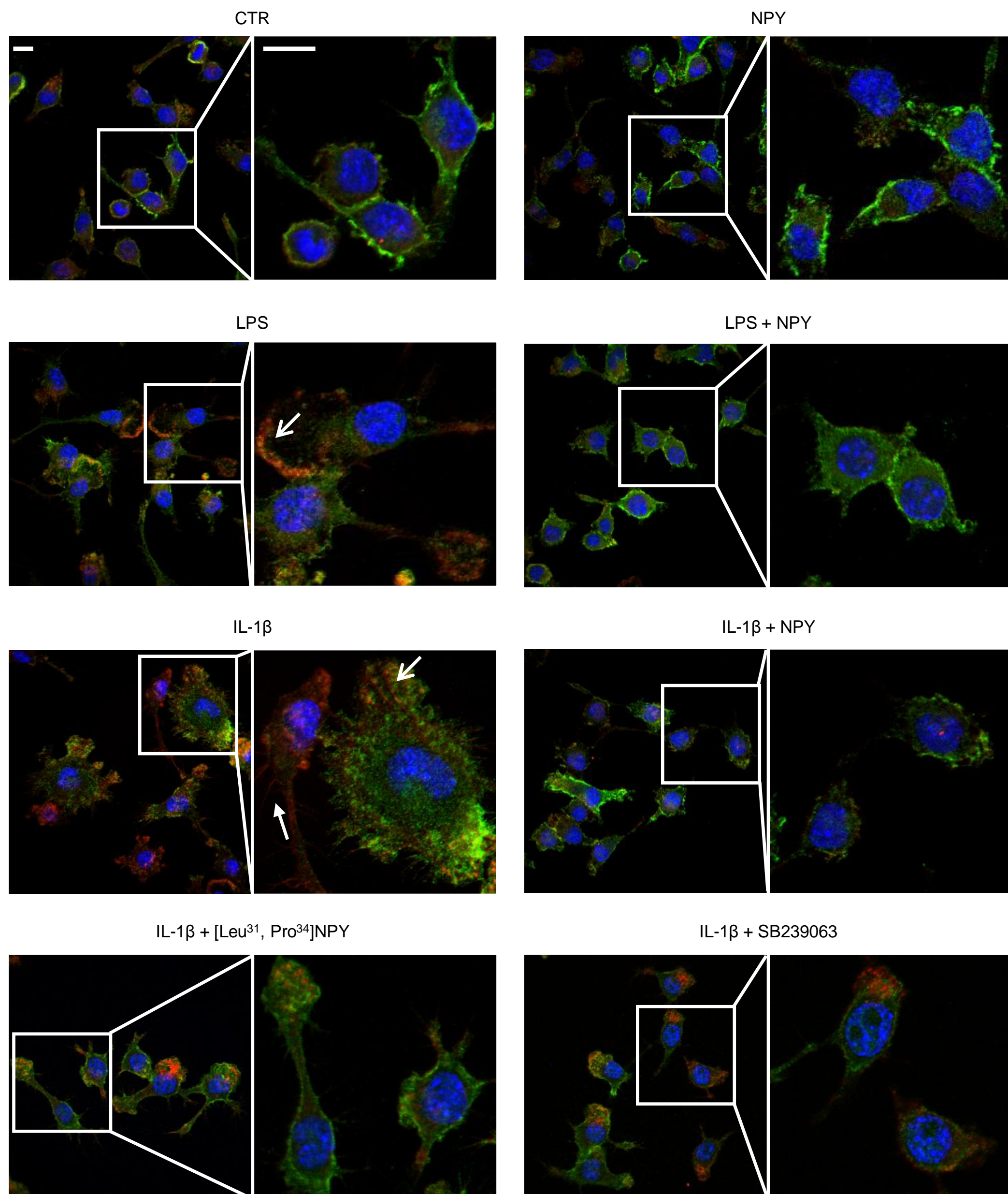


Fig. 4.6. NPY inhibits actin reorganization *via* Y1 receptor activation and through inhibition of p38 activation. (A) Representative confocal microscopy photomicrographs were taken to assess the role of LPS and IL-1β over actin mobilization. Microglial cells treated with 100 ng/ml LPS or 1.5 ng/ml IL-1β showed significant membrane ruffling (open arrowhead in LPS panel) and actin cytoskeleton rearrangement (open arrowhead points to the formation of stress fibers while closed arrowhead points to filopodia, in IL-1β panel). This effect was abolished by 1 μM NPY, or by Y1 receptor agonist, [Leu31, Pro34]NPY (1 μM), as well as in the presence of SB239063 (20 μM). Cells were stained for actin (in red), for CD11b (in green) and Hoechst 33342 (nuclei in blue). Scale bar 10 μm.

4.3. DISCUSSION

Microglial cells are perceived as the resident macrophages of the CNS and, for that reason, play a critical role in the protection and immune surveillance of the brain parenchyma. In response to brain injury, such as infection, trauma, ischemia, brain tumors or neurodegenerative diseases, microglial cells abandon their ramified resting-like morphology and become amoeboid, migrating to the site of injury and releasing several pro-inflammatory and trophic factors (Arend et al. 1990; Bernardino et al. 2005; Garden and Möller 2006; Turrin and Rivest 2006).

Growing evidence suggest that NPY plays an important role in the immune system (e. g. regulation of natural killer cell activity, T helper cell differentiation, B cell homeostasis, leukocyte trafficking), and in the regulation of inflammatory mediator release (e. g. IL-1 and IL-6) (De la Fuente et al. 2001; Bedoui et al. 2003a; Bedoui et al. 2004; Nave et al. 2004; Bedoui et al. 2007; Wheway et al. 2007a). In fact, we have recently reported a possible anti-inflammatory role for NPY concerning the release of IL-1β and the production of NO by microglial cells (Ferreira et al. 2010). These observations support the modulatory role of NPY over microglia response during the development of an inflammatory reaction. To further disclose the immunomodulatory role of NPY in the CNS, we used a LPS experimental model of inflammation to study the effect of this neuropeptide in microglial cell motility. In the present study, we showed that NPY inhibited LPS-stimulated microglia motility, through Y_1R activation. Furthermore, LPS-stimulated microglia motility was mediated by IL-1β signaling. Moreover, microglial cell motility, induced by LPS, involved p38 MAPK signaling.

We have also previously shown that murine N9 microglia cell line expresses NPY and Y_1, Y_2 and Y_5 receptors, validating the use of this biological model in the study the role of NPY on several microglial responses (Ferreira et al. 2010). Microglia respond to various stimuli such as blood-derived factors invading the brain parenchyma and subsequently to the disruption of the blood-brain barrier, extracellular ATP and ADP released from ischemic and traumatic CNS injuries, microbial signals, adhesion molecules, cytokines, complement molecules, among others (Honda et al. 2001; Forstreuter et al. 2002; Allan and Rothwell 2003; Minghetti et al. 2005; Nimmerjahn et al. 2005; Block et al. 2007). In response to activating stimuli, microglial cells migrate to the site of injury or inflammation, where they release inflammatory mediators that promote cell recruitment (including peripheral blood cells). LPS alone is a strong bacterial chemoattractant, acting through TLR4, which ultimately triggers cell migration, phagocytosis and release of inflammatory mediators (Nave et al. 2004; Chen and Pan 2009; Kawai and Akira 2010). For that reason, we stimulated cells with LPS and observed an inhibitory effect of NPY on LPS-induced motility. Using a pharmacological approach, we treated cells with selective Y_1R agonist and selective antagonists for Y_1R, Y_2R and Y_5R, and observed that the inhibitory effect of NPY was mediated by Y_1R activation.

It is also known that in rat primary microglial cultures, extracellular ATP and ADP, acting through purinergic receptors, strongly enhances microglia migration and the concomitant formation of membrane ruffles (Ferrari et al. 1997a; Honda et al. 2001). In addition, LPS-induced activation of TLR4 together with exposure to ATP activates $P2X_7$ receptors, inducing a

substantial release of IL-1β from microglial cells (Ohtani et al. 2000; Bianco et al. 2006). In the present study we showed that LPS and ATP co-administration also stimulate microglia cell motility and this effect was abolished by IL-1ra treatment, suggesting that LPS-induced motility involves IL-1β. Moreover, exogenous application of IL-1β significantly increased cell motility. In the mouse brain, IL-1β released by microglia induces the expression of intercellular adhesion molecule-1 (ICAM-1) by astrocytes, a molecule deeply involved in cell migration (Kyrkanides et al. 1999). The regulation of IL-1β mRNA and protein expression may also occur via a cell density-dependent mechanism, since IL-1β protein levels increased after LPS treatment to BV2 microglial cells at a lower seeding density (Summers et al. 2009). Interestingly, in a experimental autoimmune encephalitis (EAE) model, interleukin-1 receptor-associated kinase-1 (IRAK-1) deficient mice have decreased monocyte/macrophage infiltration to inflamed brain tissues (Deng et al. 2003). Also, when bone marrow-derived macrophages from IRAK-1−/− mice are stimulated with phorbol 12-myristate 13-acetate (PMA) they presented reduced migration (Gan and Li 2010).

NPY exerts important actions in the regulation of inflammatory mediator release, natural killer cell activity, T helper cell differentiation, B cell homeostasis and leukocyte trafficking (Bedoui et al. 2003a). In the present work, we demonstrated that NPY significantly reduced IL-1β-induced motility and this effect was mimicked again using a selective Y_1R agonist, implying that NPY acted *via* Y_1R (similarly to the results obtained under LPS stimulation). However, NPY does not play a straightforward role, having opposite outcomes depending on the cell type, concentration and stimulus duration. Using NPY concentrations ranging from 10^{-12} to 10^{-8} M, De La Fuente and colleagues observed a stimulatory effect of adherence, chemotaxis, ingestion of cells and inert particles, and production of superoxide anion in murine macrophages (De la Fuente et al. 1993). Meanwhile, NPY concentrations ranging from 10^{-10} to 10^{-5} M inhibited macrophage migration induced by *Leishmania major* (Ahmed et al. 1998). Nave and colleagues reported that NPY inhibits tissue immigration of circulating blood monocytes and suggested, through *in vitro* adhesion assays, that NPY increases cell adhesion to blood vessels through Y_2 receptor activation However, the authors showed that peripheral blood mononuclear cells only expressed Y_2 receptor when stimulated with 10 μg/ml of LPS, while pharmacological studies were performed with only 1 μg/ml LPS (where no Y_2 receptor expression was observed) (Nave et al. 2004).

Our findings suggest that microglial cell motility induced by IL-1β is a p38-dependent process. When this pathway was blocked by the inhibitor of p38 MAPK, SB239063, the stimulatory effect of IL-1β was abrogated. Activation of Y_1R also inhibited p38 MAPK phosphorylation stimulated by IL-1β, suggesting that the inhibitory effect of NPY in cell motility possibly occurs upstream to p38 activation.

There are several reports suggesting the involvement of p38 MAPK signaling in the motility of various cell types, such as smooth muscle cells, endothelial cells, neutrophils, mast cells, epithelial cells, to name a few (reviewed by (Huang et al. 2004)). In a study performed by Shen and colleagues, LPS-induced ICAM-1 expression was completely abolished when Schwann cells of sciatic nerves were previously treated with p38 inhibitors (Shen et al. 2008). Moreover, inhibition of p38 MAPK, in a murine model of pulmonary inflammation, caused a decreased

neutrophil migration and accumulation in airspaces due to reduced chemotaxic response (Nick et al. 2000). Conversely, activation of PI3-kinase/PKC/p38 MAPK signalling mediated insulin-like growth factor 1 (IGF-1) acceleration of macrophage migration (Furundzija et al. 2010). Also, migration of monocytes and subsequent activation into tissue macrophages, induced by oxidant stress, is dependent of ERK and p38 MAPK activation (Ogura and Kitamura 1998).

Furthermore, we observed that p38 MAPK is involved in microglial migration and that the inhibition of this pathway prevents the reorganization of actin cytoskeleton. The activation of p38α isoform leads to the phosphorylation of two important proteins involved in migration: caldesmon (participates in the assembly of actin filaments) and paxillin (found at focal adhesions). Alternatively, p38α regulates cell migration by inducing the expression of matrix metalloproteases (MMPs) in LPS-stimulated macrophages and TNF-α-stimulated monocytes (Woo et al. 2004; Nguyen et al. 2006). Nucleation and branching of actin filaments are implicated in vital steps of microglial response to inflammation, such as phagocytosis, vesicle and organelle motility, exocytosis and, most importantly, in the formation of membrane protrusions, adhesion and podosome formation (Ridley et al. 2003). p38 MAPK-activated kinases can act in diverse cellular processes, through the activation of different substrates. Notably, MAPK-activated protein kinase 2 (MK2), is a downstream target of p38 MAPK, and plays an important role in actin filament remodeling inducing the phosphorylation of heat-shock protein 27 (Hsp27) (Shi and Gaestel 2002).

The effect of NPY on microglial cell motility may indicate a protective role for this neuropeptide. Inflammation promotes the recruitment of cells that have the ability to release both toxic and trophic molecules. Reducing the local number of cells could restrain a possible exacerbation of the inflammatory response, thereby maintaining the neuroprotective quality of microglia. Our work unveiled a promising role for NPY in the modulation of microglial function, acting as a repressor of cell motility upon inflammatory challenge. In our study, LPS stimulation induced microglial cell motility *via* IL-1β signaling through a p38-dependent pathway. This effect was inhibited by NPY acting on Y_1R. In summary, these data highlight NPY as a potential drug target candidate concerning the therapy of innate neuroimmune conditions affecting the CNS.

CHAPTER 5. Neuropeptide Y inhibits phagocytosis by microglial cells

5.1. Introduction

In the normal CNS, microglial cells play an active role in the clearance of apoptotic vesicular material, a process that occurs without inflammation. During apoptosis, phosphatidylserine (PtdSer) residues become exposed on the outer leaflet of the plasma membrane, making them available to PtdSer recognizing receptors, expressed by microglial cells (Walter and Neumann 2009). In fact, microglia display a wide range of receptors that mount a concerted response leading to phagocytosis and removal of cell debris (fig. 5.1) (Walter and Neumann 2009).
In broad terms, phagocytosis implies the vesicular ingestion of extracellular particulate material. Phagocytes are attracted toward the site of injury where they recognize pathogens or cell damage-related antigens. Antigens then adhere to the cell membrane promoting the formation of pseudopodia, membrane protrusions that surround the particulate material. Fusion of the pseudopodia encloses the material within large vesicles called phagosomes, entering the endocytic processing pathway. Phagosomes fuse with lysosomes, which contain several hydrolytic enzymes, and form phagolysosomes. Digested contents are finally eliminated out of the cell by exocytosis (Kindt et al. 2006).

5.1.1. Phagocytosis and actin rearrangement

Phagocytosis is an elegantly coordinated process, triggered by environmental signals, and requires a highly dynamic cytoskeleton rearrangement. The core of cell cytoskeleton is composed by three main types of polymers: microtubules; a group of polymers known collectively as intermediate filaments and actin filaments (Fletcher and Mullins 2010). These polymers are distinguished from each other according to their mechanical rigidity, assembly dynamics, polarity, and type of molecular motors with which they associate. Microtubules are the most inflexible of the three polymers, although the microtubule cytoskeleton can be reorganized rapidly allowing individual microtubules to quickly explore the cellular space. Microtubules and microtubule-associated motors (members of the dynein or kinesin families) are particularly involved in mitosis, enabling the assembly of the microtubule array, in interphase, the mitotic spindle and intracellular traffic during cell division (Fletcher and Mullins 2010).
Intermediate filaments are the most flexible and can be crosslinked to either actin filaments or microtubules, by proteins called plectins. These filaments are assembled in response to mechanical stress. Intermediate filaments are not polarized and cannot support directional movement of molecular motors (Fletcher and Mullins 2010).
Actin reorganization is involved in several cell activities, such as lamellipodial and filopodial protusion, podosome formation, vesicle and organelle motility, chemotaxis and phagocytosis (Raven et al. 2004). Both actin filaments and microtubules are polarized polymers, meaning that their subunits are structurally asymmetrical at the molecular level. Myosin motors act on the

actin bundles creating stress fibers, which enable the cells to contract and sense their external environment (Fletcher and Mullins 2010). Microglial cells have specific signaling systems that regulate the rapid assembly/disassembly of the actin cytoskeleton enabling the cell to phagocyte if needed. In other words, microglial cells express distinct types of receptors whose signaling pathways trigger the distribution of actin into branched filaments that support the leading edge of the motile cell and generate the forces involved in cell shape alterations, such as those that occur during phagocytosis (Fox 2006; Walter and Neumann 2009). Ultimately, all phagocytic processes are driven by a highly controlled rearrangement of the actin cytoskeleton.

5.1.2. Microglia and phagocytic receptors

Phagocytes express several types of receptors, mainly opsonin receptors, scavenger receptors and Toll-like receptors (TLRs). In mammals, immunoglobulins bind to foreign particles rendering them more susceptible to engulfment by phagocytic cells. The Fc (fragmented; crystallized) domain of immunoglobulins is recognized by Fc receptors present on phagocytes, and the opsonized particle is internalized. Microglial complement binding receptors are mainly involved in the clearance of neuronal structures predetermined to die (Neumann et al. 2009; Walter and Neumann 2009). For instance, unwanted synapses are opsonized by complement components C1q and C3, therefore becoming tagged for elimination by microglia (Stevens et al. 2007).

Microglial cells also express scavenger receptors (e. g. macrophage receptor with collagenous structure) (MARCO), a group of receptors that recognize modified low-density lipoprotein (LDL), and bind to lipidic membranes of apoptotic cells as well as fibrillary amyloid beta (Aβ) peptide. Upon binding, scavenger receptors cluster on the membrane surface and induce rearrangement of the actin cytoskeleton through different members of the Rho guanosine triphosphate hydrolase (GTPase) family (Castellano et al. 2001; Myers and Casanova 2008).

TLRs are widely expressed by macrophages and microglia, thereby enabling these cells to respond to microbial pathogens. Recognition of pathogen-associated molecular patterns (PAMPs) and subsequent binding triggers TLR signaling through myeloid differentiation primary response gene 88 (MyD88) adapter molecule and activation of MAPKs and NF-κB (Tricker and Cheng 2008). These signaling pathways are critical in the development of the inflammatory response and in the induction of proinflammatory activity. Alternatively, TLR ligands can trigger phagocytosis through a MyD88-independent pathway *via* activation of Rho GTPases Cdc42 and Rac (Tricker and Cheng 2008).

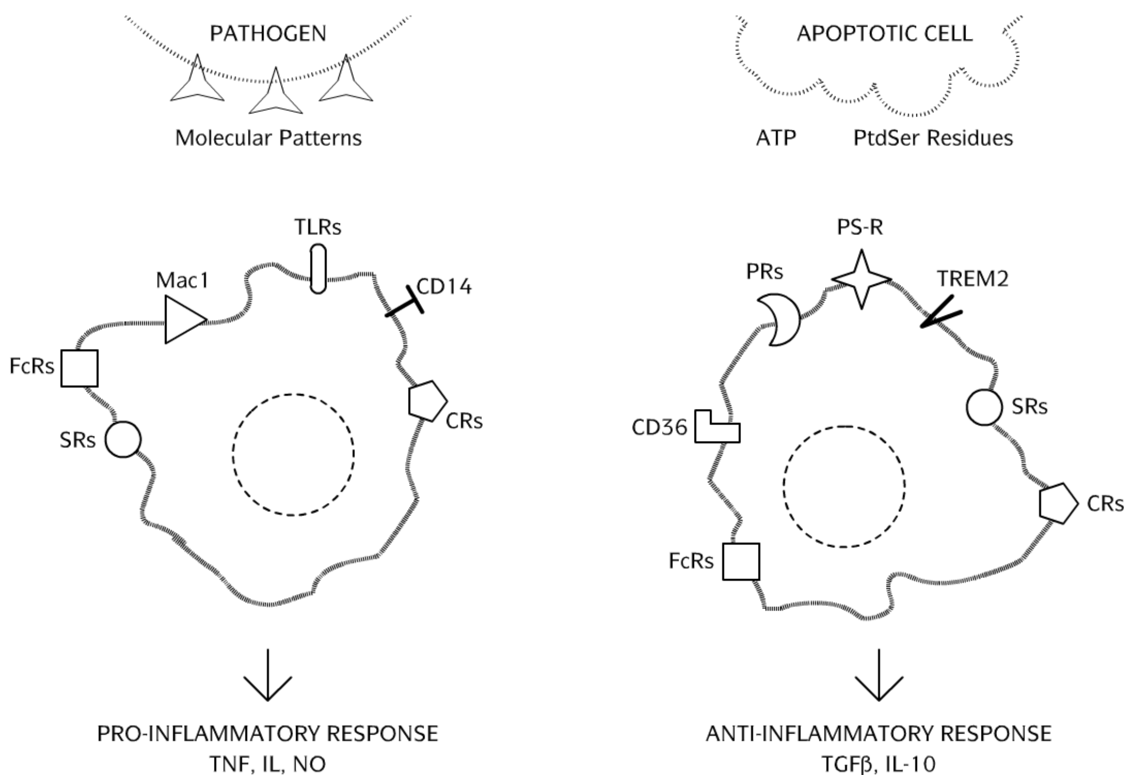


Fig. 5.1. Representative scheme of phagocytosis occurring with or without an inflammatory response. Microglial cells express several receptors able to recognize specific structural patterns present, ultimately triggering the release of pro-inflammatory cytokines. However, in the presence of apoptotic cells, microglia recognize their PtdSer residues, engaging in the phagocytosis of the dying cell, a process that induces the release of anti-inflammatory cytokines. Adapted from (Napoli and Neumann 2009).

5.1.3. Microglia phagocytosis in a pathological context

In acute CNS injury, cell death may lead to deposition of tissue debris, which does not subsist if macrophages proceed with efficient clearance. In particular, efficient remyelination depends critically on the efficiency of microglial cells in the removal of myelin debris around the axons of demyelinating neurons. In fact, insufficient microglial clearance detains oligodendrocyte differentiation and reduces the recruitment of oligodendrocyte precursor cells, suggesting that phagocytosis of myelin debris by microglia is critical for neuronal repair (Kotter et al. 2006). Moreover, active microglia/macrophages are attracted by myelin degradation products or lysosomal lipids in multiple sclerosis (Li et al. 1993; Bruck et al. 1995; Barnett et al. 2006; Merson et al. 2010). In experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, blockade of microglial triggering receptor expressed on myeloid cells-2 (TREM2) results in disease exacerbation, with increased permeability and demyelination of the brain parenchyma. These receptors are microglia/macrophage specific membrane-bound receptors involved in the reduction of inflammation and promotion of phagocytosis (Piccio et al. 2007). On the other hand, intravenous application of TREM2 positive cells promote EAE recovery of the spinal cord, suggesting that endogenous resident microglia are not enough efficient concerning debris clearance (Takahashi et al. 2007).

In chronic degenerative diseases such as Alzheimer's disease (AD), microglial cells are associated with the appearance of Aβ plaques. Microglial cells play a neuroprotective role in AD through the secretion of proteolytic enzymes that degrade Aβ deposits enabling their clearance (El Khoury et al. 2007; Meyer-Luehmann et al. 2008; Napoli and Neumann 2009). Additionally, ageing microglia are less effectively recruited and their phagocytic activity is reduced which may ultimately contribute to the exacerbation of chronic neurodegenerative diseases (e. g. AD, by increasing Aβ plaque load).
Moreover, dying cells in the CNS produce and release signaling molecules that promote the recruitment of microglia and other phagocytes. Different nucleotides such as ATP and uridine triphosphate (UTP) are released by damaged neurons. In particular, microglial $P2Y_6$ receptors recognize UDP released from injured neurons and stimulate microglial removal of cell debris (Koizumi et al. 2007). Recently, an extensive report revealed that purinergic receptor agonists ATP, adenosine diphosphate (ADP), UTP, UDP, α,β-methylene ATP and 3'-O-(4-benzoyl)benzoyl ATP increased phagocytosis of latex beads. In addition, these nucleotides up-regulated the expression of receptor involved in the recognition and internalization of apoptotic cells, such as CD11b/CD18 and vitronectin receptor (αvβ3, CD51/CD61). Interestingly, nucleotides had no effect on adhesion of viable cells (Marques-da-Silva et al. 2010).

IL-1β is one of the first inflammatory mediators released by activated microglia. This well known pro-inflammatory cytokine is involved in excitotoxicity, ischemia, brain trauma, inflammation and cell death (Bernardino et al. 2005; Vezzani and Granata 2005; Allan 2005a)As mentioned before, IL-1β is a key player of LPS-induced inflammation, namely by triggering the production of nitric oxide and enhancing microglia motility. Furthermore, as we show, neuropeptide Y (NPY) is a strong inhibitor of these microglial actions, supporting an anti-inflammatory role for this neuropeptide. In the present chapter, we assign a novel role for NPY in the regulation of important microglial responses to danger signals in the brain, involving phagocytosis.

5.2. RESULTS

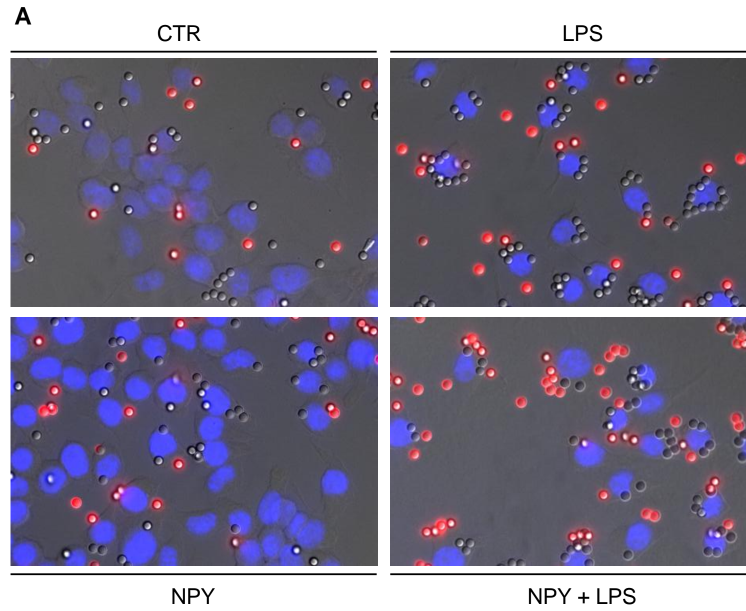
5.2.1. NPY inhibits bead phagocytosis by microglial cells

Murine N9 microglia cell line was used to disclose the role of NPY in endotoxin-induced phagocytosis. LPS is a component of Gram-negative bacteria outer membrane and binds to CD14/TLR4/MD2 receptor complex present at the cell membrane, triggering several signaling cascades (Cohen 2002). We have already used this cell line to dissect the effects of LPS over microglial physiological responses, such as production of inflammatory mediators (e. g. nitric oxide (NO) and IL-1β) and migration/motility, and we observed that NPY-mediated inhibition of microglia activation occurred via Y_1 receptor (Ferreira et al. 2010).

Prior to bead incorporation, microglial cells were challenged with LPS (100 ng/ml) and NPY (1 μM) for 6 hrs. Following opsonization, beads were added at a density of $1x10^5$ per well and left for 20 min for incorporation. After fixation, beads that remained free in the coverslip, solely adherent to the cell surface, or not completely engulfed, were available for immnunolabelling. Therefore, phagocytosed beads were distinguished from non-phagocytosed beads on account of fluorescent labeling (none versus red, respectively) (fig. 5.2. A). LPS significantly increased bead phagocytosis, while NPY inhibited this effect ($mean_{CTR} = 100 \pm 29.46\%$, $n = 5$; $mean_{LPS} = 730.50 \pm 74.02\%$, $n = 3$; $mean_{LPS+NPY} = 250 \pm 23.47\%$, $n = 3$; $p < 0.001$) (fig. 5.2. B).

5.2.2. LPS-induced phagocytosis involves IL-1β-mediated signaling

LPS and ATP co-administration induces a massive release of IL-1β (Griffiths et al. 1995; Ferrari et al. 1997b; Grahames et al. 1999; Bernardino et al. 2008). In fact, we have previously shown that murine N9 microglial cells release the biologically active form of interleukin-1β (IL-1β) upon LPS and ATP challenge (Ferreira et al. 2010). LPS activates TLR4, triggering several inflammatory responses, while ATP exposure causes $P2X_7$ receptors to form a non-selective pore leading to a massive calcium entry that consequently activates interleukin converting enzyme (ICE) (Ferrari et al. 1997a; Abreu and Arditi 2004). In our study, LPS (100 ng/ml) plus ATP (1 mM) significantly stimulated bead phagocytosis ($mean_{CTR} = 100 \pm 29.46\%$, $n = 5$; $mean_{LPS+ATP} = 699.50 \pm 58.33\%$, $n = 3$; $p < 0.001$). Interestingly, and surprisingly, this effect was completely abolished by IL-1ra treatment (150 ng/ml), suggesting the involvement of IL-1β in LPS-induced microglia phagocytosis ($mean_{CTR} = 100 \pm 29.46\%$, $n = 5$; $mean_{LPS+ATP+IL\text{-}1ra} = 113.90 \pm 19.02\%$, $n = 3$; $p < 0.001$). Accordingly, IL-1β (1.5 ng/ml) significantly increased microglial cell phagocytosis, which was also inhibited by exposure to IL-1ra ($mean_{IL\text{-}1\beta} = 685.90 \pm 36.37\%$, $n = 3$; $mean_{IL\text{-}1\beta+IL\text{-}1ra} = 198 \pm 37.10\%$, $n = 3$; $p < 0.001$) (fig. 5.3. B). In fig. 5.3. A, representative photomicrographs illustrate the stimulatory effect of IL-1β in microglia phagocytosis, as well as LPS and ATP, and the inhibitory effect of IL-1ra on in these events.



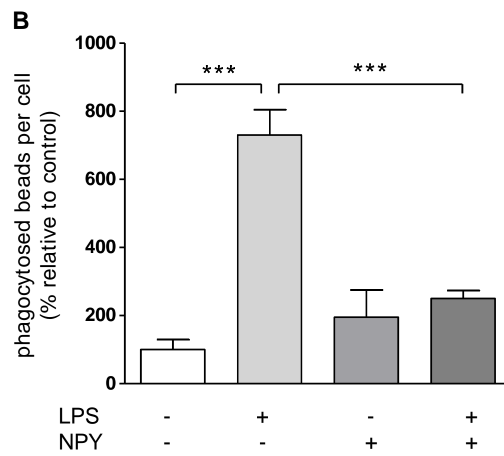
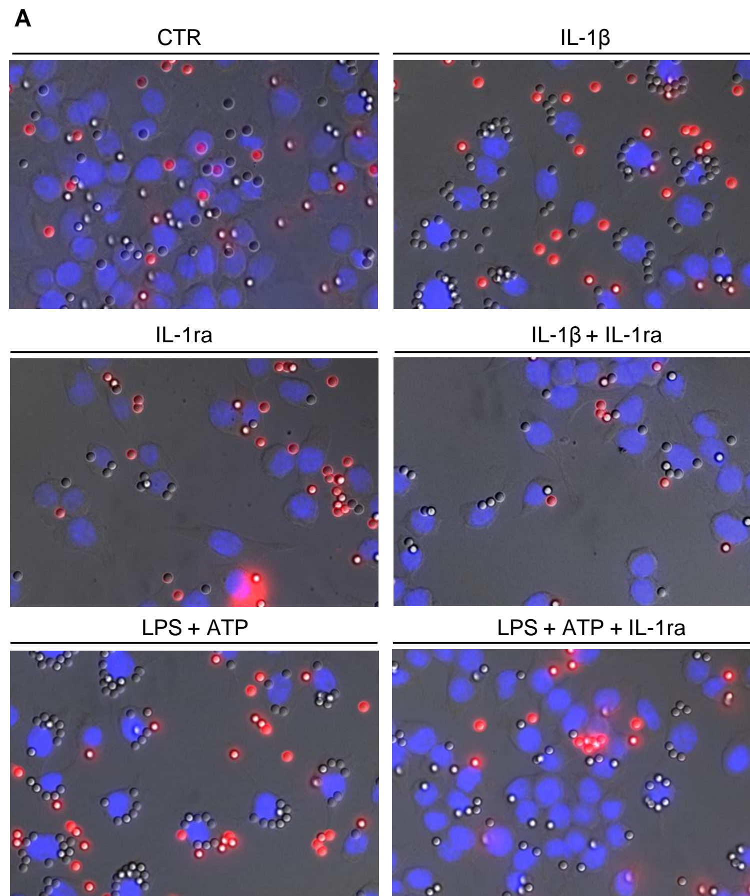


Fig. 5.2. NPY inhibits bead phagocytosis by microglial cells. (A) Representative photomicrographs illustrate the inhibitory effect of NPY on LPS-induced phagocytosis. (B) LPS (100 ng/ml) increased bead phagocytosis, while NPY (1 μM) inhibited this effect. Data are expressed as mean ± SEM (n=3-5) and as a percentage of control (***$p < 0.001$, using Bonferroni's Multiple Comparison Test).



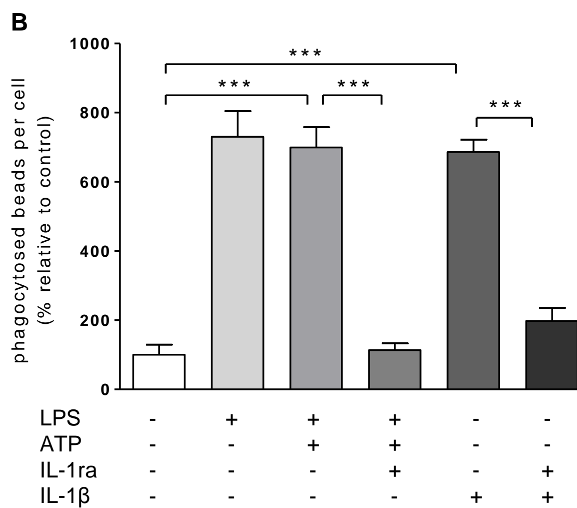
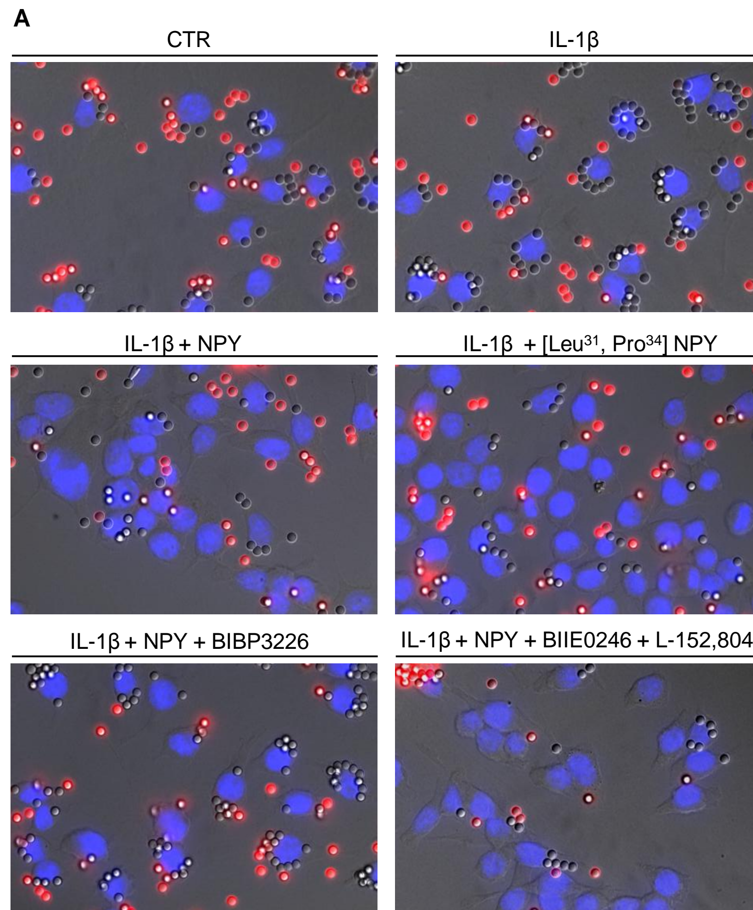
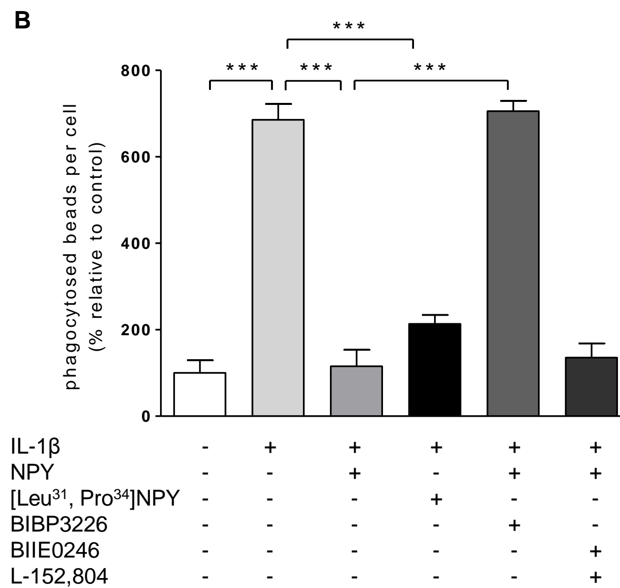


Fig. 5.3. LPS-induced phagocytosis is mediated by IL-1β release. (A) Representative photomicrographs illustrate the inhibitory effect of IL-1ra on LPS-induced phagocytosis and the stimulatory effect of IL-1β in cell phagocytosis. (B) LPS (100 ng/ml) plus ATP (1 mM) significantly induced bead phagocytosis. LPS-induced phagocytosis was prevented by IL-1ra application (150 ng/ml) suggesting the involvement of IL-1β. IL-1β (1.5 ng/ml) increased phagocytosis was completely inhibited by IL-1ra. Data are expressed as mean ± SEM (n=3-5) and as a percentage of control (***p < 0.001, using Bonferroni's Multiple Comparison Test).

5.2.3. NPY inhibits IL-1β-stimulated phagocytosis *via* Y_1 receptor activation

In accordance with the previous experiments, microglial cells were stimulated with IL-1β (1.5 ng/ml) and treated with NPY (1 μM). As a result, we observed that NPY inhibited IL-1β-induced phagocytosis ($mean_{IL\text{-}1\beta}$ = 685.90 ± 36.37%, n = 3; $mean_{IL\text{-}1\beta+NPY}$ = 115.40 ± 37.99%, n = 3; $p < 0.001$). Moreover, to assess through which receptor NPY inhibited microglial phagocytic activity, cells were treated with the Y_1 receptor agonist [Leu^{31}, Pro^{34}]NPY (1 μM) and Y_1 receptor antagonist BIBP3226 (1 μM). Y_1 receptor activation resulted in the inhibition of IL-1β-induced phagocytosis while the Y_1 receptor antagonist blocked the effect induced by NPY ($mean_{IL\text{-}1\beta+[Leu,Pro]NPY}$ = 213.70 ± 20.37%, n = 3; $mean_{IL\text{-}1\beta+NPY+BIBP3226}$ = 705.90 ± 23.89%, n = 3; $p < 0.001$). The involvement of other receptors was excluded with the use of selective antagonists for Y_2 receptor (BIIE0246, 1 μM) and for Y_5 receptor (L152-804, 1 μM), since in the presence of both antagonists NPY was still able to inhibit bead incorporation ($mean_{IL\text{-}1\beta+NPY+BIIE0246+L152\text{-}804}$ = 135.70 ± 32.65%, n = 3) (fig. 5.4. B). Representative photomicrographs illustrate the inhibitory effect of Y_1 receptor activation on IL-1β stimulated phagocytosis in microglial N9 cells (fig. 5.4. A).





IL-1β	-	+	+	+	+	+
NPY	-	-	+	-	+	+
[Leu³¹, Pro³⁴]NPY	-	-	-	+	-	-
BIBP3226	-	-	-	-	+	-
BIIE0246	-	-	-	-	-	+
L-152,804	-	-	-	-	-	+

Fig. 5.4. NPY inhibits IL-1β-induced phagocytosis *via* Y1 receptor activation. (A) Representative photomicrographs illustrate the inhibitory effect of NPY *via* Y1 receptor on IL-1β-induced cell phagocytosis. (B) Microglia cells were stimulated with IL-1β (1.5 ng/ml) and treated with NPY (1 μM). As NPY inhibited IL-1β-induced phagocytosis, a selective Y1 receptor agonist [Leu31, Pro34]NPY (1 μM) and a selective Y1 receptor antagonist BIBP3226 (1 μM) were used to determine the effect of Y1R activation in IL-1β-induced phagocytosis. The involvement of other receptors was excluded with the use of selective antagonists for Y2 receptor (BIIE0246, 1 μM) and for Y5 receptor (L152-804, 1 μM). Data are expressed as mean ± SEM (n=3-5) and as a percentage of control (***p < 0.001, using Bonferroni's Multiple Comparison Test).

5.3. DISCUSSION

Microglial cells are the resident macrophages of the Central Nervous System (CNS) and, in response to brain injury, migrate and release several pro-inflammatory and trophic factors at the site of injury (Arend et al. 1990; Bernardino et al. 2005; Garden and Möller 2006; Turrin and Rivest 2006). Increasing evidence have appointed NPY as an important regulator of immune system function and in the regulation of inflammatory mediators release (e. g. IL-1 and IL-6) (De la Fuente et al. 2001; Bedoui et al. 2003a; Bedoui et al. 2004; Nave et al. 2004; Bedoui et al. 2007; Wheway et al. 2007a). In the previous chapter, we reported the role of NPY in the inhibition of microglial cell motility induced by interleukin-1β (IL-1β). To further unveil the role of NPY in the modulation of microglial response, we used an experimental model of microglial phagocytic activity induced by LPS. In our study, we showed that NPY prevented LPS-induced microglia phagocytosis of latex beads. This process was shown to occur *via* IL-1β and was inhibited by Y_1 receptor activation.

In our study, we used murine N9 microglial cell line as a biological model to evaluate the role of NPY in phagocytosis. Microglial cells are constantly prowling the brain environment. In order to effectively identify an invading pathogen, microglia express a vast array of pattern recognition receptors, such as Toll-like receptors (Garden and Möller 2006; Block et al. 2007). Among the different members of TLR family, TLR4 is the best characterized one. TLR4 recognizes LPS, a component of the outer membrane of Gram-negative bacteria (Kawai and Akira 2010). Accordingly, we observed that LPS significantly enhanced bead phagocytosis by microglial cells, while NPY inhibited this effect. Then, we proposed to uncover if the stimulatory action of LPS was mediated *via* IL-1β. We have gathered increasing evidence concerning the modulatory role of NPY in LPS-induced inflammation and, as a result, we observed that LPS challenge triggers several microglial responses *via* IL-1β. Accordingly, upon stimulation with LPS, IL-1β is involved in the production and release of nitric oxide by microglial cells (Dinarello 2009; Pinteaux et al. 2009). Moreover, we have shown that LPS and ATP co-administration stimulate microglia cell motility and this effect is abolished by IL-1ra treatment, suggesting that LPS-induced motility involves IL-1β (Ferreira et al. 2010). Therefore, we co-treated cells with LPS and ATP, which was shown to increase bead phagocytosis, while IL-1ra treatment abolished this effect suggesting that this process is mediated by IL-1β.

Macrophage infection with *Shigella flexneri* or *Shigella typhimurium* occurs with the delivery of virulence proteins IpaB and SipB, respectively. These proteins bind and directly activate caspase-1 inducing the release of pro-inflammatory cytokines IL-1β and IL-18. In addition, activation of caspase-1 by these bacterial proteins triggers apoptosis of the infected macrophages (Weinrauch and Zychlinsky 1999). Studies made with caspase-1-deficient mice have demonstrated that the release of IL-1β and IL-18 is vital for resolving the bacterial infection (Sansonetti et al. 2000). Ultimately, bacterial infection results in the amplification of the release of IL-1β and IL-18, which culminates in local tissue damage and enhanced cell recruitment to the infection site (Hersh et al. 1999; Monack et al. 2000). Ultraviolet light induces lymphocyte apoptosis, an event followed by its phagocytosis by macrophages. Interestingly, this step induces

the production of endogenous anti-inflammatory cytokine IL-1ra, peaking at 16h after irradiation (a period during which levels of apoptotic and necrotic cells increased fivefold). Cytochalasin, which causes actin filament depolymerization, inhibited phagocytosis as well as IL-1ra production (Craciun et al. 2005). Since LPS induces the release of IL-1β, it is possible that during LPS-induced phagocytosis, the production of IL-1ra is increased to balance possible cytotoxic effects of IL-1β.

In the present study, we showed that NPY inhibited IL-1β-induced phagocytosis *via* Y_1 receptor activation. *In vitro* studies have described the role of NPY in the modulation of various functions of macrophages, such as adherence, chemotaxis, phagocytosis and superoxide anion production (De la Fuente et al. 1993; Dureus et al. 1993; Ahmed et al. 1998; Medina et al. 2000; De la Fuente et al. 2001). As described in chapter 1, we have assigned a new role for NPY in microglia motility and nitric oxide production (Ferreira et al. 2010).

In an extensive work performed by De la Fuente and colleagues, NPY was shown to modulate several peritoneal macrophage functions, including phagocytosis of latex beads. The group used different concentrations of NPY (ranging from 10^{-13} to 10^{-7} M) and mice from four age groups: young (12±2 weeks), adult (24±2 weeks), mature (50±2 weeks) and old (72±2 weeks). Results showed that NPY increased phagocytosis in macrophages from adult and from mature mice (particularly at higher concentrations), while it decreased the phagocytosis of latex beads by old mice. Interestingly, the release of IL-1β was higher in older animals, and NPY acted by stimulating the release of this cytokine in adults while inhibiting it in old mice (De la Fuente et al. 2001). The role of NPY in the regulation of phagocytosis seems to depend on the pathogen studied and their mechanism of replication. *In vitro* studies showed that NPY can increase phagocytosis of *Candida albicans* by isolated murine peritoneal macrophages (De la Fuente et al. 1993), while it inhibits the engulfment of *Leishmania major* by monocyte/macrophage murine cell line (Ahmed et al. 2001). Since infection of phagocytes is a crucial step for the replication of *Leishmania major* inhibiting phagocytosis results in a protective action (Gregory and Olivier 2005).

The effect of NPY varies according to several parameters (e. g. concentration, target cell, stimulus) and may also depend on the interaction between the type of cells present under experimental conditions. In a peritoneal cell suspension (where lymphocytes are also present) from adult mice, NPY increases phagocytosis. However, phagocytosis is inhibited by NPY in old mice. Noteworthy, isolated macrophages from adult mice respond to NPY by decreasing phagocytosis, while isolated cells from old mice respond in an opposite manner (De la Fuente et al. 2001). NPY also regulates human neutrophil ability to phagocytose gram-negative bacteria. Higher concentrations of NPY (10^{-6}-10^{-5} M) inhibit phagocytosis of *Escherichia coli*, while low concentrations of NPY (10^{-12} M) significantly enhance phagocytosis through both Y_1 and Y_2 receptor activation (Bedoui et al. 2008). Y_1 receptor is widely expressed by different cells of the immune system, including dendritic cells, macrophages, T and B lymphocytes, among others (Wheway et al. 2005). Given the bimodal role of NPY in phagocytosis carried by neutrophils and monocytes/macrophages, it will be an arduous task to ascertain which receptors are involved (Bedoui et al. 2003a; Bedoui et al. 2007; Bedoui et al. 2008).

The interaction between a pathogen and microglia/macrophage may trigger several signaling pathways, namely, tyrosine kinase (interferon-γ signaling), serine kinase (mitogen-activated protein kinase signaling), small GTPase and lipid signaling pathways. In our previous results, p38 MAPK was shown to be involved in actin reorganization, a necessary step for membrane ruffling and the formation of membrane protrusions. Since cytoskeleton remodeling is vital for cell phagocytosis, p38 could be a putative molecular target to disclose which signaling pathways are implicated in this process. There are several reports implicating p38 activation in phagocytosis, whether as a consequence of phagocytosis or as a necessary step to initiate this process (McLeish et al. 1998; Shiratsuchi and Basson 2005; Cui et al. 2009; Shinzawa et al. 2009). In fact, Blander and colleagues have shown that the use of selective p38 inhibitors impairs the ability of macrophages to phagocyte *E. coli* (Blander and Medzhitov 2004). Moreover, upon activation, phosphorylated p38 translocates to the nucleus and phosphorylates MAPK-activated protein kinase 2 (MK2) (Ben-Levy et al. 1995). Cells deficient in MK2 are unable to regulate actin reorganization and, therefore, to form membrane protrusions (Kotlyarov et al. 2002). MK2 modulation of phagocytosis may occur through small heat shock protein HSP25/27 since it regulates actin polymerization (Benndorf et al. 1994).

The work presented in this chapter is still in an early stage of development. However, the role of NPY in the regulation of microglial cell phagocytosis is promising since this neuropeptide was able to strongly inhibit LPS-induced phagocytosis. In our study, LPS stimulation induced microglial cell phagocytosis *via* IL-1β signaling and this effect was inhibited by NPY acting through Y_1 receptor activation. Therefore, NPY may act as an important regulator of microglia function in order to control the possible exacerbation of an inflammatory response occurring in the CNS.

CHAPTER 6. GENERAL DISCUSSION

In response to brain injury and inflammation, microglia cells become activated, migrating to the site of injury, where they undertake phagocytosis of cellular debris and release both neurotoxic and neurotrophic factors (Hagberg and Mallard 2005; Garden and Möller 2006; Block et al. 2007; Glezer et al. 2007; Napoli and Neumann 2009). Neuropeptide Y (NPY) is widely distributed in the Central and Peripheral Nervous System and detains important physiological roles (Silva et al. 2002). Moreover, our group has developed relevant work uncovering the neuroprotective, antiepileptic and proneurogenic role of NPY (for review see (Xapelli et al. 2006)). Additionally, growing evidence support an immunomodulatory role for NPY (Bedoui et al. 2003a; Prod'homme et al. 2006; Bedoui et al. 2007; Wheway et al. 2007a).
In that sense, we discuss in the present thesis, the involvement of NPY in the regulation of several aspects of microglial response to inflammation, namely the production of inflammatory mediators, cell motility and phagocytosis.

6.1. Role of NPY in the modulation of LPS-induced release of nitric oxide (NO) and interleukin-1β (IL-1β) and the involvement of NF-κB signaling

The inflammatory response is characterized by a very coordinated chain of events that initiate with the interaction between a pathogen and microglia/macrophage, unfolding into the activation of various signaling pathways, which ultimately lead to the expression of cytokine genes. These molecules, along with chemokines, promote the recruitment of cells to the site of infection and coordinate their responses to remove the pathogen (Rosenberger and Finlay 2003).
We investigated whether NPY could play a role in the production and release of inflammatory mediators such as NO and IL-1β, by microglial cells. Following LPS challenge (100 ng/ml), we measured the intracellular production of NO and observed that the production of this gaseous molecule was increased. However, when cells were treated with NPY (1μM), this effect was inhibited. Considering that NPY could be acting upon inducible nitric oxide synthase (iNOS) expression, we analyzed iNOS expression under the same experimental conditions. Using confocal microscopy and Western blotting analysis, we observed that NPY inhibited iNOS expression under LPS challenge.
Also, when microglial cells were co-stimulated with LPS and adenosine triphosphate (ATP, 1 mM) cells responded with a massive release of IL-1β, as measured by ELISA. Most importantly, IL-1β is a well-known pro-inflammatory cytokine with relevant actions over neuronal excitability and cell survival. In addition, IL-1β (1.5 ng/ml) stimulation induced NO production, a response prevented in the presence of a selective IL-1 receptor antagonist (IL-1ra, 150 ng/ml). Interestingly, IL-1β-induced NO production was inhibited by NPY treatment. Pharmacological studies with a selective Y_1 receptor agonist ([Leu^{31},Pro^{34}]NPY, 1 μM) and selective antagonists for receptors Y_1 (BIBP3226, 1 μM), Y_2 (BIIE0246, 1 μM) and Y_5 (L-152,804, 1 μM) demonstrated that NPY inhibition was mediated exclusively through Y_1 receptor activation.

Furthermore, we observed that NPY inhibited IL-1β release and downstream nuclear translocation of NF-κB, a transcriptional factor implicated in iNOS expression and subsequent NO synthesis. NF-κB activation is also involved in human inflammatory diseases such as rheumatoid arthritis, atherosclerosis, asthma, multiple sclerosis among others (Tak and Firestein 2001).

6.2. Role of NPY in the modulation of IL-1β-induced microglial motility and the involvement of p38 MAPK signaling pathway

Adequate chemotaxis allows the phagocyte to reach and accumulate at the inflammatory focus and it is an essential step for later accomplishing phagocytosis of foreign or damaged molecules.
We sought to investigate whether NPY could be involved microglial cell motility and which signaling pathways underlined this process. Using a scratch wound assay, we observed that LPS (100 ng/ml) stimulation of microglial cells stimulated motility, and that this effect was blocked by NPY treatment (1 μM). Interestingly, co-stimulation with LPS (100 ng/ml) and ATP (1 mM) also resulted in increased cell motility, an effect in turn inhibited by IL-1ra (150 ng/ml) application. To further disclose the involvement of IL-1β in motility, cells were treated directly with IL-1β (1.5 ng/ml). Accordingly, IL-1β induced microglial motility.
We also investigated the involvement of p38 MAPK signaling pathway in cell motility. Consequently, we observed that IL-1β-induced microglial motility was inhibited by SB239063 (20 μM), a selective inhibitor of p38 MAPK. IL-1β (1.5 ng/ml) also induced p38 MAPK phosphorylation and translocation to the nucleus. p38 MAPK signaling pathway is implicated in several physiological processes such as angiogenesis and cell differentiation, as well as pathological conditions such as cancer, heart failure, metabolic and neurodegenerative diseases (Cuenda and Rousseau 2007). Since aberrant cell migration can lead, for instance, to tumor growth, invasion and metastasis, p38 and its substrates become interesting targets to control disease development. Using confocal microscopy and Western blotting analysis, we were able to observe that NPY inhibited IL-1β-induced p38 activation *via* Y_1 receptor activation. Given the relevant role of p38 in cell motility and considering that this event requires the reorganization of actin cytoskeleton, we sought to uncover the effect of p38 MAPK inhibition in cell morphology. As expected, SB239063 (20 μM) treatment decreased the extent of actin filament reorganization occurring during plasma membrane ruffling.

6.3. Role of NPY in the regulation of IL-1β-stimulated microglial cell phagocytosis

CNS environment is constantly under surveillance by microglia, which express multiple and diverse membrane receptors, enabling the discrimination between subtle differences in molecules from different pathogens. Consequently, the appropriate response to remove the threat can be mounted.

Given the effect of LPS and IL-1β in actin reorganization and membrane ruffling, we investigated whether LPS or IL-1β could stimulate bead phagocytosis in our biological model. We observed that LPS (100 ng/ml), as well as co-administration of LPS (100 ng/ml) and ATP (1 mM) increased latex bead phagocytosis. When cells were pre-treated with IL-1ra (150 ng/ml), bead phagocytosis was inhibited which strongly suggested the involvement of IL-1β. Moreover, direct application of IL-1β (1.5 ng/ml) augmented the number of phagocytosed beads. These actions were inhibited in the presence of NPY (1 μM). Again, performing a pharmacological approach to disclose which NPY receptor was responsible for the inhibition of phagocytosis, we observed that NPY inhibited IL-1β *via* Y_1 receptor activation.

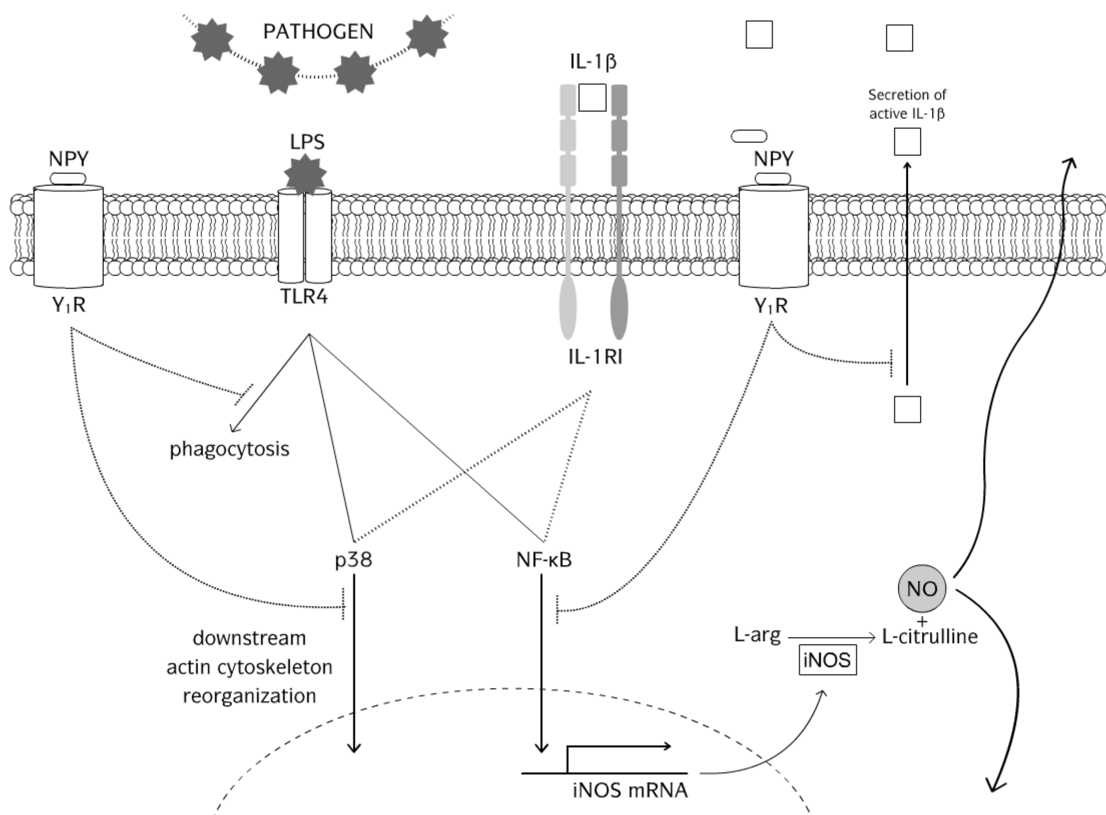


Fig. 6.1. Integrative scheme illustrating main findings. LPS binds to TLR4, triggering the activation of NF-κB signaling pathway, ultimately leading to its nuclear translocation and transcription of iNOS mRNA. iNOS catalyzes arginine conversion to citrulline followed by the release of nitric oxide (NO). LPS stimulation also promotes the release of IL-1β, which is then free to bind to IL-1RI. NPY, acting on Y_1 receptor, inhibits both IL-1β release and NF-κB nuclear translocation, affecting iNOS synthesis and NO production. LPS binding to TLR4 also results in the activation of p38 signaling, a pathway involved in the promotion of cell motility. IL-1β signaling is also involved in LPS-induced motility. NPY inhibits both LPS- and IL-1β-induced p38 activation. Additionally, Y_1 receptor activation inhibits LPS-stimulated phagocytosis.

Uncovering the anti-inflammatory role of NPY may extend our understanding over the crosstalk between the CNS and the immune system. For this reason, regulating microglia function may unveil therapeutic targets for the prevention of neurological dysfunction in a variety of CNS injuries and chronic diseases.

6.4. MAIN CONCLUSIONS

6.4.1. LPS increases the expression of inducible nitric oxide synthase (iNOS), as well as the production of NO in N9 murine microglial cells.

6.4.2. LPS and ATP co-stimulation of N9 murine microglial cells lead to a massive release of IL--1β.

6.4.3. LPS and ATP co-stimulation induce NO production, a response prevented in the presence of selective IL-1 receptor antagonist (IL-1ra), strongly suggesting the involvement of IL-1β.

6.4.4. Direct IL-1β stimulation increases the expression of inducible nitric oxide synthase (iNOS), production of NO and promotes nuclear translocation of nuclear factor-kappaB (NF--κB) in N9 murine microglial cells.

6.4.5. LPS-induced NO production mediated by IL-1β occurs through a nuclear factor-kappaB (NF-κB)-dependent pathway.

6.4.6. NPY inhibits LPS- and IL-1β-induced NO production, as well as nuclear translocation of NF-κB and iNOS expression, *via* Y_1 receptor activation.

6.4.7. In N9 murine microglial cells, LPS increases cell motility in a process that involves IL-1β signaling.

6.4.8. IL-1β-induced cell motility is inhibited by SB239063, a selective inhibitor of p38 MAPK.

6.4.9. IL-1β-induced p38 MAPK phosphorylation and nuclear translocation is inhibited by NPY *via* Y_1 receptor activation.

6.4.10. p38 MAPK inhibition decreases actin filament reorganization and membrane ruffling induced by IL-1β. Actin cytoskeleton reorganization is also prevented in the presence of NPY, acting through Y_1 receptor.

6.4.11. LPS stimulation increases latex bead phagocytosis by N9 murine microglial cells.

6.4.12. LPS-stimulated bead phagocytosis is blocked by IL-1ra application, suggesting the involvement of IL-1β in this process.

6.4.13. IL-1β-induced phagocytosis is inhibited by NPY, acting through Y_1 receptor activation.

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