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Involvement of the Prokineticin system in animal models of inflammation/neuroinflammation

PhD thesis of:

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…to my father

SUMMARY

1. INTRODUCTION

1.1. Role of cytokines and chemokines in peripheral inflammation and neuroinflammation

Immune and nervous system have evolved to provide regulation of physiological homeostasis and to protect against threats (*González H. et al., 2014*): the role of immune system is to defend the organism against infection and injury, whereas, the nervous system integrates biological functions and provides a nearly instantaneous homeostatic control mechanism by releasing neurotransmitters and other regulatory molecules (*Chavan S. S. et al., 2017*). This cross-talk is suggested by the presence of neurotransmitters receptors (such as acetylcholine and adrenergic receptors) on the surface of many immune cells (like macrophages, dendritic cells, T cells and others) and, in turn, the ability of immune cells to synthesize and release substances classically designated as neurotransmitters and neuromodulators (acetylcholine, dopamine and other catecholamines) (*Rosas-Ballina et al., 2011; Kawashima et al., 2012; Marino and Cosentino, 2013*).

To defend the organism from damages, the immune system can launch two types of responses:

- -innate
- -adaptive

Innate response includes all defence mechanisms that are encoded in the germline genes and are mediated by Pattern Recognition Receptors (PRRs, such as Toll-like receptors, TLRs) (*Futosi et al., 2013*). It acts very quickly, because the recognition molecules are already expressed on the surface of many cells. Adaptive immune response is characterized by somatic immunoglobulin chains rearrange which allows

a very specific interaction between the target antigen (not only prokaryotic and eukaryotic but also viral antigen) and the antigen-specific receptors expressed on the surfaces of T and B lymphocytes (*Schatz, D. G. et al., 1992*). It is slower acting when compared to the innate response: initially, it produces a small number of cells able to recognize specific antigens that progressively proliferate until a sufficient number to conduct an effective immune response is reached.

Although the innate and adaptive immune systems are described as contrasting separate arms of the host response, they usually act together: in both systems many of the soluble mediators, which regulate and amplify the ~~immune~~ response, are the same. These mediators are proteins and small peptides that could be constitutively present in biologic fluids (such as the component of complement system) or released from activated immune cells (*Medzhitov R., 2007*).

The principal soluble mediators of two responses are cytokines and chemokines: cytokines (cyto, from Greek "κύτος" kytos "cavity, cell" plus kines, from Greek "κίνηση" kinisi "movement") are a broad category of small proteins (about 5–20 kDa) that are important in cell signalling, and chemokines (Greek -kinos, movement) belong to cytokines family with interferons (INF) interleukins (IL), lymphokines (L) and tumour necrosis factors (TNF); their name is derived from their ability to induce directed chemotaxis in nearby responsive cells. Cytokines and chemokines are produced by several types of cells (including macrophages, T and B lymphocytes, mast cells, endothelial cells and fibroblasts) and they are usually classified by structural characteristics; for cytokines:

1) the **four-α-helix bundle family**: the members have similar three-dimensional structures with four bundles of α-helices. This group is divided in others three subfamilies: IL-2 (that includes IL-4, IL-7, IL-9, IL-15, IL-21 erythropoietin and thrombopoietin), IFN (that includes IFN-α, IFN-β, IFN-γ, IFN-ε, IFN-κ and IFN-ω) and IL-10 (that includes IL-19, IL-20, IL-22, IL-24);

2) the **IL-1 family**, which primarily includes IL-1 and IL-18;

3) the **IL-17 family**, which has yet to be completely characterized but it has a key role in inducing and mediating of proinflammatory responses, mainly in autoimmune diseases;

4) the **cysteine-knot cytokines**: include members of the Transforming-Growth-Factor-beta superfamily, including TGF-β1, TGF-β2 and TGF-β3.

Also for the chemokines, it is possible to recognize four groups and the classification depends on the spacing of their first two cysteine residues:

1) **CXC group** (or α-chemokines): from N-terminal sequence, it is possible to identify a sequence CXC (Cys-X random amino acid residue-Cys) where one amino acid residue separates the two cysteine residues. In mammals, there have been described 17 different CXC chemokines, which are subdivided into two categories: those with ELR sequence (glutamic acid–leucine–arginine, called ELR-positive chemokines) preceding the CXC sequence (chemoattract for neutrophils, such as IL-8) and those without an ELR motif (ELR-negative, chemoattract for lymphocytes, like CXCL13);

2) **CC group** (or β-chemokines): the first two cysteine residues are adjacent to each other. Members of this family chemoattract monocytes/macrophages (CCL2 or MCP-1), basophils, eosinophils and T lymphocytes (CCL5 or RANTES) but have little or no effect on neutrophils;

3) **C group** (or γ chemokine): containing only two of the four conserved cysteine residues and the only members of this family are lymphotactin-α and -β (or XCL1 and XCL2), which are known to chemoattract T lymphocytes;

4) **CX3C group** (or δ chemokine): the only CX3C chemokine discovered until now is called fractalkine (or CX3CL1). It is characterized by the presence of three amino acids between the first two cysteine residue. It has double action as chemoattractant and adhesion molecule.

Chemokines exert their biological effects through cell-surface receptors that belong to G protein–coupled receptors (GPCRs) family. There have been characterized approximately 19 different receptors and their nomenclature follows that of chemokines: CXCRn, CCRn, XCRn and CX3CRn.

The structure of chemokine receptors is a single polypeptide chains of about 350 residues spanning 7 times the membrane, three intracellular and three extracellular hydrophilic loops, a short amino-terminal (N-terminal) extracellular domain and a serine/threonine-rich intracellular carboxyl-terminal (C-terminal) domain, important for receptor regulation. In the first two extracellular loops are present 2 of 4 conserved cysteine residues that allow the formation of the first disulphide bound required for the definition of the molecular structure, whereas, the second one is due to the bound between the N-terminal domain and the third extracellular loop structure (*Bonecchi et al., 2009*). Chemokine receptors activate various signalling pathways, such as the Mitogen-Activated Protein Kinase (MAPK) pathway, the Phospholipase C (PLC) pathway resulting in Ca^{2+} influx, and the phosphatidyl inositol-3 kinase (PI3K) pathway (*Bajetto et al., 2002; Cartier et al., 2005*), leading to varied functional outcomes, including adhesion, polarization and chemotaxis.

Functionally, chemokines are divided in two groups: homeostatic (such as CCL14, CCL19, CCL20, CCL21, CCL25, CCL27, CXCL12 and CXCL13, they are constitutively present in certain tissues) and inflammatory (such as CXCL-8, CCL2, CCL3, CCL4, CCL5, CCL11, CXCL10) that they are product in response to pro-inflammatory stimuli.

1.2. Peripheral inflammation

Inflammation is a primary host response that is triggered by noxious stimuli and conditions, such as pathogens, tissue injury, irritant substances, undegradable foreign bodies or cellular debris. In periphery, inflammation is characterized by five cardinal

signs: redness (*rubor*), heat (*calor*), swelling (*tumor*), pain (*dolor*) and loss of function (*functio laesa*) (*Gallin et al., 1992*).

Redness and heat are the results of an increase of blood flow, swelling is due to an increased vascular permeability, pain occurs through activation and sensitization of primary afferent nerve fibres and *functio laesa* is the result of these alterations. These modifications happen following the activation of both immune and peripheral nervous system: indeed, they have a common integrate protective function in host defence and in the response to tissue injury (*Chiu I.M. et al., 2012*).

During the effector phase of inflammation, many cellular changes occur: initially, around the site of damage, the vascular blood vessels respond by increasing blood flow and enhancing vascular permeability trough vasodilation; the latter is mediated by nitric oxide (NO) and vasodilatory substances such as Prostaglandins (PGI_2), Calcitonin Gene-Related Peptide (CGRP) and Substance P (SP). NO is produced from L-arginine through the action of nitric oxide synthase (NOS).

The purpose of the vasodilation is to make easier the penetration of soluble mediators and inflammatory cells. Neutrophils are the first and most abundant leukocytes to be recruit to a site of injury or inflammation by chemokines (CXCL8 family, including CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8) and their transmigration process consists in a series of stages:

- Margination
- Rolling
- Adhesion
- Extravasation or transmigration

When neutrophils reach the afflicted tissue site, they become activated (either by direct contact with inducer or through the actions of cytokines secreted by tissue-resident cells) and try to eliminate the inducer agents by releasing of high potent effector contain in their granules (*De Oliveira S. et al., 2016; Smolen J. E. et al., 2000; Sharwood E.R., 2004*).

This response does not discriminate between microbial and host targets, thus collateral damage to host tissues is unavoidable with cellular debris production. (*Nathan C. et al, 2006*). The elimination of the inducer agents is usually followed by a resolution and repair phase (Figure 1): production of arachidonic acid metabolites such as Lipoxin A_4 (LXA_4) and Lipoxin B_4 (LXB_4) inhibit the neutrophils recruitment and promote the recruitment of monocytes, which remove, together to tissue-resident macrophages, dead cells and initiate tissue remodelling (*Serhan, C. N. and Savill, J., 2005*). The initiation of tissue repair is mediated by macrophage products such as resolvins, protectins, TGF- β and others growth factors (*Medzhitov R., 2008*).

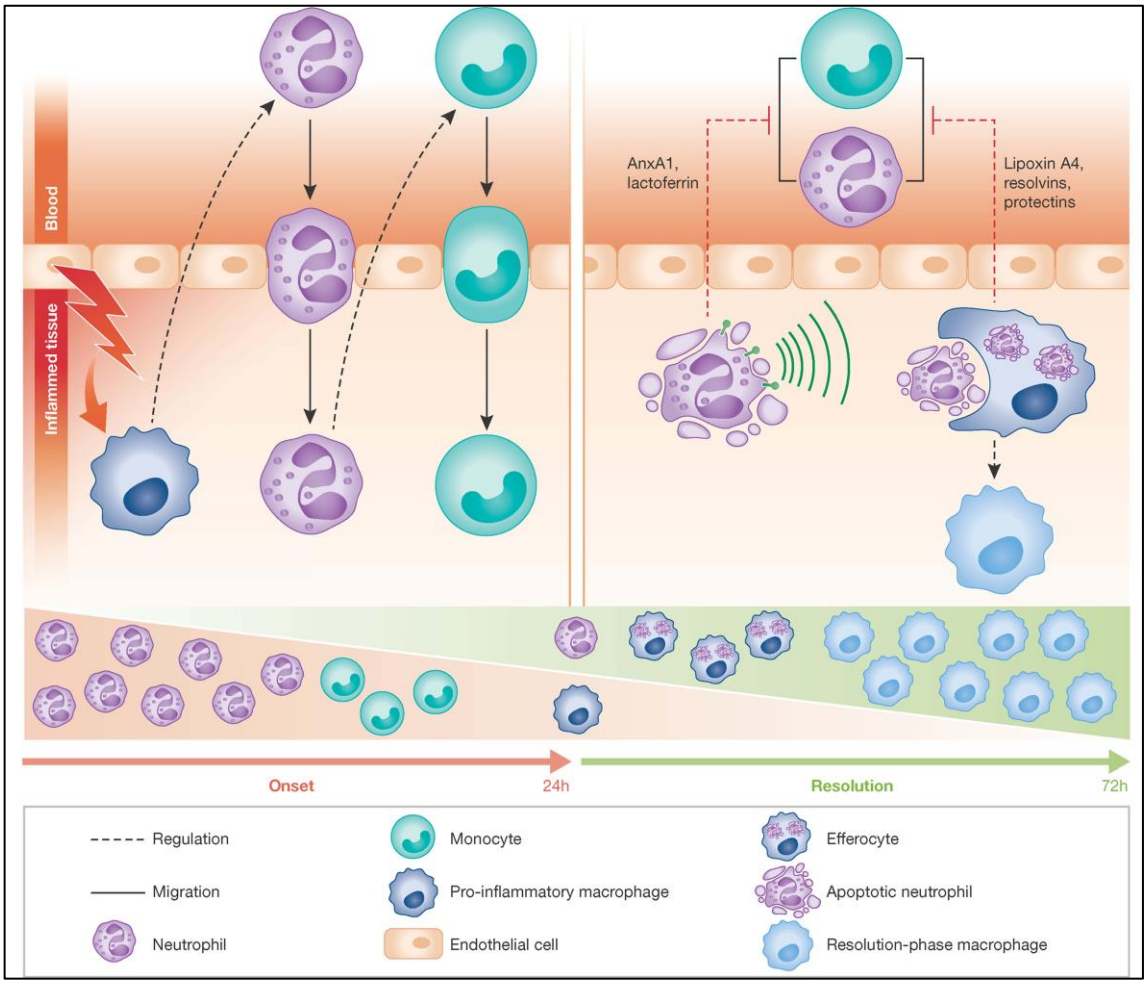


Figure 1. Cellular interplay during resolution of inflammation. Overview of cellular processes during onset (left) and resolution (right) of inflammation. (*Ortega-Gómez A et al., 2013*).

In addition to immune cells, a relevant role is played by primary afferent sensory neurons; indeed, they are involved in signals transduction associated with pain sensation and are classified in:

- Aβ-fibres: large-diameter myelinated fibres, involved in conduction of non-nociceptive input, such as a simple touch, vibration or movement. Following persistent nociceptive stimulation, the afferent impulses of Aβ fibres can be interpreted as pain (allodynia);
- Aδ fibres: medium-to-small-diameter, myelinated fibres, respond to mechanical and thermal stimuli, involved in the "first pain" response;
- C fibres: small-diameter, nonmyelinated fibres, involved in responses to mechanical, chemical or thermal stimulation; they are responsible for "second pain", because respond to nociceptive stimulation only when they become sensitized, such as following inflammation.

During inflammation, the release of cytokines and chemokines by immune cells or the activation of danger signal receptors (such as Transient Receptor Potential cation channels, TRPV1, TRPM8, and TRPA1) on the surface of afferent neurons can activate signalling mechanisms that increase their membrane excitability (Figure 2).

For instance, excitations of C fibres result in the generation of action potentials conduct not only orthodromically to the central nervous system (CNS) but also antidromically into inactive branches of the afferent fibre, inducing release of soluble mediators such as cytokines, chemokines, SP, CGRP and neuropeptide Y.

These molecules rapidly activate various cell types, including immune and endothelial cells with consequent increase of vascular permeability, vasodilation and protein extravasation. The entire process may be self-amplifying, because cytokines, chemokines and neuropeptides can operate both in autocrine or paracrine fashion in primary sensory neurons and lower their threshold for further neurotransmitter and

neuropeptide release, process called "sensitization" (*Xanthos D.N. and Sandkühler J., 2014; Chiu I. M. et al., 2012*).

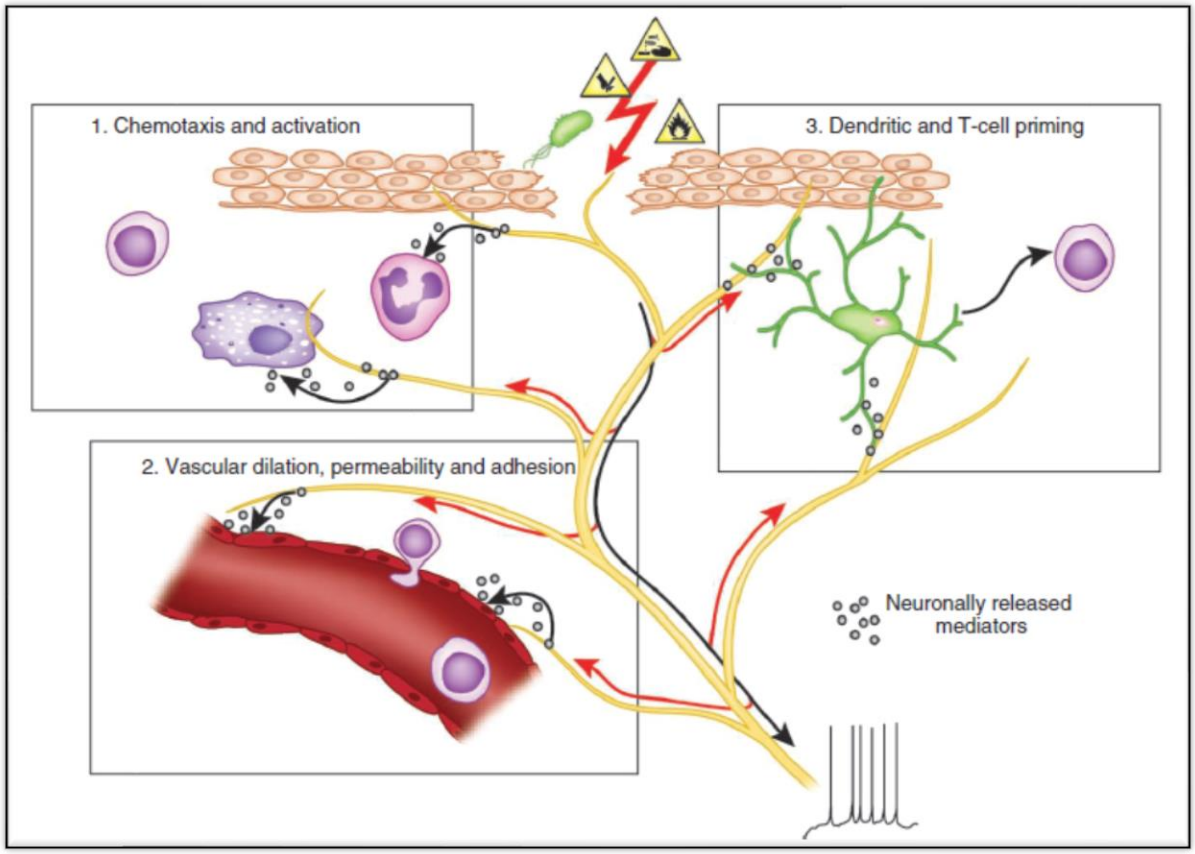


Figure 2. Noxious stimuli, microbial and inflammatory recognition pathways trigger activation of the peripheral nervous system. Sensory neurons possess several means of detecting the presence of noxious or harmful stimuli. (1) Cytokine receptors recognize factors secreted by immune cells, which activate MAP kinases and other signalling mechanisms to increase membrane excitability. (2) Danger signal receptors, including TRP channels, recognize exogenous signals from the environment (for example, heat, acidity, chemicals) or endogenous danger signals released during trauma or tissue injury. (3) Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), recognize pathogen-associated molecular patterns (PAMPs) released by invading bacteria or viruses during infection (*Chiu I. M. et al., 2012*).

1.2.1. Peripheral inflammation: Inflammatory Bowel Diseases (IBDs) overview

Peripheral inflammation, in addition to its role in host defence, contributes to the pathophysiology of many chronic diseases. For instance, inflammatory bowel diseases (IBDs), is a group of inflammatory conditions of the colon and small intestine

characterized by chronic relapsing intestinal inflammation. Crohn's disease and ulcerative colitis are the two majors form of IBDs. Their incidence is increased over the years (*Molodecky, N. A. et al. 2012*) and, for this reason, they represent a big issue for the worldwide health-care (*Lakatos P.L., 2006*). Although their aetiology remains largely unknown, as reporter by Fiocchi, the onset of these disease is linked to a complex interaction between the genetic, environmental, microbial factors and the immune responses (*Fiocchi C., 2015*).

A combination of these IBDs risk factors seems to initiate alterations in epithelial barrier function thereby allowing the translocation of luminal antigens (for example, bacterial antigens from the commensal microbiota) into the bowel wall. Subsequently, the aberrant and excessive activation of mucosal immune cells, such as macrophages and T cells, induced cytokine/chemokine release that give rise to a subclinical or acute inflammatory state (*Strober W. et al., 2002*). In particular, many studies conducted both in human and in rodents reported an increase expression of pro-inflammatory cytokines IL-1β and TNF-α (*Reinecker H. C. et al, 1993; Antoniou E. et al., 2016; Derrick D. E. and Kusum K. K., 2017*).

Interestingly, a therapy with biologic agents (such as Infliximab or Adalimumab) is currently used in therapy to block the action of TNF-α (*Swoger J. M. et al., 2010; Billiet T. et al., 2013; Peng J. C. et al., 2014*). This confirm that pro-inflammatory cytokines exert a key role in the development of IBDs. However, an inflammatory response is characterized by an ongoing infiltration of circulating immune cells.

This cell-trafficking is regulated by chemokines and several studies showed an increased colonic expression of CXCR1 and CXCR2 (the receptors of CXCL8) both in animal models of colitis (*Buanne, P. et al., 2007*; *Bento et al., 2008*) and in biopsy tissues from mucosa of IBD patients (*Mazzucchelli et al., 1994*; *Puleston et al., 2005*). Moreover, CCL5 (RANTES) that is able to attract CCR1 and CCR5 expressing cells into the mucosa (*Kunkel E.J. et al., 2002; Oki M. et al., 2005*) is increased in the peripheral monocytes of Crohn's disease patients (*Schwarzmaier D. et al., 2013*). At the same way,

several studies identified elevated CCL2 mRNA and protein expression in the colon of IBDs patients (*Aomatsu T. et al., 2012*).

Although the studies reported above prove the involvement of cytokines and chemokines in the development of IBDs, our knowledge is still far from the understandings of the complete mechanism of diseases development.

1.3. Neuroinflammation

For decades, the brain has been viewed as an "immune-privileged" organ, where inflammation can only occur through direct infection or after the breakdown of the blood–brain barrier (BBB) and subsequent infiltration of peripheral immune cells. Recent studies have demonstrated how this concept is not completely exact and the new term "neuroinflammation" was born. As suggested by the name, it indicates a process that involve nervous tissue: indeed, it is characterised by activation of microglial cells, astrocytes, neurons and endothelial cells, consequent changes in the permeability of the BBB followed by the infiltration of peripheral immune cells into the CNS parenchyma, secretion of inflammatory cytokines and chemokines and, finally, neuronal damage and death (*González H. et al., 2014*).

Several studies strongly suggest that neuroinflammation is a pivotal process involved in the progression of many neurodegenerative diseases, such as Parkinson's disease (*De Virgilio A. et al., 2016*; *Vivekanantham S. 2015*), psychiatric disorders (*Na KS. Et al., 2014; Jones K.A. and Thomsen C., 2013*), mood disorders (*Hurley L.L. and Tizabi Y. 2013; Young J. et al., 2014*), multiple sclerosis (MS) (*Naegele M. and Martin R., 2014*) or Alzheimer's disease (*Heneka M.T. et al., 2015; Pimplikar S.W., 2014; Heppner F.L., 2015; Liu C. et al., 2014*).

During neuroinflammation, three mainly events occur:

- **synaptic impairment**: is one hallmark of neurodegenerative disorders and of the early progression of dementia (*Masliah et al., 2001*). It is manifested through

loss of synaptic function and impairment of synaptic plasticity, resulting in alteration of memory formation and consolidation: indeed, neurons are damaged and inadequate for neurotransmission and astrocytes are not able to preserve the synaptic homeostasis (*Faissner et al., 2010*);

- **inhibition of neurogenesis**: in adults occurs in subgranular layer (SGZ) of the dentate gyrus (DG) in the hippocampus as well as other areas such as the subventricular zone of the lateral ventricles and the amygdala (*Bernier et al., 2002*). Studies showing an age-related decline in hippocampal neurogenesis suggest that it may contribute to the cognitive deficits observed in the early and later stages of neurodegenerative diseases, particularly in dementia (*Kuhn et al., 1996*). During neuroinflammation, neurogenesis is a negatively modulated process: indeed, pro-inflammatory cytokines such as IL-6, TNF-α and IL-18 (Liu et al., 2005) and microglia activation by Toll-like receptors (TLRs) can inhibit neurogenesis (*Rolls A. et al., 2007*);
- **neuronal death**: may be necrotic or apoptotic. During neuroinflammation, many pro-apoptotic pathways are activated which can accelerate long-term neurodegeneration (*Lyman M. et al., 2014*). In addition to cytokines, nitric oxide (NO) also plays a key role in this process: NO synthesis can be increased by the enzyme inducible Nitric Oxide Synthase (iNOS) from astrocytes and microglia, NO causes apoptosis by inhibiting neuronal respiration, which increases glutamate release, resulting in NMDA receptor-mediated excitotoxic cell death (*Bal-Price A. and Brown G. C., 2001*).

The main cellular actors in neuroinflammation are neurons, microglia and astrocytes. Neurons are about 100 billion and represent the chief type of cell in the brain. Traditionally, were believed to be passive viewers in neuroinflammation, whereas, more recent studies suggest that neurons themselves can produce inflammatory mediators such as COX-2-derived prostanoids (*Davis S. and Laroche S., 2003*), cytokines such as IL-1β, IL-6, and TNF-α (*Gong C. et al., 1998; Murphy P. G. et al., 1999*) and

inflammatory induced enzyme iNOS (*Heneka M. T. et al., 2001*). However, as mentioned above, also microglia and astrocytes contribute in the onset and progression of AD.

Microglia is a cellular population of brain with myeloid origin and, in physiological condition, represent about 10-15% of all cells of Central Nervous System (CNS). Depending on their localization, they acquire a compact or ramified phenotype. The latter, thanks to the high number of process, can facilitate the interaction between neighbouring blood vessels, neurons and astrocytes (*Wake H. et al., 2009*) an important process for cerebral tissue maintenance (*Paolicelli R. C. et al., 2011*) and neuronal plasticity (*Parkhurst C. N. et al., 2013*).

Depending on the integration of regulatory signals, microglial activation seems to be a highly regulated process (although is not yet fully understood); however, microglia may undergo to distinct kinds of activation:

- M1-like phenotype: neurotoxic phenotype, similar to peripheral macrophages that generate a detrimental microenvironment for neurons by producing inflammatory cytokines and Reactive Oxygen Species (ROS);
- M2-like phenotype: a neuroprotective phenotype that secrete neurotrophic factors and anti-inflammatory mediators, thus inducing a supportive microenvironment for neurons (*Kettenmann H. et al., 2011*).

After CNS injury or infection, there is an initial inflammatory response mediated by M1-like microglia: this early activation plays a beneficial role, involving microbicide and phagocytic activity of cellular debris, which is a necessary condition for reparation of lesions. At the same time, M2-like microglia participate in attenuating inflammation induced by M1-like microglia and produce neurotrophic factors, thus promoting tissue reparation (*Shechter, R. et al., 2013*). If M1-like microglia undergo to over-activation, inflammation can become a chronic condition which results in the production of cytokines, chemokines and neurotoxic factors that can lead to neuronal loss over time (*Burguillos M. A. et al., 2011*).

For the brain homeostasis, astrocytes play a key role (Figure 3): they represent about 20-40% of all glia cells but the precise proportion depends from the different cerebral areas (*Verkhratsky A. and Butt A. M., 2013*).

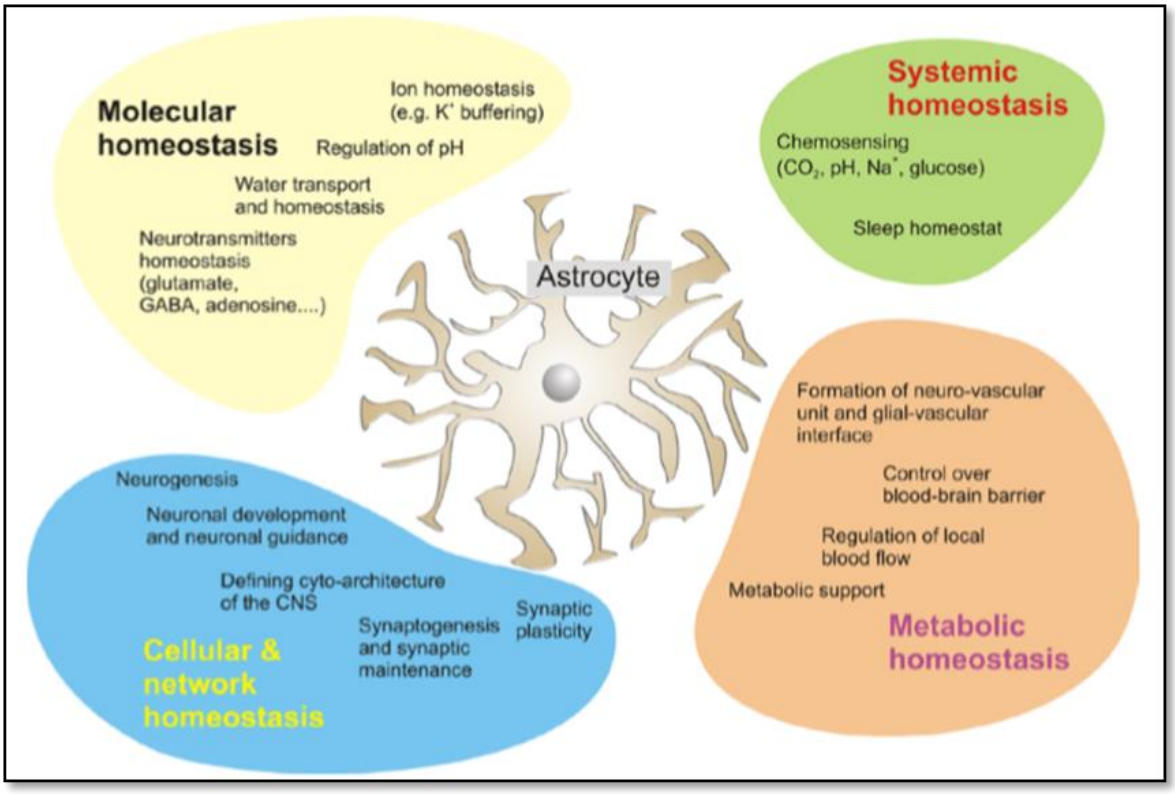


Figure 3. Astrocytes as central homeostatic elements in CNS. Astrocytes represent a highly heterogeneous cell population, they provide structural support for other cellular elements of the CNS and control homeostasis and turnover of several key neurotransmitters and neuromodulators (*Verkhratsky A. et al., 2014*).

As well as remember their name, astrocytes have a characteristic star-shape and they perform many functions: biochemical support of endothelial cells that form blood–brain barrier (BBB), maintenance of ion balance and regulate pH of microenvironment, contribute to synaptogenesis, modulate information processing and signal transmission, regulate neural and synaptic plasticity, supply of nutrients, maintain excitability and connectivity of neuronal network (*Zlokovic B. V. 2008*; *Iadecola C. and Nedergaard M. 2007*; *Walz W., 2000*; *Deitmer J. W. and Rose C. R.,1996*; *Perea G. et al., 2009*;

Sofroniew M. V. 2014). In case of injury, astrocytes quickly change in activate phenotype increasing GFAP and neurotrophic factor S100β production (*Mrak R. E. and Griffin W.S., 2001a and 2001b*).

1.3.1. Neuroinflammation: Alzheimer's Disease (AD) overview

At beginning of last century, the psychiatrist Alois Alzheimer presented a clinic pathological case of dementia in a fifty-years old woman, named Auguste D. He followed her case until she died and, in the 1906, he reported publicly on it (*Alzheimer A., 1907*). During the next years, other scientists reported similar cases in the medical literature and some of them used the term "Alzheimer's disease" (AD), name still now used (*Berchtold N. C. and. Cotman C. W., 1998*). AD is a chronic progressive neurodegenerative disorder characterised by three primary groups of symptoms:

- cognitive dysfunction (memory loss, language difficulties and executive dysfunction);
- psychiatric symptoms and behavioural disturbances (depression, hallucinations, delusions, agitation);
- comprises difficulties with performing activities of daily living (driving, shopping, and managing self-care) (*Burns A. and Lliffe S., 2009*).

The cause of AD is not yet understood but the onset is associated with neuronal death resulting by neuroinflammation. The pathological hallmarks of the disease are:

1. alterations in the production or clearance of amyloid beta (Aβ) peptides: Aβ peptides derive from the amyloid precursor protein (APP). APP is cleaved by α, β and γ secretase that are involved in non-amyloidogenic and amyloidogenic pathway. In the non-amyloidogenic pathway, α-secretase cleaves APP generating a soluble fragment of APP and a membrane-bound carboxyl-terminal fragment; whereas, in the amyloidogenic pathway (Figure 4), β- and

γ-secretase (or β-site APP cleaving enzyme, BACE-1) cleave APP in fragments of 30-51 amino acid residues (*Mu Y. and Gage F. H., 2011*). The most common peptides are $A\beta_{1-40}$ and $A\beta_{1-42}$ that have fibrillogenic activity and can aggregate into insoluble oligomeric form that leads production of extracellular Aβ plaques (*Hamley I. W., 2012*);

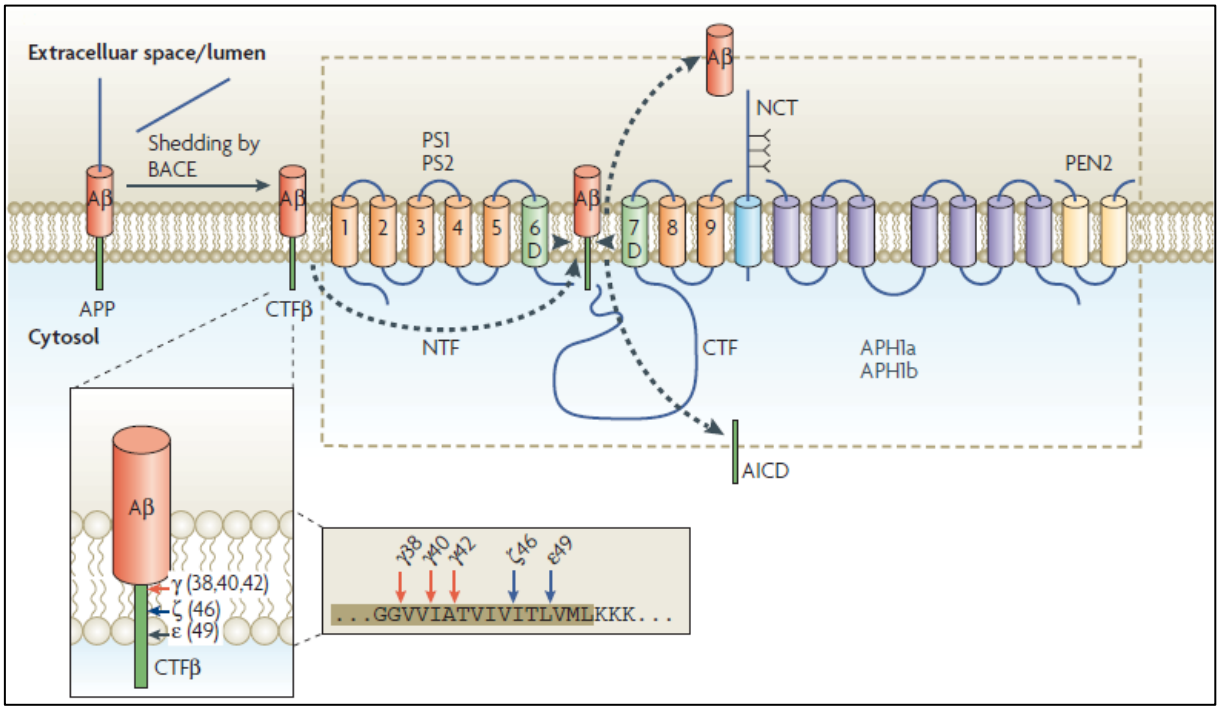


Figure 4. Amyloid β-protein generation by normal proteolytic processing of β-amyloid precursor protein (APP). In the amyloidogenic processing of APP are involved β-site APP-cleaving enzyme (BACE) and the γ-secretase complex. Full-length APP is first processed by BACE, and the large ectodomain is secreted, whereas, the remaining peptide (CTFβ) binds to a docking site on the surface of the γ-secretase complex and then, transferred to the active site that includes transmembrane domains 6 and 7 of presenilin-1 (PS1) or PS2. Subsequently, PS1 and PS2 are both activated by presumed autoproteolytic cleavages, which create their N-, and C-terminal fragments (NTF and CTF). These bind to each other and also to 3 other essential γ-secretase components, APH1a (or APH1b), PEN2 and nicastrin (NCT) forming the core complex required for γ-secretase activity. The γ-secretase cleavage occurs in the middle of the membrane and liberates amyloid β-protein (Aβ). In the frame, are highlighted various proposed sites of intramembrane proteolysis by γ-secretase (*Haass C. and Selkoe D. J., 2007*).

2. hyperphosphorylation of Tau protein (a microtubule-associated protein) that provokes intracellular neurofibrillary tangle (NFT) formation (*Haass C. and*

Selkoe D. J., 2007). Although NFT may better correlate with the decline in cognitive skills in AD, it looks to be a downstream event of Aβ accumulation (Figure 5), however, some experimental evidences indicate that Aβ plaques have direct toxic effects and may promote neurodegeneration by the activation of microglial cells and astrocytes (*Regen F. et al., 2017; Heneka M. T et al., 2015*).

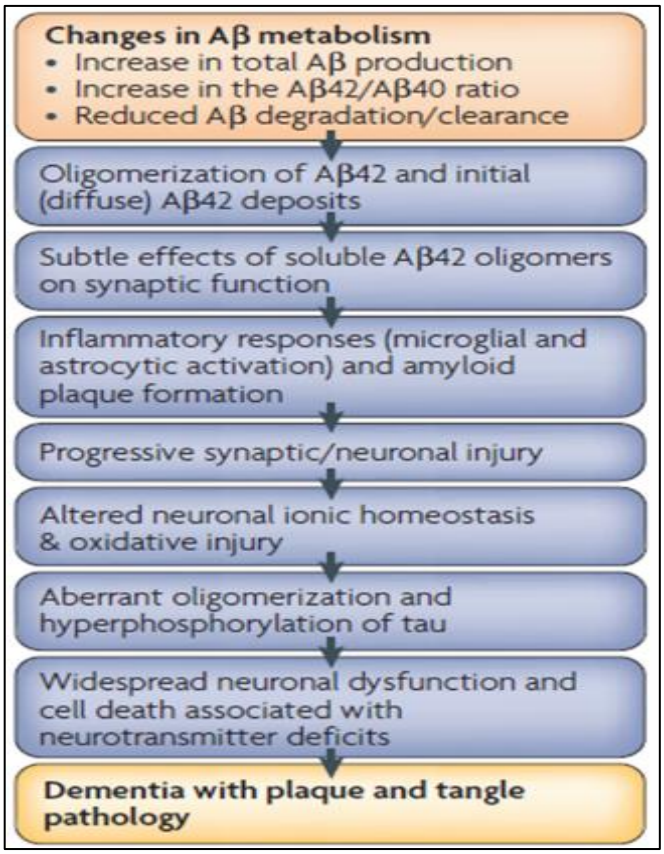


Figure 5. The amyloid β-protein (Aβ) cascade (*Haass C. and Selkoe D. J., 2007*).

Many years ago, it was demonstrated that amyloid peptides and their precursor protein APP are potent glial activators (*Barger S. W. and Harmon A. D., 1997*): indeed, trough TLR4, aggregated amyloid-β (Aβ) stimulates microglial cells to produce strong levels of pro-inflammatory factors such as NO, IL-6 and TNF-α (*Walter S. et al., 2007*) and TLR4 polymorphism has been associated with elevated risk of AD (*Balistreri C. R. et al., 2009*). Astrocytes have a controversial role for Aβ clearance and degradation: indeed, astrogliosis occurs in AD, probably because astrocytes can form a protective

barrier between Aβ deposits and neurons but, in response to chronic stress (such as pro-inflammatory cytokines) they could overexpress BACE1 enzyme becoming a source of Aβ themselves (*Roßner S.et al., 2005*). Nevertheless, several studies underline the role of chemokines in AD: for example, increased levels of CXCL8 have been detected in patients with AD (*S. Franciosi, et al., 2005*) as well as augmented expression of CCL5 in astrocytes which has modulatory effect on microglia activation (*Skuljec J. et al., 2011*) but its role in AD is still controversial (*Tripathy D. et al., 2010*). Moreover, CCL2 induces activation of astrocytes influencing Aβ peptide accumulation and production of proinflammatory cytokines and chemokines causing neuronal death (*Lee Y. K. et al, 2009*).

2. Bv8 AND PROKINETICIN SYSTEM: A NEW FAMILY OF CHEMOKINES

About twenty years ago, for the first time by our research group, a small peptide of 77 amino acid was isolated from skin secretions of *Bombina variegata* frog and called Bv8, to indicate its origin and molecular mass of 8 KDa. Its homologues were founded in spiders (atracotoxin-Hvf17), snakes (Mamba Intestinal Toxin-1, MIT1) rodents and humans (PK1 or Endocrine Gland-derived Vascular Endothelial Grow Factor, EG-VEGF and mammalian Bv8 or PK2). Zhou and colleagues, termed the corresponding proteins "prokineticin 1" (PK1) and "prokineticin 2" (PK2) again mainly referring to their ability to contract guinea pig ileum *in vitro*, a property shared by this group of proteins (*Mollay C. et al., 1999*; *Negri L. et al. 2002*; *Kaser A. et al., 2003*). The amino acid sequence of Bv8 is similar to MIT-1: indeed, they have 58% of sequence identity (*Negri L. et al., 2002*). Furthermore, PK1 have an overall identity of 58% and homology of 76% with human PK2 and murine Bv8 and 43% identity with amphibian Bv8 (*Masuda Y. et al., 2002*). These proteins have some commons structural characteristics (Figure 6):

- identical amino-terminal (N-terminal) sequence important for the biological activity and receptor recognition (alanine, valine, isoleucine, threonine, glycine and alanine, AVITGA sequence), for this reason, they are also named 'AVIT proteins' (*Bullock C. M. et al., 2004*; *Negri L. et al., 2005*; *Kaser A. et al. 2003*);
- 10 cysteine residues with identical spacing that define a five disulphide bridges, motif called a colipase fold, that confer to the molecule a compact three-dimensional conformation and high protection from enzymatic degradation (*Kaser A. et al., 2003*);
- tryptophan (W) residue in position 24, very important for receptor binding.

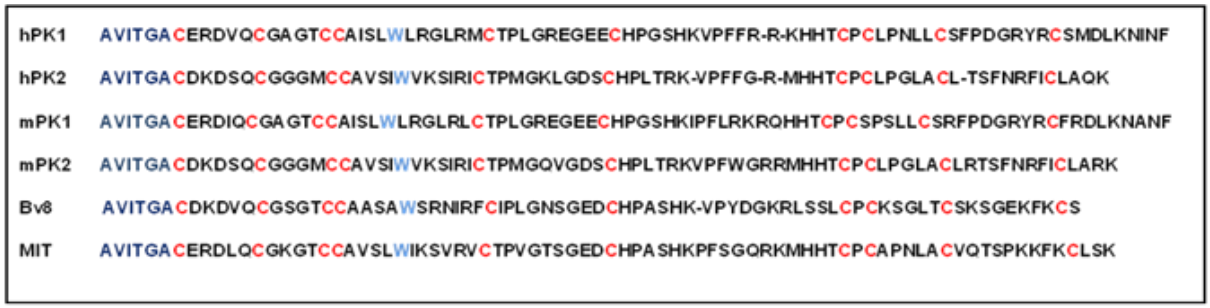


Figure 6. Amino acid sequences of human and mouse PKs and their homologues from frog and snake. AVITGA - dark blue; cysteine (C) - red; tryptophan (W) - light blue.

PK1 and PK2 share only 44% amino acid identity and most of this homology resides in the N-terminal signal peptide and the distinct AVITGA sequence motifs that are highly preserved across species (fish, frog, snake, and various mammalian species) (*Li M. et al., 2001*; *Kaser A. et al., 2003*). As mentioned above, the AVITGA sequence is implicated in receptor recognition and the high degree of disulphide cross-linking gives rise to a remarkably stable compact protein that is highly resistant to protease degradation (*Bullock C. M. et al., 2004*; *Boisbouvier J. et al., 1998*). Both proteins fold into a polarized ellipsoid structure with one side containing a net positive charge and the opposite with hydrophobic residues (Protein Data Bank, accession number 1IMT). The C- and N- terminal ends are exposed on the surface, whereas the more charged residues are buried inside the molecule (*Boisbouvier J. et al., 1998*). The gene that encodes PK1 is located on murine chromosome 3 and human chromosome 1p21. It is composed from three exons encoding a precursor protein of 105 amino acids and a mature form of 86 amino acids, with no known alternative splicing product (*LeCouter J. et al., 2003a*; *Li M. et al., 2001*). The pk2 gene maps to murine chromosome 6 and human chromosome 3p21.1 and it is composed of four exons (Figure 7), which give rise to two mature proteins: PK2 (81 amino acids, exons 1, 2 and 4) and a splice variant with a 21-amino acid insert called long PK2 (PK2L, 102 amino acids, exons 1, 2, 3 and 4) (*Jilek A. et al., 2000*). The secreted PK2L, contains the additional 21 basic amino acids between Lys-47 and Val-48 of the mature PK2 protein and it is supposed to be

processed, by furin proteolytic cleavage into a smaller peptide called PK2β, but the role of PK2L and PK2β has not yet been elucidated.

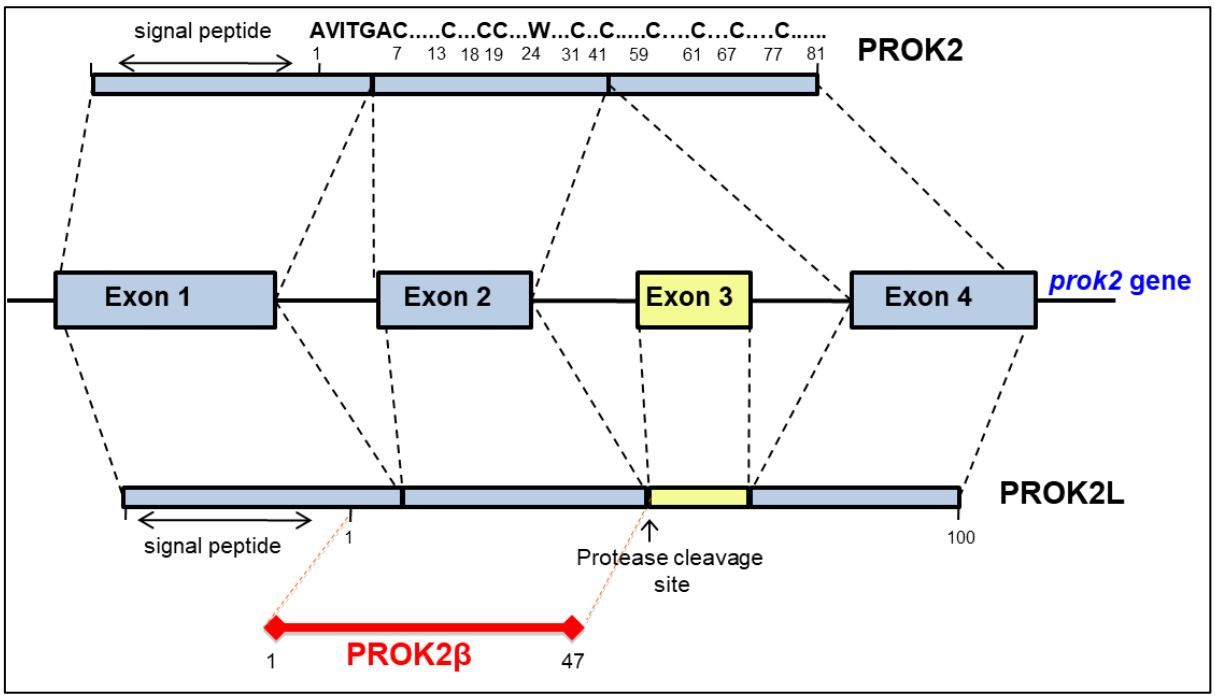


Figure 7. PK2 and its splice variant PK2L. Gene structure of PK2 and the differential exon use by PK2 and PK2L mRNA. In red is showed PK2β peptide, the product of PK2L proteolytic cleavage.

The structural elements essential for the function of PKs have been also investigated by deletion, insertion, and substitution mutations (*Bullock C. M. et al., 2004*), as well as proteolytic fragmentation (*Negri L. et al., 2005*). Any disruption of the N-terminal hexapeptide sequence renders human PK1 inactive (*Bullock C. M. et al., 2004*), whereas deletion of the N-terminal alanine residue of frog Bv8 results in significant but not complete loss of biological activity (*Negri L. et al., 2005*).

The amount of data obtained in these first 20 years of research has allowed to classify Prokineticins belong to chemokines class, despite that a phylogenetic study (a method that analyses the evolutionary relationships between proteins family and their members) revealed higher similarity between defensins and Prokineticins than with chemokines. Usually, proteins are classified as chemokines according to common

structural characteristics, biological functions and kind of receptors that they bound to exert their biological effects. These characteristics fit in very well with Prokineticins: indeed, together with chemokines, Prokineticins are small secreted peptides (8-10 KDa), are highly basic proteins and bind sulphate proteoglycans, they both contain cysteine residues and they are potent chemoattractant (*Monnier J. and Samson M., 2008*).

2.1. Bv8/Prokineticin receptors

Prokineticins exert their biological functions through activation of two closely related G-Protein Couple Receptors (GPCRs), called Prokineticin Receptor 1, PKR1, and Prokineticin Receptor 2, PKR2 (*Lin D. C. H. et al., 2002; Masuda Y. et al., 2002; Soga T. et al., 2002*).

PKR1 and PKR2 belong to the Neuropeptide Y (NPY) receptor class, have an overall identity in their amino acid sequences of 85%, diverge mainly at the N-terminal and are about 80% identical to the previously described mouse orphan receptor gpr73 (*Parker R. et al., 2000*). They are located on 2p13.1 and 20p12.3 human chromosomes for PKR1 and PKR2 respectively.

In cultured cells that express PKR1 or PKR2 exogenously, data obtained from binding experiments show that the non-mammalian peptides Bv8 and MIT display an affinity for PKRs at least one order of magnitude higher than that of PK2 and two orders of magnitude higher than that of PK1. Except MIT, a clearly PKR2-preferring ligand, all the other natural PKs show no selectivity for either receptors (*Negri L. et al., 2009*).

Nevertheless, Chen and co-workers showed that, in PKR1- and PKR2-expressing cells, unlike PK1 and PK2, PK2β showed high affinity for PKR1 and very low affinity for PKR2 (*Chen J. et al., 2005*).

Displacing Ligand	*Radioligand (pM)*	*PKR1 (Ki, nM)*	*PKR2 (Ki, nM)*
Bv8	^{125}I-Bv8, 10	0.34	0.78
	^{125}I-MIT, 4	0.69	0.71
MIT	^{125}I-MIT, 100	4.1	0.67
	^{125}I-MIT, 1.9	-	0.003
PK1	^{125}I-MIT, 100	250.0	81.0
	^{125}I-PK1, 2000	104.0	34.0
PK2	^{125}I-MIT, 100	6.9	7.6
	^{125}I-PK2, 100	4.5	6.4
PK2β	^{125}I-PK2, 100	34.6	>1000

Table 1. Binding affinity of Bv8/PK proteins to PKR1 and PKR2. Data obtained from binding experiments using Chinese hamster ovary (CHO) cultured cells that express PKR1 or PKR2 exogenously.

Several studies show that PKR1 and PKR2 are associated with $G\alpha_q$, $G\alpha_i$ and $G\alpha_s$ proteins and, as consequence of this redundancy, Prokineticins signalling depends on tissues-specific expression of the ligands, receptors and associated G proteins and which possible signalling pair is involved in specific physiological or behavioural process (Figure 8). Indeed, in PKRs transfected neuronal and specific endothelial cell lines, the activation of PKRs stimulates intracellular calcium mobilization through several mechanisms. One of them is via $G\alpha_q$ coupling that activates Phospholipase C (PLC)-β and subsequent formation of inositol 1,4,5-trisphosphate (*Lin D. C. H. et al., 2002b*) and calcium release from intracellular stores. Intracellular calcium stimulation by PK1 also activates the calcineurin pathway, which induces dephosphorylation of the transcription factor, NFAT (Nuclear Factor of Activated T cells), followed by nuclear translocation and regulation of gene transcription (*Cook I. H. et al., 2010*). Whereas the stimulation of calcium mobilization upon receptor activation is

dependent on $G\alpha_q$, activation of the MAPK (Mitogen-Activated Protein Kinase) pathway is pertussis toxin–sensitive, proving that PKRs may also couple to $G\alpha_i$ protein. The coupling of PKRs with $G\alpha_i$ proteins can lead also to ERK (extracellular-signal-regulated kinase) phosphorylation (*Lin D. C. H. et al., 2002b*). It has been demonstrated that in the Dorsal Root Ganglia (DRG), prokineticin receptors increase $[Ca^{2+}]i$ by activation of the TRPV1 channels in a dose-dependent fashion and are followed by subsequent translocation of PKC to the neuronal membrane (*Vellani V. et al., 2006*). Cross-talk between the glial cell line-derived neurotrophic factor (GDNF)/Ret, transient receptor potential vanilloid 1 (TRPV1) and prokineticin signalling have also been reported (*Hu W. P. et al., 2006*; *Ngan E. S. et al., 2008*).

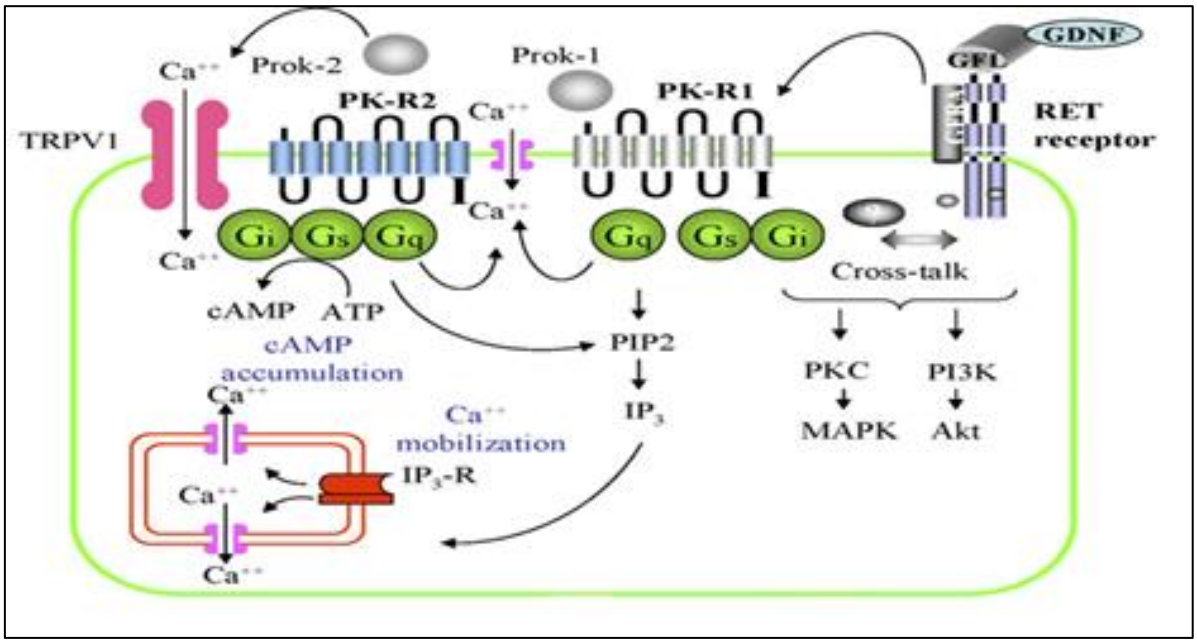


Figure 8. Prokineticin Signalling. PK1 and PK2 bind two G-protein coupled receptors (PKR1 and PKR2) which are coupling to $G\alpha_i$, $G\alpha_s$, $G\alpha_q$ to activate MAPK/Akt, cAMP accumulation and calcium mobilization, respectively (*Ngan E. S. W. and Tam P.K., 2008*).

2.2. Prokineticin receptor antagonists

As mentioned before, in all members of the Bv8/PK family, the highly conserved N-terminal sequence AVITGA and the Tryptophan (Trp or W) residue in position 24 are

necessary for biological activity (*Bullock et al., 2004*; *Negri et al., 2005*); indeed, AVITGA family members can interact with PKR1/PKR2 by orienting the protein region that comprises the AVITGA sequence and the conserved Trp residue in position 24 (*Miele R. et al., 2010*).

Deletions and/or substitutions in these conserved residues produces peptides that act like antagonist molecules: to give some instances, the N-terminal deletion of the first two amino acids in amphibian Bv8 molecule (dAV-Bv8), yields an analogue lacking any biological activity but still able to bind the receptors, or the substitution of Trp with Ala in position 24, yields a molecule, Ala-24, that preferentially binds and activates PKR2 (*Negri L. et al., 2005; Lattanzi R. et al., 2012*). Despite peptides represent an excellent starting point for the design of novel therapeutics, they have some weaknesses, such as chemically and physically instability, prone to proteolytic hydrolysis, short half-life and fast elimination (*Fosgerau K. and Hoffmann T., 2015*), therefore, Balboni and colleagues have synthesized and developed several nonpeptidic prokineticin antagonists (*Balboni G. et al., 2008; Lattanzi R. et al., 2014*). PC1 (Figure 9), the lead compound, is characterized by a triazinic group which contain the following substitutions: N1 and N5 link a 4-ethylbenzyl and a 4-methoxybenzyl, respectively; C2 links an amino-ethyl-guanidine.

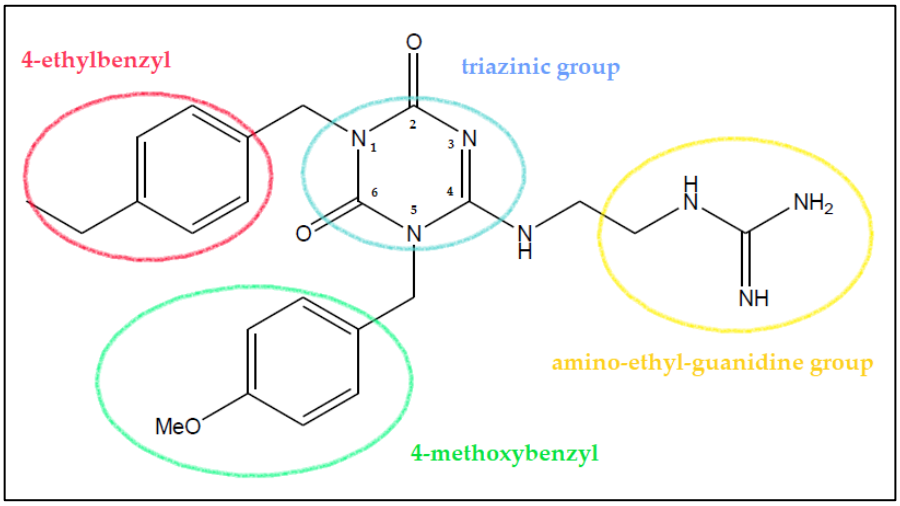


Figure 9. PC1, chemical structure.

PC1 mimics the structural features required for Bv8 receptor binding: indeed, the triazine-guanidine moiety of PC1 mimics the N-terminal AVIT sequence, whereas the methoxybenzyl moiety is oriented as the Trp residue in position 24 (*Balboni G. et al., 2008*). PC1 acts as a preferentially PKR1 ligand as demonstrated by *in vitro* and *in vivo* experiments conducted in our laboratory. *In vitro* binding experiments demonstrated that PC1 has 30 times higher affinity for PKR1 (IC_{50} = 104 nM) than for PKR2 (IC_{50}=3200 nM) and it behaves as an antagonist because it blocks the Bv8-induced $[Ca^{2+}]i$ mobilization in G-protein coupling PKR1- and PKR2-transfected CHO (Chinese Hamster Ovary) cells (*Balboni G. et al., 2008*). Another analogue of PC1 has been synthesized: the 4-ethylbenzyl group in position 5 of PC1 structure was replaced with a 4-fluorine atom to give PC7 (Figure 10). *In vitro* assays show that PC7 results about 100 times more selective for PKR1 than for PKR2 (IC_{50} = 36 nM IC_{50} = 4400 nM, respectively) and displays 4 times higher affinity for PKR1 than the lead compound PC1 (*Lattanzi R. et al., 2014*).

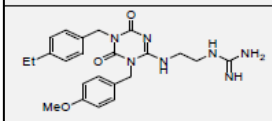
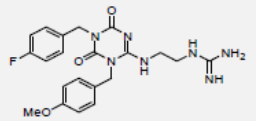
Triazinediones	**PKR_1 IC_{50} (nM)**	**PKR_2 IC_{50} (nM)**	**Selectivity (IC_{50} PKR_2 / IC_{50} PKR_1)**
 **PC-1** ***(reference)***	*144 ± 15*	*2964 ± 215*	*20.6*
 **PC-7**	36 ± 6.1	4399 ± 340	122

Figure 10. Affinity and selectivity of PC1 and PC7 expressed as IC_{50} values. The affinity and selectivity of PC1 and PC7 was evaluate *in vitro*, using the BRET (Bioluminescence Resonance Energy Transfer) technology in neuroblastoma SHSY5Y cells expressing luminescent PKR1 or PKR2 and fluorescent $G\beta_1$ subunit (*Lattanzi R. et al., 2014*).

2.3. Distribution and functions of Prokineticins and their receptors

Several studies show that both prokineticin receptors are distributed throughout the body: PKR1 is mainly expressed in peripheral tissues, including endocrine glands and organs of the reproductive system, spleen, gastrointestinal tract, lungs, heart and immune cells (such as neutrophils and macrophages) whereas, in the central nervous system (CNS), PKR2 is more abundantly expressed and PKR1 is present only in discrete brain areas (*Soga T. et al., 2002*; *Lin D. C. et al., 2002*; *Cheng M. Y. et al., 2006*; *Negri L. et al., 2007*).

Distinct expression patterns have been reported in various tissues, which provide the clue for their tissue-specific biological functions. In addition, differential G-protein expression pattern and multiple G-protein coupling of the receptors further increase the functional complexity of the system, allowing cells to perform different physiological functions, in response to the same ligand stimulation.

The name "Prokineticins" was assigned to PK1 and PK2 by Zhou and colleagues (*Li M. et al., 2001*) and reflects their ability to induce specific and potent contractions on the smooth muscle of the gastrointestinal tract. Subsequently, other functions are identified. As far as the reproductive system is concerned, PK1 is predominantly expressed in steroidogenic tissues, including the ovary, uterus, placenta and adrenals in response to the hormonal changes across the menstrual cycle and during pregnancy; whereas, PK2 is mainly (but not exclusively) expressed in non-steroidogenic cells of the testis, is undetectable in human ovary and in the endometrium its expression remains constant across the menstrual cycle (*Maldonado-Perez D. et al., 2007*; *Ngan E. S. et al., 2006*; *Denison et al., 2008*).

The PKs/PKRs are expressed in the endothelial cells of vascular tissues. PK1 and PK2 both exert vascular effects through activation of PKRs and, more specifically, PKR1 activation acts to enhance cell proliferation and survival; whereas, PKR2 is implicated in regulating of endothelial cell permeability (*LeCouter J. et al., 2003a*; *Lin D. C. et al., 2002*; *Kisliouk T. et al., 2003*). PKR1 is also strongly expressed in endothelial cells of

arterioles and vessels and signalling through Gαq11, it induces formation of vessel-like structures by human aortic endothelial cells; whereas PKR2 is the only prokineticin receptor expressed by hepatic sinusoidal endothelial cells (*Guilini et al., 2010*). In the disease state, PK1 is highly expressed in many kinds of cancers and is thought to be partly responsible for neoplastic angiogenesis (*Pasquali et al, 2006*; *Monnier J. and Samson M., 2010*).

In 2004, LeCouter and co-worker. reported the expression of PK2 as well as of PKR1 and PKR2 in multiple cell lineages from the bone marrow, while PK1 is undetectable. PK2 is able to promote differentiation, survival and mobilization of granulocytic and monocytic lineages and both PK1 and PK2 can induce formation of granulocytic and monocytic colonies in human and mouse hematopoietic stem cells (*LeCoulter J. et al., 2004*).

In the gut, PKR1 is more abundantly expressed than PKR2: PKR1 is localized in epithelial cells, in submucosal and myenteric neurons of ileum and colon (*Wade P. R. et al., 2010*). PK2 is expressed in normal liver, but only in Kupffer cells, the liver resident macrophages (*Monnier J. et al., 2008)*. In the mouse embryonic gut, a key role is carry out by PK1: is expressed in the mucosa and mesenchyme and is able to modulate proliferation and differentiation of enteric neural crest cells (*Ngan E. S. et al., 2008*).

As mentioned above, in the CNS, PKR2 is the receptor more abundantly expressed and PK2 acts as an endogenous neurotrophic factor to support neuronal survival (*Cheng M. Y. et al., 2002*). Primary cultured neurons, astrocytes and microglia, prepared from cerebrum of mouse, indicate that neurons express PKR2 and PK1, whereas cultured astrocytes and microglia express PKR1 e PK2 (*Koyama Y. et al., 2006*). PK1 is also express in the brainstem, with high abundance in the *nucleus tractus solitarius* (*Cheng et al., 2006*). PK2 and PKR2 are also abundantly localize in suprachiasmatic nucleus where they control the behavioural circadian rhythm (*Cheng et al., 2002*). In the olfactory bulbs (OBs) PK2/PKR2 signalling plays a key role in the neurogenesis: indeed, PK2 works as a chemoattractant for neuronal progenitors derived from the

subventricular zone (SVZ) and regulates OBs morphogenesis; PK2- or PKR2-null mice display a marked reduction in the size of the OBs, a loss of normal OBs architecture, and an accumulation of neuronal progenitors in the rostral migratory stream (*Matsumoto et al., 2006; Ng et al., 2005*).

2.4. Prokineticin and pain perception

During the first years after Prokineticins discovery, several evidences showed their involvement in nociception and pain transmission. Indeed, both PKR1 and PKR2 are localized in the main stations of pain pathway, such as in peripheral terminals of nociceptor axons, in the Dorsal Root Ganglia (DRG), in outer layers of the dorsal horns of the spinal cord and at the supraspinal level in the Peri-Aqueductal Grey (PAG) and Rostral Ventromedial Medulla (RVM) nuclei (*Negri L. et al., 2006*; *deNovellis V. et al., 2007*). In particular, systemic Bv8 injection shows a biphasic time-course: an initial rapid phase of hyperalgesia peaks after 1 hour and is followed by a secondary phase peaking at 4-5 hours. The initial phase of hyperalgesia is due at least in part to a local action on nociceptors, whereas, the second phase is due to release of neuropeptides implicated in pain processing, such as Calcitonine Gene Related Peptide (CGRP) and substance P in the spinal cord (*De Felice et al., 2012*).

The increase in nociceptor excitability results from functional interaction between PKR1 and TRPV1, two co-expressing receptors in the DRG. A previous study conducted from our group demonstrate that in primary cultures of DRG neurons, about 70% of TRPV1 expressing neurons co-express PKR1, whereas a smaller proportion (~9.5%) co-express PKR2. Other evidences of PK system involvement in pain perception are given by mice lacking PKR1, PKR2 or PK2 gene that exhibit impaired pain perception to various stimuli (thermal, mechanical, and capsaicin) (*Negri L. et al., 2006*; *Hu W. P. et al., 2006*). In the central nervous system, the Bv8 intra-PAG administration exerts a pro-nociceptive action by increasing the intrinsic GABA-

ergic tone which, in turn, is responsible for the inhibition of PAG antinociceptive output neurons impinging on RVM neurons (*deNovellis V. et al., 2007*).

Previous studies conducted in rodents by our group highlight a critical role for PK2 in granulocyte-mediated inflammatory pain. In animal model of inflammatory pain induced by injection of complete Freund's adjuvant (CFA) in the hind paw, there is a strong activation of the PK system: PK2 expression forcefully increase in mouse and rat hind paw skin and this increase is PKR1-mediated.

Moreover, PKR1 is the mainly receptor involved in hyperalgesia (*Negri L. et al., 2006*) and the PK2 increase in the inflamed paw correlates with the development and duration of pain. Interestingly, the inflammatory pain response has higher intensity and longer duration in rats than in mice and this effect is probably due to the larger expression of PK2L, (the splice variant of PK2) (*Giannini E. et al., 2009*). PK2L is susceptible to proteolytic cleavage that give rise to a small peptide called PK2β that selective binds PKR1 (*Chen J. et al., 2005*).

PK system is involved in chronic pain conditions, such as neuropathic pain. Indeed, it is a pain caused by damage or disease affecting the somatosensory nervous system and its development and maintenance involves interactions between neurons, inflammatory immune cells, glial cells, as well as a wide cascade of cytokines and chemokines (*Austin P. J. and Moalem-Taylor G, 2010*). The key role of PK2 in neuropathic pain is well establish in several animal models, such as chronic constriction injury (CCI) of the sciatic nerve, spared nerve injury (SNI) of the same nerve and diabetic neuropathy induced by Moderate Low Doses of Streptozotocin (MLD-STZ).

In the CCI, neuropathic pain is induced by three loose ligatures–and it is a model associated with infiltrating cells. As reported by studies from my laboratory, sciatic nerve ligature is followed by infiltrating cells and PK2 increase is responsible of thermal hyperalgesia and contributed to develop of tactile allodynia. Administration of PKRs antagonist (PC1 or PC7) prevent the injury-induced overexpression of PK2, microgliosis and astrocytes activation in the spinal cord and restored the physiological

levels of proinflammatory and anti-inflammatory cytokines (*Maftei D. et al., 2014*; *Lattanzi R. et al., 2015*).

Comparable results are showed in SNI model: neuropathy is obtained by transection of two of the three terminal branches of the sciatic nerve. Mice develop both thermal hyperalgesia and mechanical allodynia that are reverted by PC1 treatment. Moreover, PC1 is able to inhibit the PK2-driven glial and microglial activation (*Guida F. et al., 2014*).

In MLD-STZ animal model, diabetic neuropathy is induced by intraperitoneal (i.p.) administration of streptozotocin for three consecutive days. Animals develop mechanical allodynia and PK2 seems to be implicated both in the early stage of allodynia development as well as in its maintenance. The use of PC1 is effective in relieving diabetes-induced hypersensitivity, eliminating the PK2 overexpression and the peripheral inflammatory status (*Castelli M. et al., 2016*).

2.5. Prokineticins in inflammation and neuroinflammation

PK system conducts a key role, not only in nociception and pain transmission, but also in inflammation/neuroinflammation response. Indeed, elevated levels of PK2 expression have been detected in infiltrating neutrophils at sites of inflammation (*LeCouter et al., 2004*).

In a previous study from our group, in the CFA-induced paw inflammation, in situ hybridization assays (performed on inflamed paw sections) and qPCR experiments (performed on FACS sorted cells) showed that neutrophils (PMN) are the major source of PK2 and, more notably, the CFA-induced inflammatory response amplifies PK2 gene transcription in PMN cells, not only locally in the paw but also systemically (*Giannini E. et al., 2009*). The mechanism of inflammation-induced increase of PK2 in granulocytes may depend by the early and rapid increase in plasma levels of Granulocyte Colony-Stimulating Factor (G-CSF) in CFA inflamed animals (*Bobrowski*

W. F. et al., 2005). Ferrara's group demonstrated that G-CSF is the only cytokine able to activate PK2 transcription in CD11b+Gr1+ bone marrow-derived cells (*Shojaei F. et al., 2007*). G-CSF is one of principal regulator of granulopoiesis and neutrophil mobilization from the bone marrow; thus, the early increase of G-CSF levels in plasma of CFA-inflamed animals could explain the systemically enhanced PK2 transcription in spleen and paw granulocytes. PK2, released in inflamed tissues, triggers further recruitment of macrophages and is able to induce a pro-inflammatory macrophage phenotype, increasing cytokines, such as IL-1β and IL-12, and reducing the IL-10 production (*Martucci C. et al., 2006*).

As well as innate response, PK system is involved in adaptive immunity: in fact, PK2, trough PKR1, is able to modulate T cell function by reducing T helper (Th)-2 cytokine levels, such as IL-4 and IL-10 production, thus indirectly switching the cells towards a Th1 and proinflammatory state (*Franchi S. et al., 2008*).

As reported in several studies, Prokineticin system dysregulation is involved in *in vivo* and *in vitro* models of inflammatory and neuroinflammatory state, such as: Rheumatoid Arthritis (mouse model of collagen-induced arthritis, CIA), Multiple Sclerosis (MS, mouse model of Experimental Autoimmune Encephalomyelitis, EAE), inflammatory colitis and in *in vitro* model of Aβ-induced neurotoxicity.

In CIA mice, PK2 expression is significantly increased and correlates with the severity of arthritis; moreover, immunohistochemical staining of PKRs show that PKR1-positive cells are predominantly neutrophils infiltrating in the synovial membrane and PKR2-positive cells are found in the synovium but associated with macrophage-like mononuclear cells (*Kurosaka D. et al., 2009; Ito H. et al 2016*).

According to Abou-Hamdan and colleagues, PK2 is an important mediator of MS. Indeed, both in mice sera and spinal cord and also in patients sera with MS, PK2 mRNA is increased, compared to healthy control. In EAE mice, the use of PC7 prevents or reduces inflammation and demyelination, decreasing the production of interferon-γ and interleukin (IL)-17 (*Abou-Hamdan M. et al., 2015*).

Since their discovery, Prokineticins have been shown to modulate contraction of smooth muscle derived from various regions of the gastrointestinal (GI) tract and, a few years later, it was proven that PK1 is able to stimulate upper GI transit (*Wade P. R. et al 2009*). Successively, in 2011, Watson and colleges have demonstrated that PK2 is strongly increased in the GI tract during inflammation, both in biopsy samples collected from patients with ulcerative colitis and in tissues samples taken from various preclinical models of colitis; this PK2 up-regulation is a direct consequence of inflammation: indeed, PK2 mRNA levels positively correlated with IL-1β expression. Moreover, authors assume that PK2 modulates the visceral nociception, acting on PKRs express by sensory neurons in the gut (*Watson R. P. et al., 2012*).

Furthermore, in an *in vitro* model of Aβ-induced neurotoxicity, it has recently been demonstrated that PK2 plays a role in Aβ-mediated neuronal death in cortical primary cultures: indeed, following Aβ stimulation, PK2 and its receptors are significantly increased at both mRNA and protein level, suggesting that modulation of prokineticin system could be a general response to Aβ injury. In addition, the functional involvement of the PK system following Aβ stimulation is further demonstrated by the ability of Bv8 to induce apoptosis comparable to that induced by Aβ: the Bv8 neurotoxic activity in cortical brain cultures is achieved with picomolar concentrations, indicating that such a low concentration could be compatible with the small amount of PK2 eventually released by Aβ stimulation. The use of PC1 significantly prevents neuronal toxicity by inhibiting the Aβ-induced PK2 increase (*Severini C. et al, 2015*). A recent study indicates also that Aβ insult up-modulates the kainate-induced currents in primary cortical cultures and this effect is blocked by PC1 (*Caioli S. et al., 2017*).

3. AIMS OF THE STUDY

The immune system constitutes the first line defence against infection or injury and it relies on a large family of Pattern Recognition Receptors (PRRs), which trigger intracellular signalling cascades ultimately culminating in the expression of a variety of proinflammatory molecules, such as cytokines, chemokines, neuropeptides and metabolite of the arachidonic acid cascade (*Mogensen T. H. et al., 2009*). Cytokine and chemokines are involved in peripheral inflammation and neuroinflammation, conditions that differ for the kind of cells and areas involved. Among the mediators responsible of these inflammatory and neuroinflammatory responses, we can now include the Prokineticin system.

Even though these inflammatory states are considered a beneficial response, if dysregulated, they can become harmful conditions. Several studies have showed the correlation between inflammatory states and many pathologies, including cancer, metabolic syndrome, psoriasis, asthma, migraine, Inflammatory Bowel Disease (IBDs), major depressive disorder or Alzheimer's disease (AD).

Given that both PK2 and its receptors are highly expressed in inflammatory and neuroinflammatory states, during my Ph.D. I worked on three projects aimed at:

I. **to investigate if the PK system is involved in the protection mechanisms of CORT-nursed rats from TNBS-induced experimental colitis**

   IBDs is the acronym to indicate a group of pathologies that affect colon and small intestine and they are characterized by chronic relapsing intestinal inflammation. Crohn's disease and ulcerative colitis are the two-major form of IBDs and, although the aetiology of IBDs remains largely unknown, the onset involves a complex interaction between the genetic, environmental, microbial

factors and the immune responses. In addition to the involvement of cytokines, several studies have also indicated that chemokines expression is consistently increased during the acute phase of the diseases (*Mazzucchelli et al., 1994; Puleston et al., 2005*).

To better understood the onset mechanisms of IBDs, many animal models have been developed and one of them is the intracolonic infusion of TNBS (2,4,6-trinitrobenzene sulfonic acid).

TNBS induces a strong inflammation through its interaction with colon tissue proteins, rendering these proteins immunogenic to the host immune system (*Morris G.P. et al., 1989*).

In a recent study, Petrella and co-workers have demonstrated that adult male rats exposed to low doses of corticosterone during lactation (CORT-nursed rats) had a reduced vulnerability to TNBS-induced experimental colitis and this protection was probably due to the corticosterone-induced alterations in intestinal permeability (*Petrella C. et al., 2014*).

Watson and colleagues showed that PK2 mRNA was increased in human biopsy samples from colitic patients and similar changes were found in rats with TNBS-induced experimental colitis, asserting that PK2 is a key mediator of the inflammatory response. The authors also presumed that the elevated levels of PK2 induced visceral pain via PKR1 activation, the major receptor expressed in the gut. Moreover, they claimed that this effect was due not only to PK2 but also to PK2β, a peptide derived from proteolytic cleavage of PK2L, the splice variant of PK2 (*Watson et al., 2012, Giannini et al., 2009*).

Based on these premises, the first aim of my Ph.D. was to better investigate the role of PK system in the mechanisms involved in the protection of CORT-nursed rats from TNBS-induced experimental colitis.

To address this issue, the mRNA and protein expressions of PK2, PK2L and PKRs were evaluated by quantitative Polymerase Chain Reaction (qPCR) and

Western Blot (WB) assays in colonic tissues collected from control and CORT-nursed rats (both healthy and colitic animals) after 4 days from TNBS intracolonic instillation.

II. **to characterize the small ligand PK2β, the splice variant of PK2**

Despite the numerous studies about Prokineticins, the biological role of PK2L and its correlate small peptide PK2β are not well established.

Chen and co-workers demonstrated that the secreted PK2L protein is processed into PK2β peptide by proteolytic cleavage which, *in vitro*, is a selective ligand for PKR1 (*Chen J. et al., 2005*).

A previous study from my laboratory demonstrated that in rats and mice bearing CFA-induced chronic inflammation in the hind paw, PK2 and PK2L expression strongly increased and they are PKR1-mediated.

Moreover, PK2 and PK2L increase in rat paw correlates with the development and duration of inflammatory pain. The pain response has higher intensity and longer duration in rats than in mice and this effect was probably due to the higher expression of PK2L protein which give rise to PK2β (*Giannini E. et al., 2009*).

Based on these data, the second aim of my Ph.D. was to evaluate the *in vivo* effects of PK2β peptide. For this purpose, I used two nociceptive tests in Wild Type (WT) and mice lacking the pkr1 or pkr2 gene:

- Hot Plate test, to measure thermal hyperalgesia (mediated by the afferent nociceptive C-fibres, which express mainly PKR1) and
- Von Frey filaments, to measure tactile allodynia (mediated by the activation of large diameter myelinated Aβ fibres, which express mainly PKR2)

Furthermore, I investigated the signalling pathway activated by PK2β in organotypic cultures of Dorsal Root Ganglion obtained from WT mice.

III. **to investigate the role of the Prokineticin system in a non-transgenic animal model of Alzheimer's disease**

Alzheimer's disease (AD) is a severe neurodegenerative disorder characterized by irreversible memory decline and impaired cognitive functions, correlated with the degree of neuronal loss in the brain (*Niikura T. et al., 2006*). Although the aetiology is not yet known, several studies have demonstrated that neuroinflammation plays a significant role in the pathogenesis of AD and one of the probable cause is represented by the deposition of extracellular aggregated amyloid-β (Aβ) in plaques.

During neuroinflammation numerous mediators are released, such as chemokines, that modulate glia activation and, in turn, the neuronal loss.

Currently, there is no cure for AD and drugs used in therapy can only slow down the progression of cognitive and behavioural impairment (*McNaull B.B. et al., 2010*); therefore, most of the researchers have focused their attention to understand the onset mechanisms of AD to develop specific drugs.

Recently, it has been demonstrated that $A\beta_{1-42}$ treatment induces up-regulation of PK2 and its receptors in primary cortical cultures (CNs) and the PKRs antagonist, PC1, prevents the neuronal loss by reducing the Aβ-induced PK2 up-regulation (*Severini C. et al., 2015*). In addition to the PK2 increase, $A\beta_{1-42}$-induced alteration in glutamatergic transmission increasing the AMPA currents in CNs and a similar effect is observed with Bv8 (the amphibian homologue of PK2) stimulation. This up-modulation of AMPA currents is blocked by PC1 treatment (*Caioli et al., 2017*).

To investigate the involvement of Prokineticin system and the potential effects induced by PC1 treatment *in vivo*, $A\beta_{1-42}$ peptide was intracerebroventricular (i.c.v.) infused in rats and brain samples were collected at given time points.

PK2/PKRs mRNA and protein levels were evaluated by qPCR, immunofluorescence (IF) and WB assays.

4. RESULTS AND DISCUSSION

I) Investigation of the role of PK system in the mechanisms involved in the protection of CORT-nursed rats from TNBS-induced experimental colitis

I.1. Effects of TNBS-induced colitis on colonic PK2/PKRs mRNA and protein expression

Rats were sacrificed at day 4 after TNBS instillation and PK2, PKR1 and PKR2 mRNA and protein levels were evaluated by qPCR and Western Blot (WB) assay. In colitic control rats, PK2 mRNA levels were significantly increased compared to healthy rats, whereas no significative differences (but only a small tendency to increase) were observed in CORT-nursed colitic animals compared with CORT-nursed healthy rats (Figure 11).

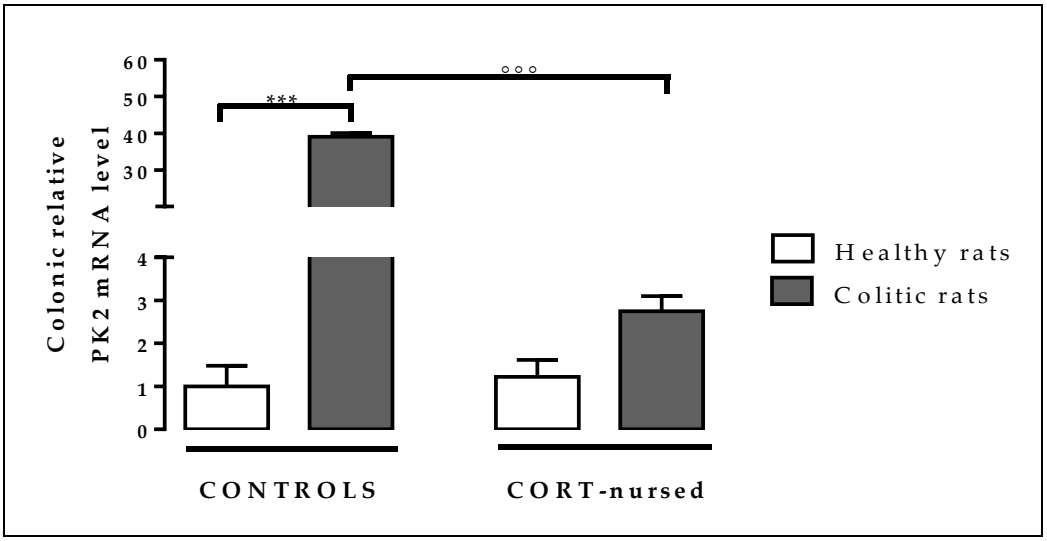


Figure 11. Effects of TNBS-induced colitis on colonic PK2 mRNA. The colonic relative mRNA levels are expressed in relation to β-actin and presented as fold increase relative to control rats. Data are express as mean ± SEM, two-way ANOVA followed by Fisher's LSD post-hoc, ***p<0.001, °°°p<0.001.

These data were confirmed by western blot analysis. Interestingly, the increase of PK2 protein levels was lower in CORT-nursed colitic rats than in control colitic rats (Figure 12). No differences were found between control and CORT-nursed healthy animals.

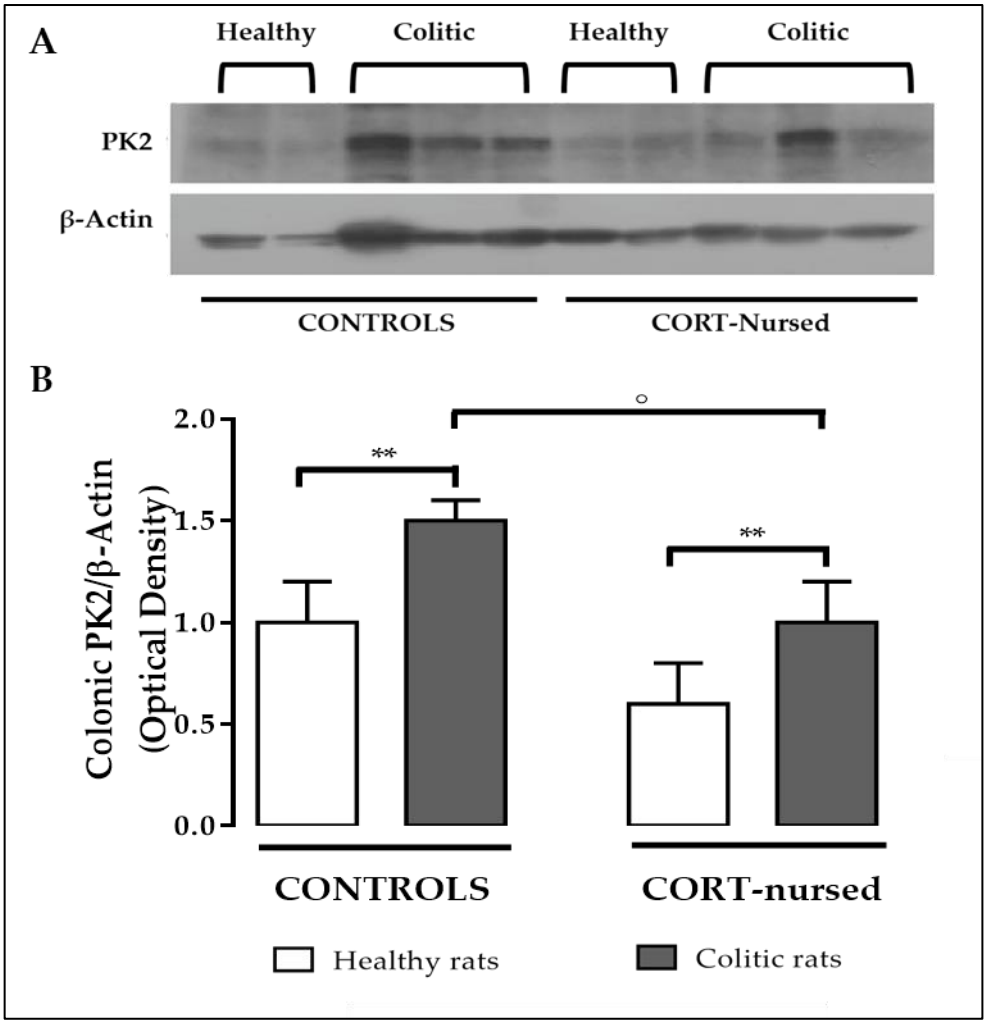


Figure 12. Effects of TNBS-induced colitis on colonic PK2 protein expression.
(**A**) Representative WB analysis showing PK2 protein amount in colonic tissues. (**B**) Optical density (OD) of corresponding WB bands expressed as the ratio of PK2 and β-actin signal. Data are express as mean ± SEM, two-way ANOVA followed by Fisher's LSD post-hoc, **p<0.01, °p<0.05

In agreement with the PK2 mRNA results, PK2L mRNA levels were increased in control colitic rats compared to control healthy animals while no difference was found in CORT-nursed colitic animals compared to CORT-nursed healthy rats (Figure 13).

It is noteworthy that significant reduction in PK2L mRNA was also observed in CORT-nursed colitic rats respected to control colitic rats and no differences were observed between controls and CORT-nursed healthy animals. The latter result has not been confirmed by WB assay, because a specific antibody for PK2L (or for PK2β, the peptide product of proteolytic cleavage of PK2L) it was not available.

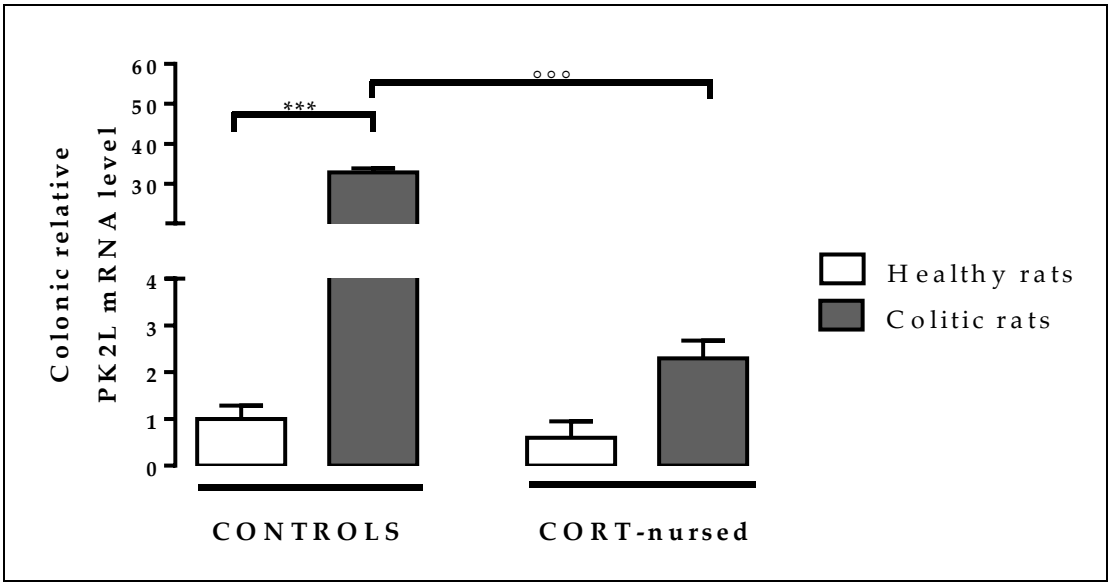


Figure 13. Effects of TNBS-induced colitis on colonic PK2L mRNA expression. The colonic relative mRNA levels are expressed in relation to β-actin and presented as fold increase relative to control rats. Data are express as mean ± SEM, two-way ANOVA followed by Fisher's LSD post-hoc, ***$p<0.001$, °°°$p<0.001$.

PKR1 mRNA levels and protein expression were not affected by colitis and CORT treatment: indeed, as showed in the Figure 14, there were no differences for PKR1 mRNA and protein between experimental groups.

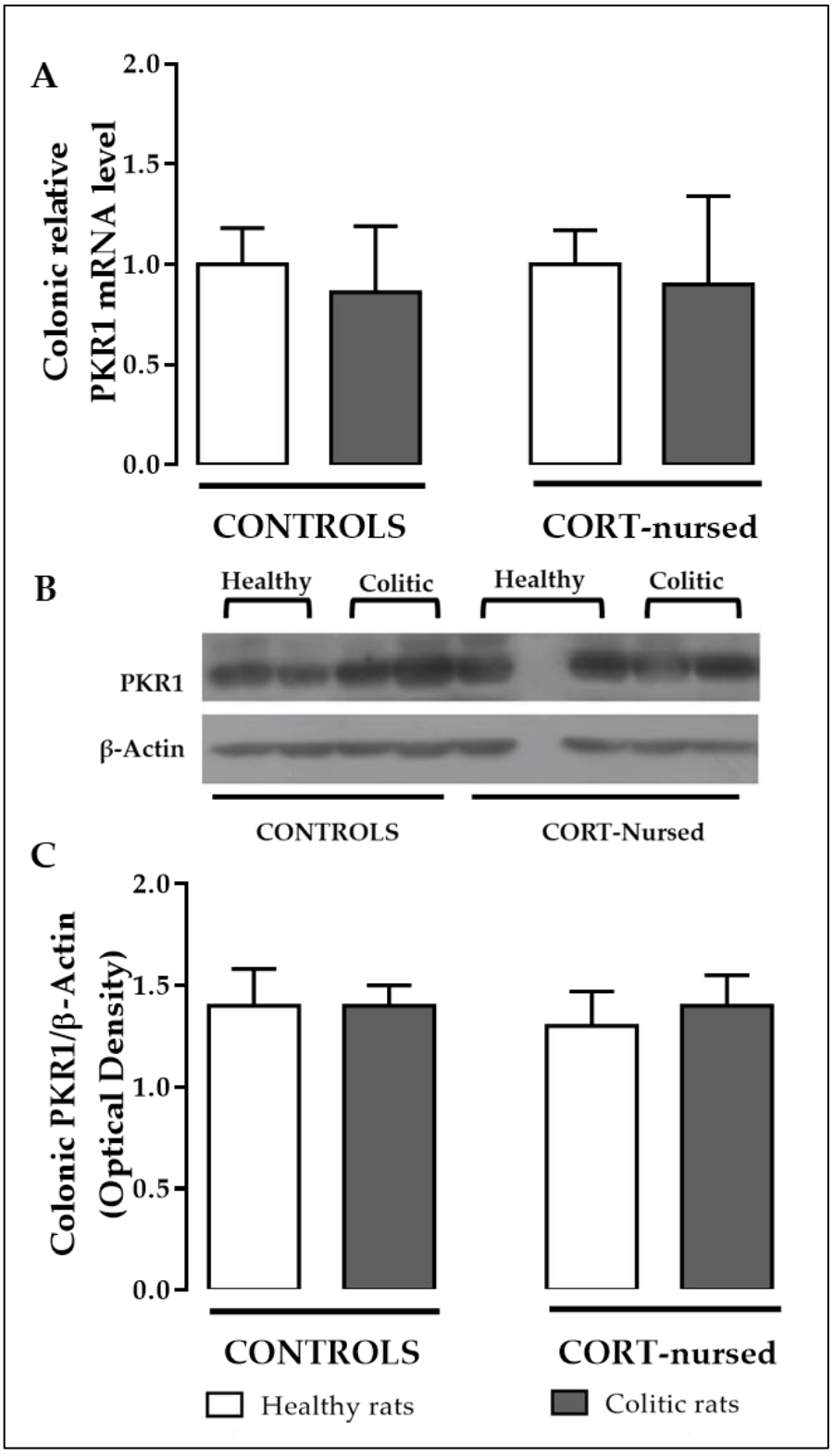


Figure 14. Effects of TNBS-induced colitis on colonic PKR1 mRNA and protein expression. (**A**) The colonic relative mRNA levels are expressed in relation to β-actin and presented as fold increase relative to control rats. Data are express as mean ± SEM. (**B**) Representative WB analysis showing PKR1 protein amount in colonic tissues. (**C**) Optical density (OD) of corresponding WB bands expressed as the ratio of PKR1 and β-actin signal. Data are express as mean ± SEM.

Regarding PKR2, its mRNA was significantly increased in colitic rats compared to healthy animals and no significant effect of CORT treatment was observed (Figure 15).

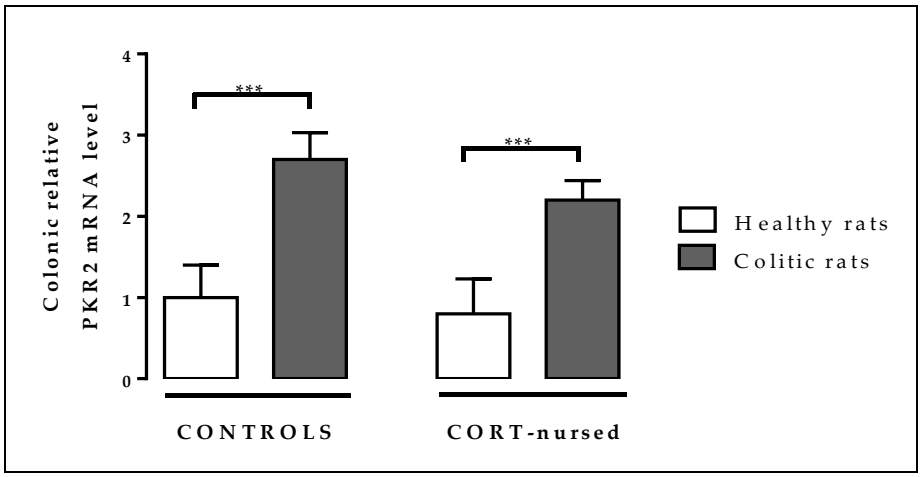


Figure 15. Effects of TNBS-induced colitis on colonic PKR2 mRNA expression. The colonic relative mRNA levels are expressed in relation to β-actin and presented as fold increase relative to control rats. Data are express as mean ± SEM, two-way ANOVA followed by Fisher's LSD post-hoc, ***p<0.001.

Conversely, there were no differences in PKR2 protein expression between groups (Figure 16).

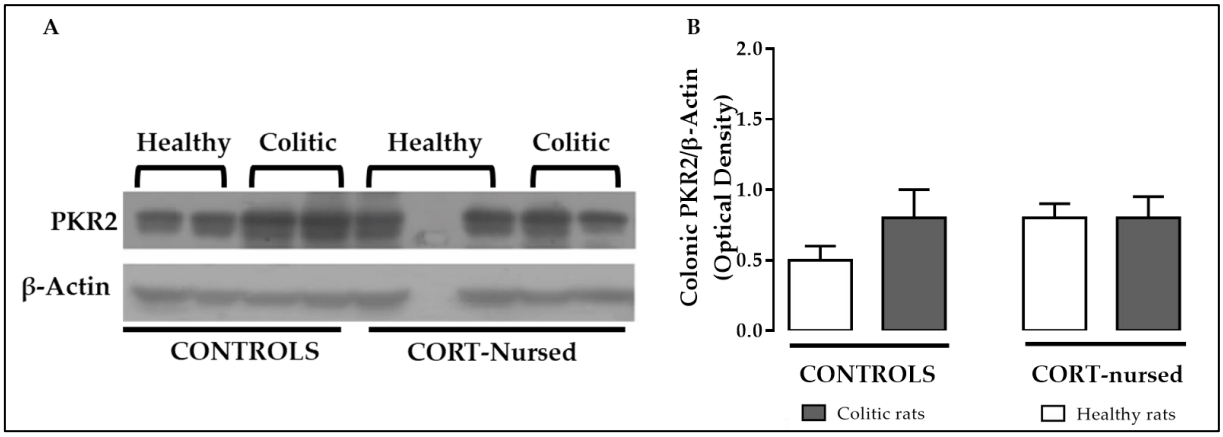


Figure 16. Effects of TNBS-induced colitis on colonic PKR2 protein expression. (**A**) Representative WB analysis showing PKR2 protein amount in colonic tissues. (B) Optical density (OD) of corresponding WB bands expressed as the ratio of PKR2 and β-actin signal. Data are express as mean ± SEM.

I.2. Effects of TNBS-induced colitis on the pro-inflammatory cytokines IL-1β and TNF-α

qPCR analysis showed that inflammatory colitis induced a significant increase in the mRNA levels of IL-1β (Figure 17 A) and TNF-α (Figure 17 B) in control colitis rats, compared to control healthy animals, while no modification of these pro-inflammatory cytokines was observed in CORT-nursed colitic rats, in comparison with CORT-nursed healthy rats. Moreover, CORT-nursed colitic rats had lower levels of IL-1β and TNF-α mRNA, with respect to control colitic animals. No differences were observed between control and CORT-nursed healthy animals.

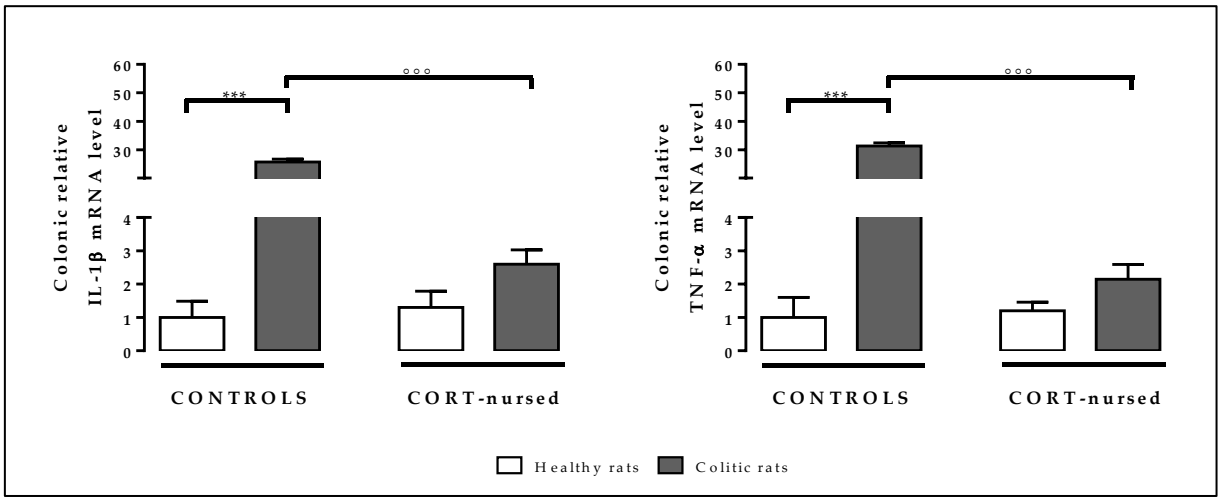


Figure 17. Effects of TNBS-induced colitis on colonic IL-1β and TNF-α mRNA expression.
The colonic relative mRNA levels of IL-1β (**A**) and TNF-α (**B**) are expressed in relation to β-actin and presented as fold increase relative to control rats. Data are express as mean ± SEM, two-way ANOVA followed by Fisher's LSD post-hoc, ***p<0.001; °°°p>0.001.

I.3. Discussion

The results here presented are a section of a project conducted in collaboration with Dr. Casolini's group and my assignment was to investigate the involvement of PK

system in the protection mechanisms of CORT-nursed rats from TNBS-induced experimental colitis (for any further details, please refer to *Zinni et al., 2017*).

The term "CORT-nursed" is used to indicate offspring nursed from mothers with mild hypercorticosteronaemia developed by drinking water supplemented with corticosterone (0.2 mg/ml). The corticosterone assumed through breast milk is easily absorbed by the gastrointestinal tract of the pups. Once adults, these rats showed improved learning capabilities, reduced fearfulness in anxiogenic situations, persistent hypo-reactivity of the hypothalamus-pituitary-adrenal axis due to an increased number of glucocorticoid receptors (GR) in the hippocampus and resistance to ischemic neuronal damage (*Casolini P. et al., 2007*; *Catalani A. et al., 2011*).

A recent study demonstrated that adult CORT-nursed rats are protected against 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced experimental colitis: indeed, they showed an improvement in some indices of pathology (such as loss of body weight and food intake, increased colonic myeloperoxidase activity and mast-cell degranulation) with respect to colitic control animals (adult male offspring whose mothers drank tap water during lactation). Here, we have investigated the cellular actors involved in the protection mechanisms of CORT-nursed rats in TNBS-induced experimental colitis.

Glucocorticoids (GCs) are able to activate the cytosolic GR, assembling a ligand-receptor complex that translocates into the nucleus where interacts with the promoter regions of different genes such as Glucocorticoid-Induced Leucine Zipper (GILZ). GILZ has immuno-modulatory activity that involves a physical interaction between GILZ itself and the transcription factors that regulate the expression of pro-inflammatory genes (*Ronchetti S. et al., 2015*) such as the transcription Nuclear Factor κB (NF-κB). Data from literature show that NF-κB promotes the transcription of pro-inflammatory genes such as IL-1β and TNF-α (*Tak P. P. et al., 2001*) and this effect is inhibited by the physical interaction of GILZ with the p65 subunit of NF-κB (*Ayroldi E. et al., 2001; Riccardi C. et al., 2001*). In the study conducted by Zinni, it has been

demonstrated that GILZ is significantly increased in CORT-nursed colitic rats and its over-expression reduced the colonic NF-κB activation (data not show, remind to *Zinni et al., 2017*) and, in turn, the expression of the pro-inflammatory cytokines IL-1β and TNF-α, as show here by qPCR analysis.

During inflammatory processes, cytokines are released together with other molecules such as chemokines belonging to the Prokineticins family (*Negri L. and Lattanzi R., 2011*). Indeed, it is already demonstrated that in inflammatory conditions, PK2 and its splice variant PK2L are strongly augmented (*Giannini E. et al., 2009*) and that the increase of IL-1β is correlated with the up-regulation of PK2 (*Franchi S. et al., 2008*). This PK2 increase is also confirmed by Watson in TNBS-induced colitis (*Watson R. P. et al., 2012*).

In this study, PK2 (both mRNA and protein) and PK2L mRNA expressions are increased in colitic rats and these increases are of minor entity in colitic CORT-nursed rats compared to control colitic rats. This protection could be ascribed to the relationship between NF-κB and the Prokineticin system. Indeed, it has been demonstrated that NF-κB is able to regulate the expression of the Bo8 gene (a Bv8 homologue from *Bombina orientalis*) and that the consensus sequence for NF-κB identified in the Bo8 promoter is similar to those present in the promoter regions of mammalian homologs PK2 (*Marsango S. et al.,2009*).

Concerning the prokineticin receptors, PKR1 is the major receptor express in the GI tract and data from qPCR and WB assays show no differences in PKR1 expression after TNBS intracolonic instillation, while PKR2 mRNA expression is increased after TNBS-induced colitis; this is in accordance with previous evidence that suggested PKR2 as the inducible receptor (*Maftei D. et al., 2014*; *Kisliouk T. et al., 2005*). Conversely, PKR2 protein expression levels are unchanged. There are two possibilities that can explain this discrepancy: the first is that PKR2 mRNA and protein expression are performed at only one-time point (four days after TNBS instillation), without performing a time-course and it cannot exclude that PKR2 mRNA up-regulation has just started at day 4

after TNBS intracolonic instillation whereas the protein expression can occur later. The second theory regards the transcription mechanisms of mRNA in protein: indeed, there is no direct relationship between the concentration of a transcript and the concentration of its protein, as reported by Liu and colleagues (*Liu Y. at al., 2016*).

II) Characterization of the small ligand PK2β

II.1. Evaluation of thermal hyperalgesia and tactile allodynia

The effect of PK2β on nociceptive thresholds to thermal and tactile stimuli was evaluated using two behavioural tests: Hot Plate test and Von Frey filaments in wild-type (WT) mice and PKR1- or PKR2-null mice (Figure 18).

In WT mice, PK2β intraplantar (i.pl.) injection induced a dose-dependently thermal hyperalgesia (Hot Plate test, Figure 18 A) which peaked 30 minutes after i.pl. administration and lasted about two hours: the dose of 10 fmol induced a slight non-significant thermal hyperalgesia, whereas at 30 fmol nociceptive threshold to thermal stimuli was decreased by 28%.

The higher dose of 60 fmol diminished the nociceptive threshold by 43%. These effects were comparable to those induced by PK2 i.pl. administration (Figure 18 B). Indeed, PK2 30 and 60 fmol decreased the nociceptive threshold to thermal stimuli by 24% and 40%, respectively. The same doses of PK2 also induced tactile allodynia (evaluated by Von Frey filaments, Figure 18 D), which peaked 60-90 min after administration and lasted about three hours.

Conversely, PK2β 30 and 60 fmol were almost ineffective in inducing sensitization to tactile stimuli (Figure 18 C). Indeed, the higher dose of 60 fmol was able to significantly decrease the nociceptive threshold to tactile stimuli by only 25%, whereas the same dose of PK2 induced a strong and long-lasting tactile allodynia.

Mice lacking PKR1 or PKR2 are less sensitive than wild-type mice to PK2β (Figure 18 E).

Administration of PK2β 600 fmol (a dose ten times larger) induced a significant decrease (36%) of thermal nociceptive threshold in PKR2-KO mice. In PKR1-null mice, 600 fmol of PK2β was almost ineffective in inducing thermal hyperalgesia.

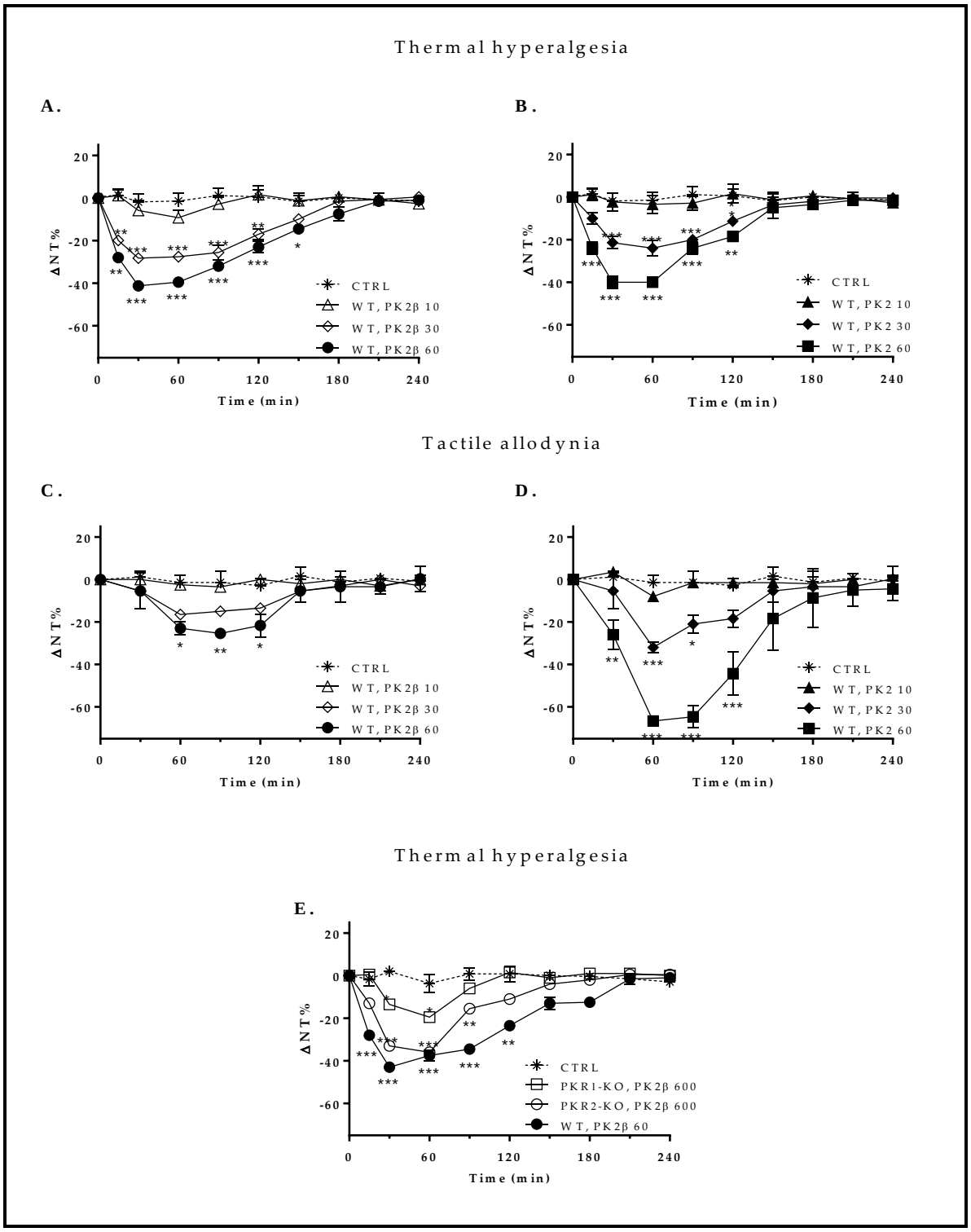


Figure 18. Time-course of percentage decrease in nociceptive threshold (% ΔNT) elicited in mice after i.pl. injection of PK2β and PK2.Effect of intraplantar injection of 10, 30 and 60 fmol of PK2β (**A**, **C**) and PK2 (**B**, **D**) on the nociceptive threshold to thermal (Hot Plate test) and tactile stimuli (Von Frey filaments). Effect of intraplantar injection of PK2β 600 fmol in PKR1- and PKR2-KO mice compared to WT mice (**E**). The data represent means ± SEM of six mice. Two-way ANOVA was used for statistical evaluation, followed by the Bonferroni's test. *** $p<0.001$, **$p<0.01$, * $p<0.05$ compared with CTRL group.

II.2. Evaluation of PK2β-induced PKR1 activation in organotypic cultures of DRG

To assess the ability of PK2β to activate PKR1 coupled to $G\alpha_i$ protein, we evaluated the STAT3 phosphorylation by WB assay in mice DRG organotypic cultures (Figure 19). The cultures were stimulated for 1 hour with PK2, PTX ($G\alpha_i$ protein inhibitor) (*Burns D. et al., 1988*) plus PK2 or PK2β. As shown in the Figure, PK2 induces tyrosine phosphorylation of STAT3, according to literature, whereas PK2β does not induce phosphorylation.

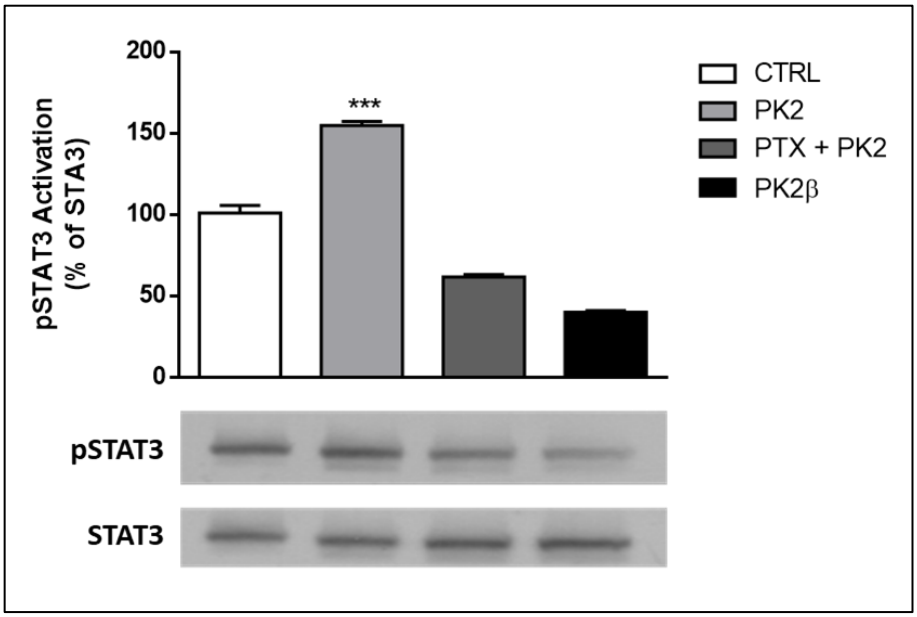


Figure 19. PK2β does not induce STAT3 activation. Western blot analysis showing representative bands of phospho-STAT3 (p-STAT3) and STAT3 protein. Values are means ± SEM of three replications for each antibody, performed on three separate pools. One-way ANOVA was used for statistical evaluation, followed by Tukey's test for multiple comparisons. *** $p<0.001$ PK2 vs CTRL.

II.3. Discussion

Alternative splicing is a fine-regulated process during gene expression which allows to a single gene to encode for multiple proteins increasing the cellular biodiversity. It occurs as a normal phenomenon in eukaryotes and several mechanisms have been observed.

Prokineticins are proteins capable of generating a wide variety of responses, thanks to specific tissue distribution and/or cellular targets in different organisms and cell types (*LeCouter J. et al., 2003b*). Two isoforms arising from a PK2 gene through alternative splicing have been identified in humans and mice, and named PK2 and PK2L (*Wechselberger C. et al., 1999*; *Chen J. et al, 2005*): PK2 is formed by 3 exons (exons 1, 2 and 4) whereas PK2L is formed by 4 exons (1, 2, 3 and 4 exons) which contain 21 additional basic residues. PK2 mRNA is found in all tissues, whereas PK2L mRNA expression is found to be highest in the lung and spleen, barely detected in the brain, and undetectable in the kidney (*Negri L. et al., 2007*).

It is known that the ligand PK2 has diverse physiological effects depending on tissues expression (*Guilini C. et al., 2010*), but the physiological role of PK2L/PK2β is not well established. The information so far available indicate that PK2L has a very poor biological activity and, when it is cleaved by extracellular proteases in the smaller peptide PK2β, results a selective ligand for PKR1 *in vitro* (*Chen J. et al., 2005*).

In this study, the activity of PK2β is better investigated. Previous data have shown that intraplantar injection of Bv8/PK2 produces an intense nociceptive sensitization to both thermal and tactile stimuli (*Negri L. et al., 2002*).

Here, we demonstrate that local injection of PK2β behaves like Bv8/PK2, being capable of strong decrease the nociceptive threshold of mice only when they are subjected to thermal but not tactile stimuli. Considerable evidences indicate that thermal hyperalgesia and tactile allodynia are mediated through different neuronal pathways (*Yeomans D. C. and Proudfit H. K, 1996*; *Ossipov M. H. et al., 1999*). Thermal hyperalgesia is most likely mediated by the afferent nociceptive C-fibres, which express mainly PKR1, whereas tactile allodynia seems to be mediated by the activation of large diameter, myelinated Aβ fibres, which express mainly PKR2 (*Ossipov M. H. et al., 1999*; *Negri L. et al., 2006*; *Vellani V. et al., 2006*). Moreover, in a previous study is demonstrated that tactile allodynia is equally evoked by Bv8 in WT and PKR1-null mice, suggesting that PKR2 receptor is involved in nociceptor sensitization to punctate

stimuli (*Negri L. et al., 2006*). All these data allow us to argue that PK2β is able to bind and activate PKR1 *in vivo*. Indeed, these results are further confirmed by pain behavioural experiments on PKRs-KO mice. Indeed, we demonstrate that the dose of PK2β that can induce strong thermal hyperalgesia in PKR2-KO mice is ineffective in PKR1-KO mice.

In this work we demonstrated (data not shown) that in different yeast strains expressing selectively PKR1 coupled to $G\alpha_s$, $G\alpha_q$ or $G\alpha_i$ subunit, PK2 was able to activate all of them subunits whereas, PK2β was capable to activate PKR1 coupled with $G\alpha_s$ or $G\alpha_q$ but not $G\alpha_i$. To confirm this data, we analysed STAT3 phosphorylation in mice DRG organotypic cultures. STAT3 is a transcriptional factor activate in response to cytokines and grow factors and its phosphorylation is due to activation of subunit $G\alpha_i$ protein. As demonstrated by WB assay, PK2β is not able to induce STAT3 activation in mice organotypic cultures of DRG.

These results contribute to the understanding of the PK ligands and receptors function and which different responses will be generated in different cell types, depending on which ligand-receptor-G protein combination is expressed.

III) Investigation of the role of Prokineticin system in a non-transgenic animal model of Alzheimer's disease

III.1. Biochemical assays in prefrontal cortex (PFC) and hippocampus

To investigate the role of the Prokineticin system in an *in vivo* animal model of Alzheimer's disease, time course analysis of PK2 and PKRs mRNA levels in prefrontal cortex (PFC) and hippocampus were performed by qPCR. Tissues were collected at 1, 7, 14 and 35 days after $A\beta_{1-42}$ or vehicle i.c.v. infusion (Figure 20).

At day 35 after i.c.v. infusion, PK2 and PKRs protein expressions were evaluated by immunofluorescence (IF) assay in PFC and by IF and Western Blot (WB) in hippocampus.

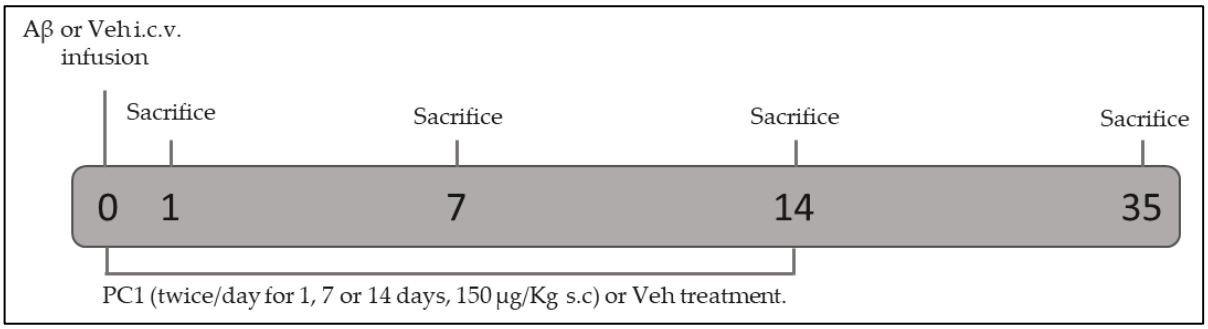


Figure 20. Experimental timeline. Rats received infusion of $A\beta_{1-42}$ or Vehicle into the left lateral ventricle. PC1 treatment (150 µg/kg s.c. 2 times a day for 14 days) started on the day of surgery and ended at day 14. Tissues were collected at time-points indicated in the image.

III.2. Evaluation of PK2/PKRs in prefrontal cortex (PFC)

In Vehicle infused rats, basal levels of PK2, PKR1 or PKR2 mRNA were negligible.

$A\beta_{1-42}$ i.c.v. infusion modulated PK2 and PKRs mRNA expression levels. Indeed, PK2 and PKR2 mRNA levels were significantly increased already at day 1 (Figure 21) and remained at the same level up to 35 days. PKR1 mRNA showed a constant tendency

to increase up to 14 days, reaching the statistical significance at day 7 after $A\beta_{1-42}$ infusion.

PC1 administration (150 μg/kg, s.c. 2 times a day for 1, 7 or 14 days, starting at the day of $A\beta_{1-42}$ infusion) had no effect on PKRs mRNA overexpression, but it tended to reduce PK2 mRNA levels even if the values did not reach the statistical significance.

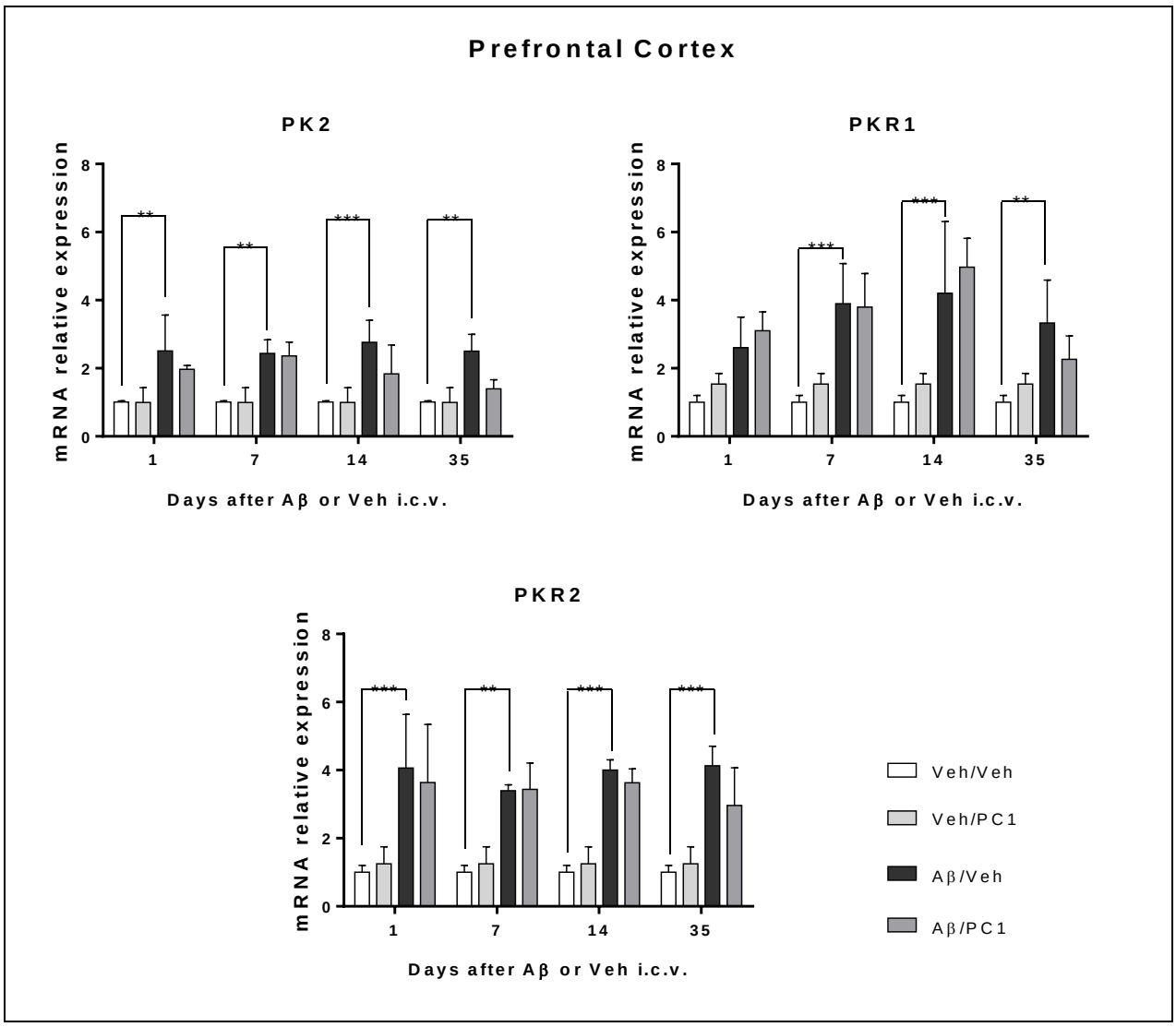


Figure 21. Time-course analysis of PK2, PKR1 and PKR2 mRNA expressions in prefrontal cortex of $A\beta_{1-42}$ or Veh i.c.v. infused rats. Data represent means ± SEM of five rats. Analysis were performed with Two-way ANOVA, followed by Bonferroni's test. **p < 0.01, ***p < 0.001.

Immunofluorescence analysis, performed 35 days after Aβ1-42 infusion, showed an increase of PK2 immunofluorescence signal (Figure 22 A) in neurons of prefrontal cortex, as demonstrated by the colocalization with NeuN (neuronal marker). PKR1 immunofluorescence signal was slightly increased after Aβ1-42 infusion in PFC neurons, as demonstrated by the colocalization with NeuN, compared with Vehicle infused rats where the signal was undetectable (Figure 22 B).

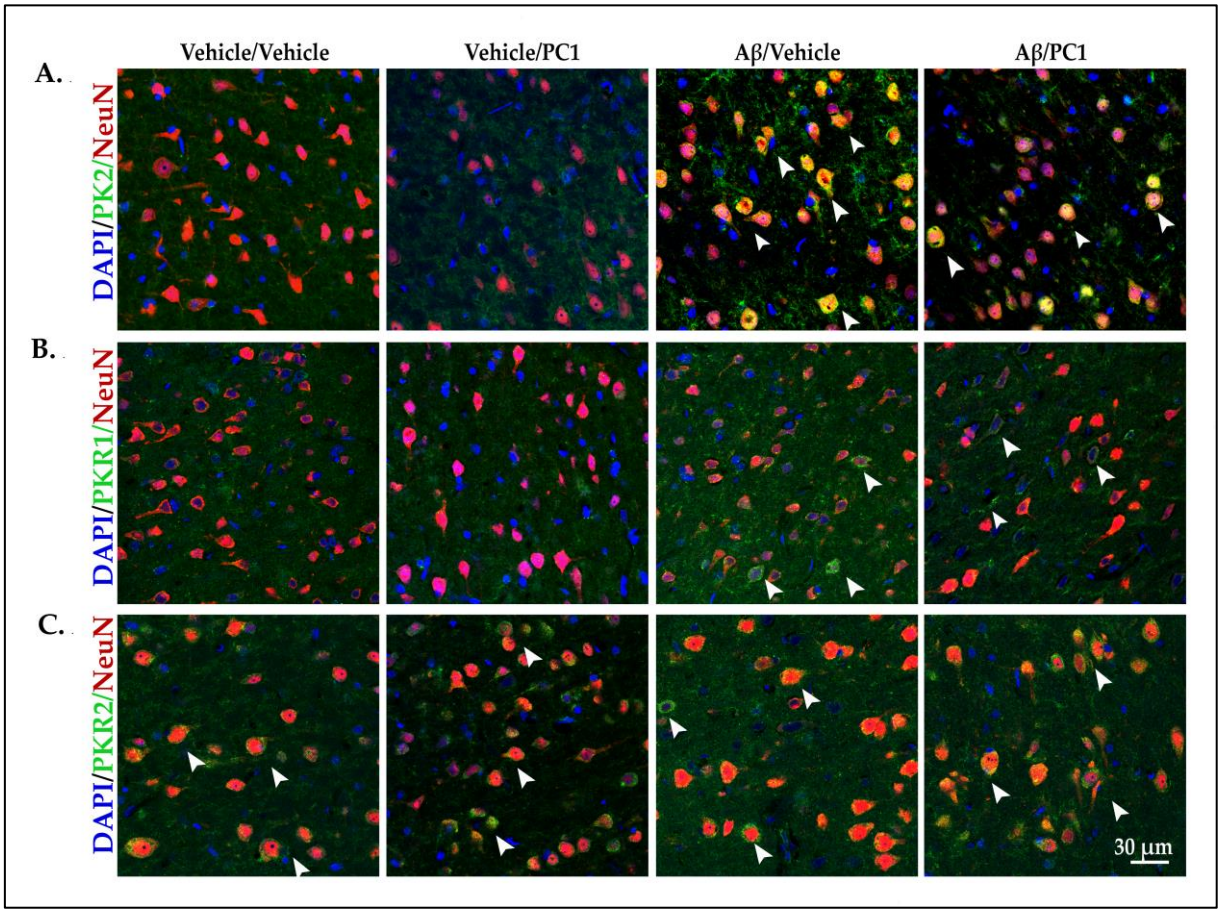


Figure 22. Expression of PK2, PKR1 and PKR2 in the PFC of Vehicle/Vehicle, Vehicle/PC1, Aβ1-42/Vehicle and Aβ1-42/PC1 rats. Immunofluorescence double-staining of PK2 (**A**), PKR1 (**B**) and PKR2 (**C**) - green with NeuN (neuronal marker) – red. Cell nuclei were counterstained with DAPI (blue fluorescence). Scale bar, 30 μm.

In the same area, PKR2 immunofluorescence signal (Figure 22 C) was present in neurons of Vehicle infused rats, as demonstrated by the colocalization with NeuN.

$A\beta_{1-42}$ infused rats, PKR2 immunofluorescence signal only slightly increased in PFC neurons.

PC1 treatment displayed a tendency to decrease the PK2 immunofluorescence signal but had no effect on PKRs.

III.3. Evaluation of PK2/PKRs expression in hippocampus

In hippocampus, time-course analysis of mRNA expression showed a strong activation of PK system (Figure 23).

In $A\beta_{1-42}$ injected rats, PK2 mRNA levels were increased already at day 1 after i.c.v. infusion and reached the maximum after 14 and 35 days,

PKR1 mRNA showed ups and downs expression pattern with a not significant increase at day 1, a decrease at day 7 followed by a significant increase at day 14 and then a decrease at day 35.

PKR2 mRNA expression showed the similar ups and downs expression pattern of PKR1 with a significant increase at days 1 and 14 and a decrease at days 7 and 35 after $A\beta_{1-42}$ infusion.

PC1 administration (150 μg/kg, s.c. 2 times a day for 1, 7 or 14 days) significantly decrease PK2 mRNA levels starting from day 7 after $A\beta_{1-42}$ i.c.v. infusion and had no effects on PKR2 mRNA expression levels.

Interestingly, PC1 treatment increased PKR1 mRNA levels, compared to $A\beta_{1-42}$ injected rats even if the values did not rich a statistical significance.

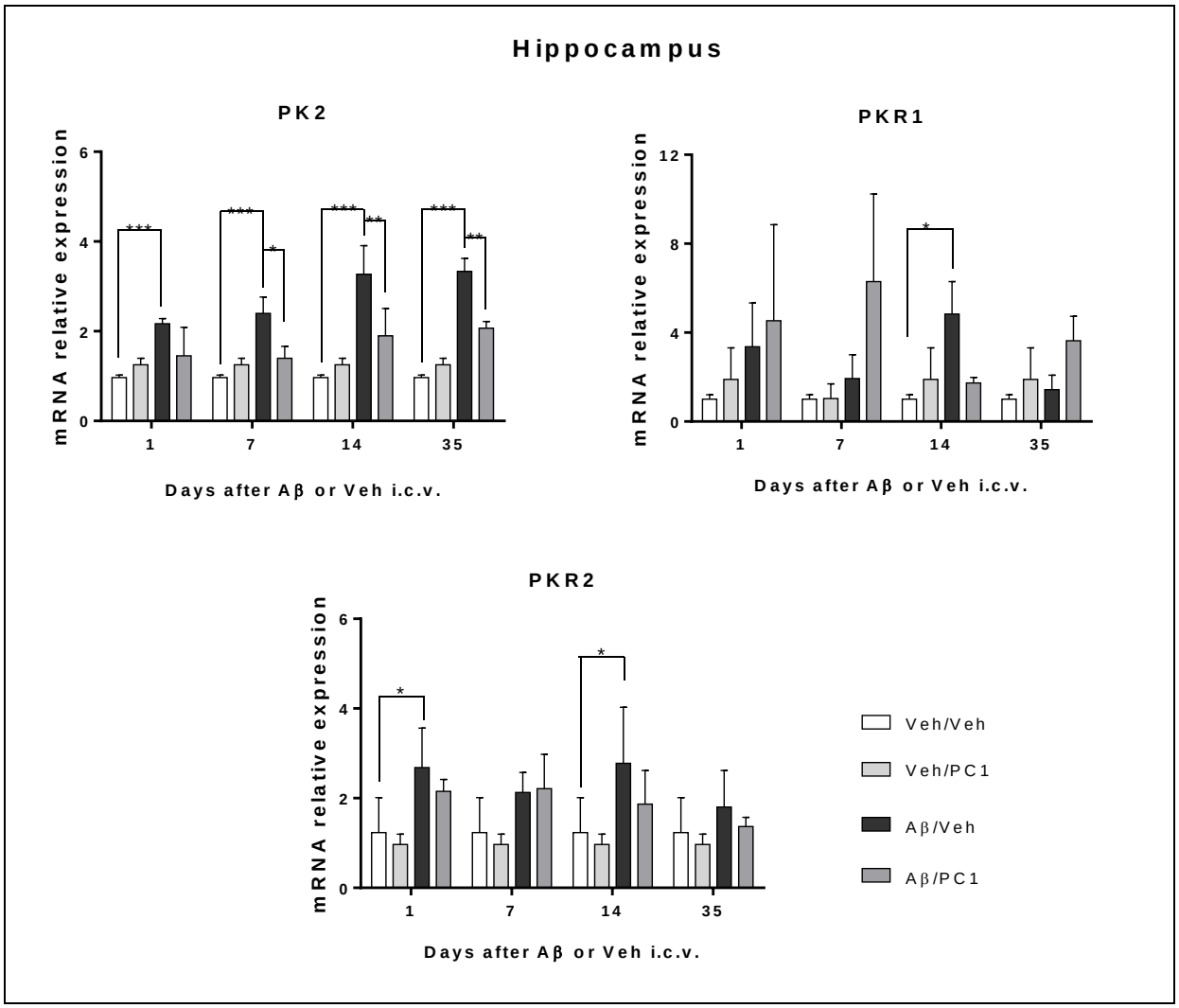


Figure 23. Time-course analysis of mRNA PK2, PKR1 and PKR2 expression in hippocampus of Aβ or vehicle i.c.v. infused rats. Data represent means ± SEM of five rats. Analysis were performed with Two-way ANOVA, followed by Bonferroni's test. *p < 0.05; **p < 0.01, ***p < 0.001.

Proteins analysis of PK2 and PKRs were performed on samples collected at day 35 after $A\beta_{1\text{-}42}$ or vehicle i.c.v. infusion by western blot (WB) and IF assays.

In hippocampus of $A\beta_{1\text{-}42}$ injected rats, WB analysis showed a significant increase of PK2 (Figure 24) and PKR1(Figure 26) protein levels compared to Vehicle injected rats, confirming the results obtained by mRNA analysis at day 35.

Regarding PKR2 (Figure 28), no differences in protein levels were found between the experimental groups. PC1 treatment significantly reduced PK2 protein levels (Figure 24) and strongly increased PKR1 protein expression (Figure 26) in hippocampus.

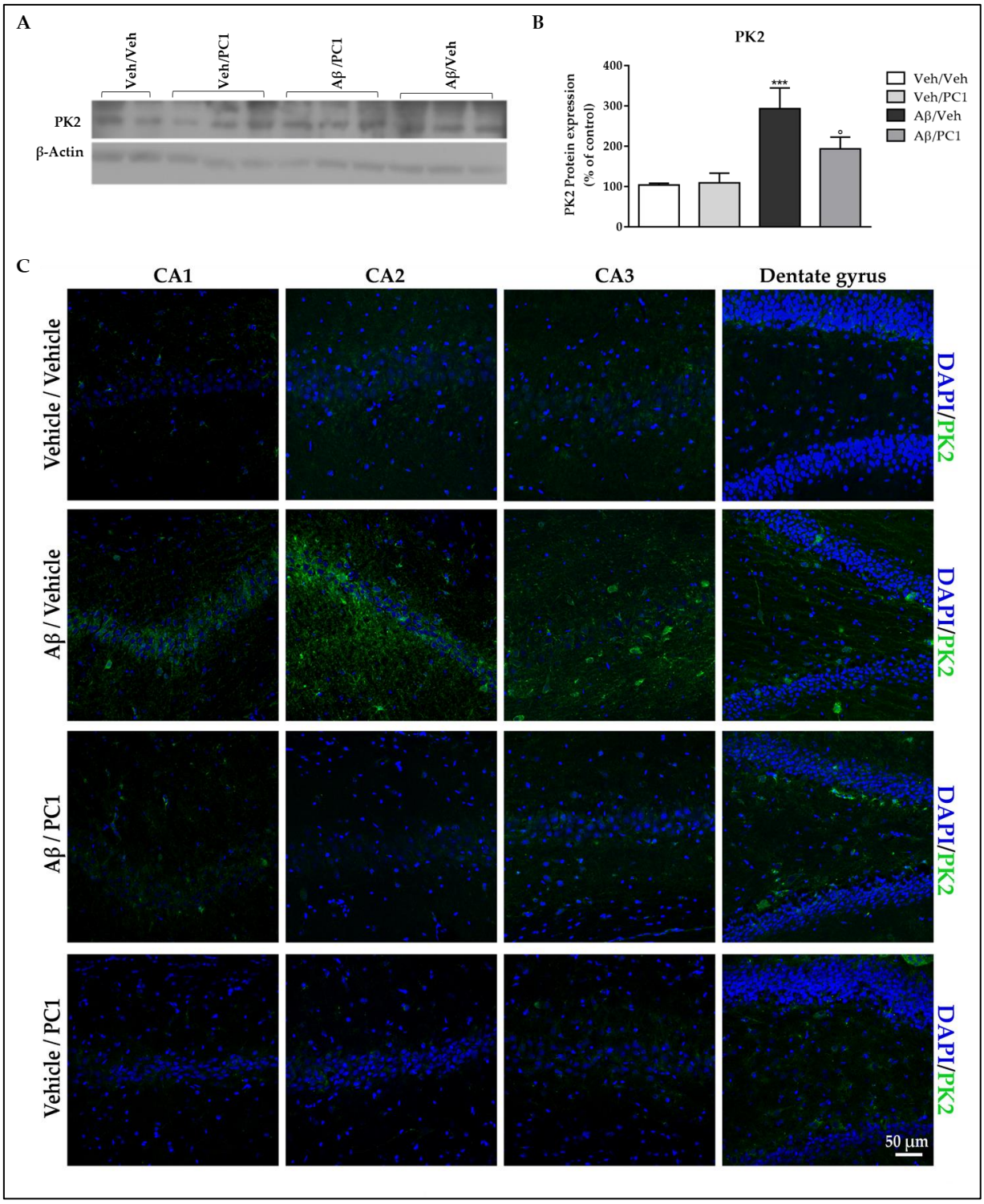


Figure 24. PK2 protein expression in hippocampus of Vehicle/Vehicle, Aβ$_{1-42}$/Vehicle, Aβ$_{1-42}$/PC1 and Vehicle/PC1 rats. (**A**) Representative WB analysis showing PK2 protein amount in hippocampus. (**B**) Optical density (OD) of corresponding WB bands expressed as the ratio of PK2 and β-actin signal. Data are express as mean ± SEM. (**C**) Immunofluorescence staining of PK2 (green) in CA1, CA2, CA3 and DG. Cell nuclei were counterstained with DAPI (blue fluorescence). Scale bar, 50 µm.

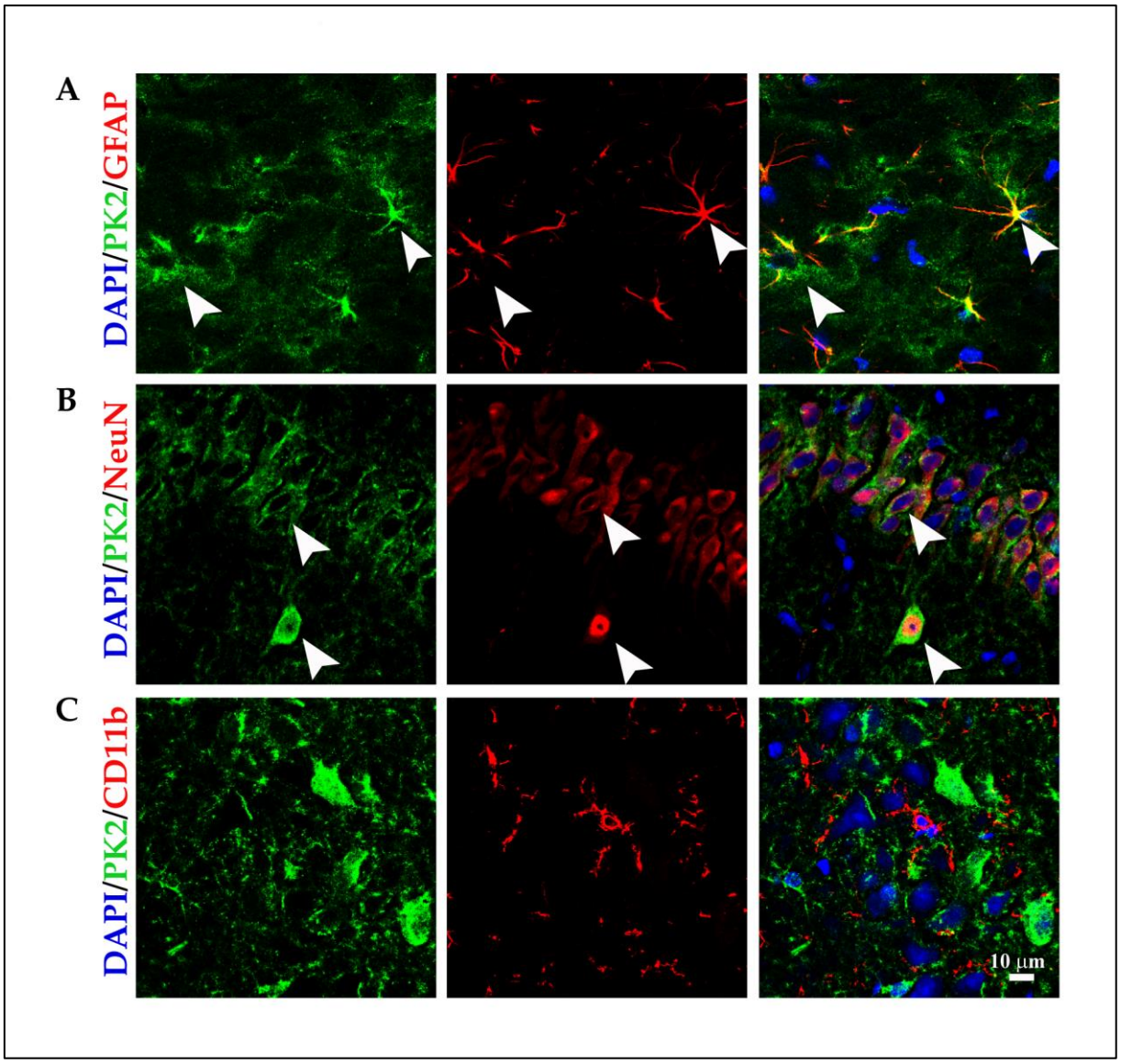


Figure 25. PK2 cellular localization in the hippocampus. Immunofluorescence double-staining of PK2 – green - and (**A**) GFAP (astrocytic marker), (**B**) NeuN (neuronal marker) and (**C**) CD11b (microglial marker) – red. Cell nuclei were counterstained with DAPI (blue fluorescence). Scale bar, 10 μm.

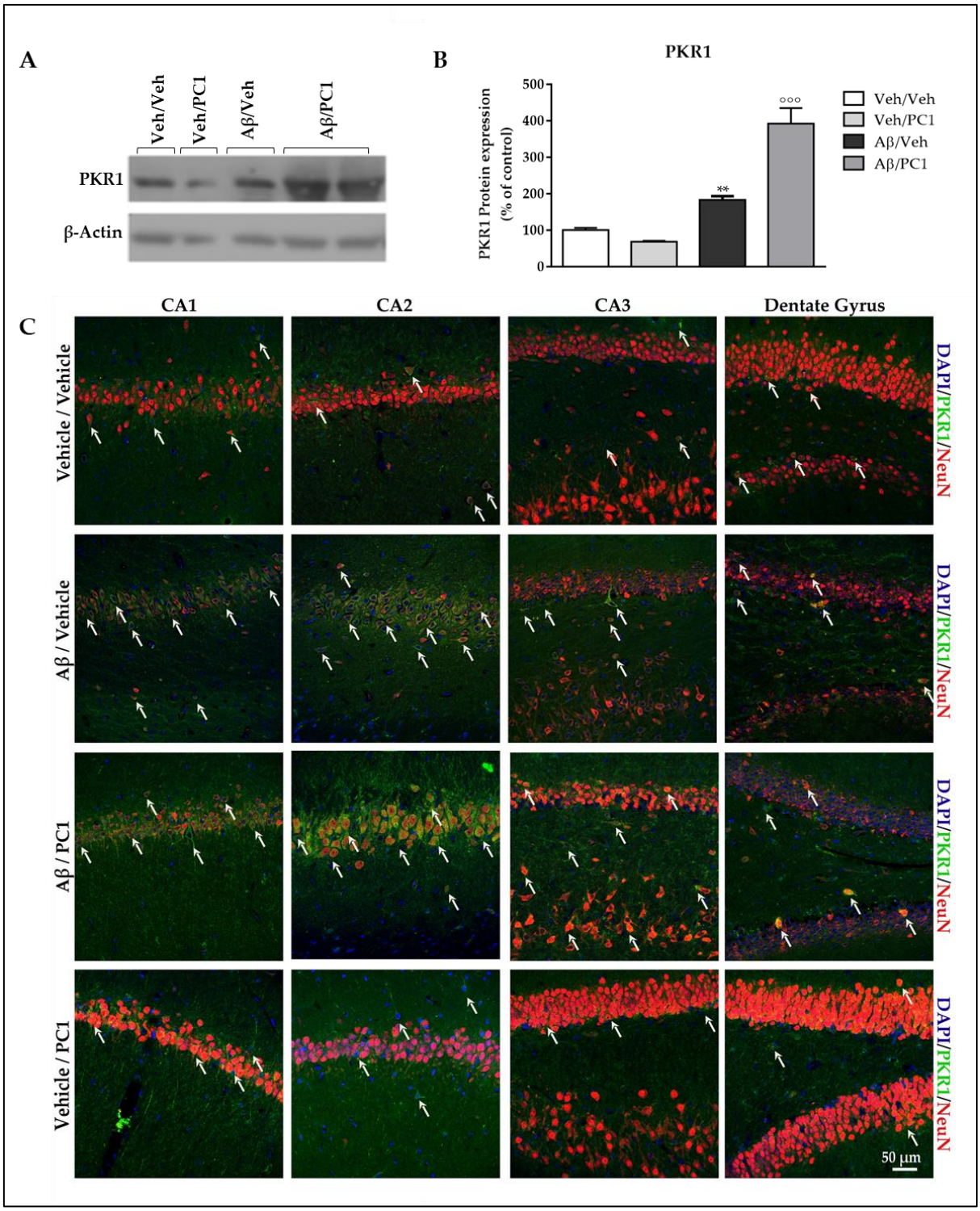


Figure 26. PKR1 protein expression in the hippocampus of Vehicle/Vehicle, Aβ1-42/Vehicle, Aβ1-42/PC1 and Vehicle/PC1 rats.

(**A**) Representative WB analysis showing PKR1 protein amount in hippocampus. (**B**) Optical density (OD) of corresponding WB bands expressed as the ratio of PKR1 and β-actin signal. Data are express as mean ± SEM.(**C**) Immunofluorescence double-staining of PKR1 - green with NeuN (neuronal marker) – red in CA1, CA2, CA3 and DG. Cell nuclei were counterstained with DAPI (blue fluorescence). Scale bar, 50 μm.

IF studies were performed on hippocampus and expression levels of PK2 and PKRs in CA1, CA2, CA3 and dentate gyrus (DG) were evaluated. In these areas, PK2 immunofluorescence signal (Figure 24) was strongly increased in CA1, CA2 and CA3 but not DG of $A\beta_{1-42}$ injected rats, compare to Vehicle i.c.v. injected animals (where the PK2 signal was faint), confirming the results obtained by WB assay.

PK2 immunoreactivity was localized in neurons and some astrocytes (as demonstrated by the colocalization with NeuN and GFAP, neuronal and astrocyte markers, respectively, Figure 25) but not in microglia. PC1 treatment was able to decrease the PK2 immunofluorescence signal in these areas.

In Vehicle infused rats, PKR1 immunofluorescence signal was faint in all the hippocampal regions investigated (Figure 26) and IF signal was localized mainly in the neurons, as demonstrated by colocalization with NeuN (Figure 27).

$A\beta_{1-42}$ infusion increased PKR1 signal in neurons and astrocytes mainly in CA1 and CA2 areas. Interestingly, as already showed by WB assay, PC1 treatment increased PKR1 immunofluorescence signal, mostly in CA2 and CA1 regions.

In Vehicle infused rats, PKR2 signal was localized in neurons of CA1, CA2 and CA3 but not DG (Figure 29), as demonstrated by the colocalization with NeuN, but not in astrocytes or microglia.

$A\beta_{1-42}$ i.c.v. infusion slowly increase the PKR2 immunofluorescence signal in neurons and PC1 treatment did not change the PKR2 immunofluorescence signal in hippocampus, according to WB assay.

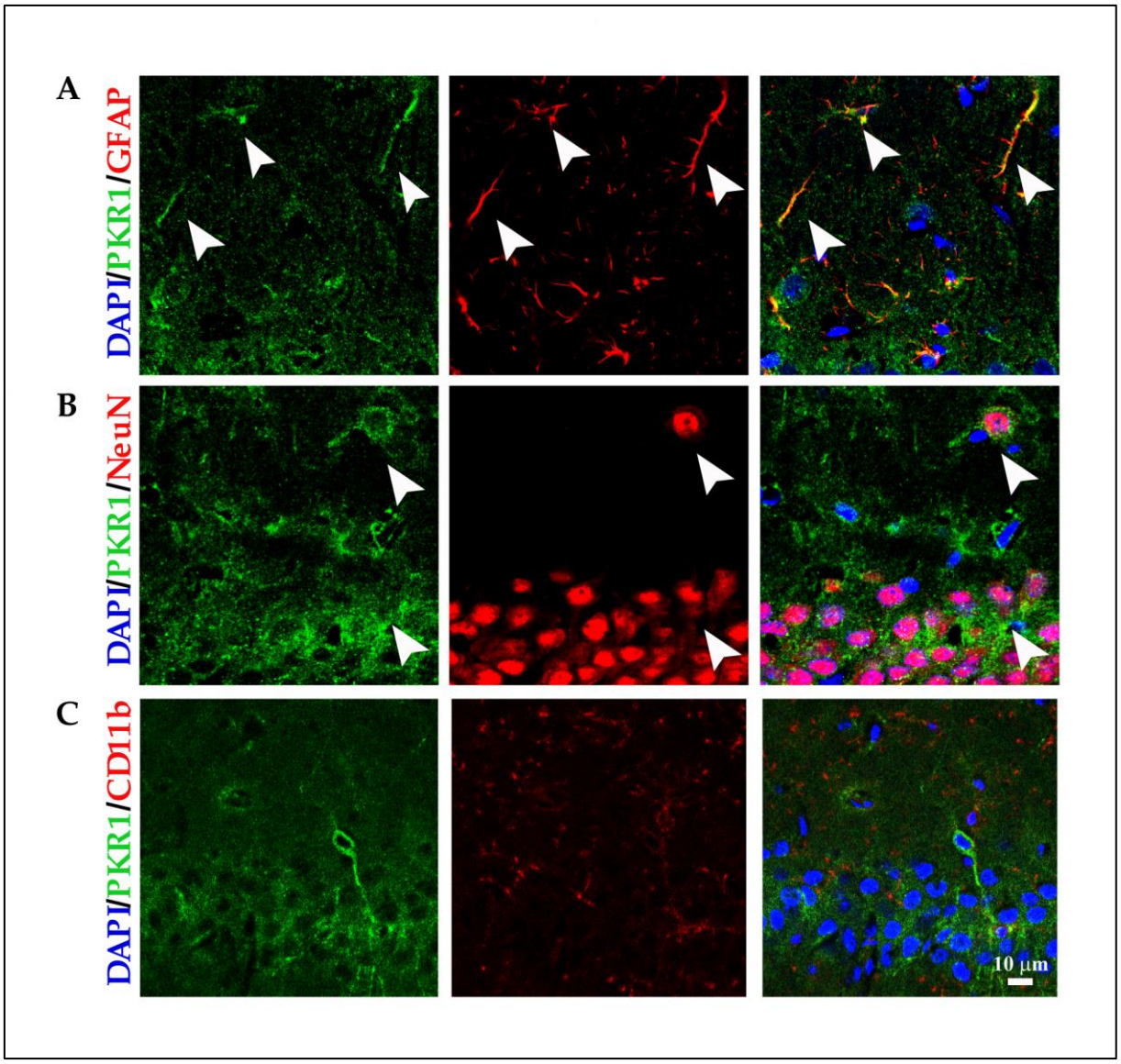


Figure 27. PKR1 cellular localization in the hippocampus. Immunofluorescence double-staining of PKR1 – green - and (**A**) GFAP (astrocytic marker), (**B**) NeuN (neuronal marker) and (**C**) CD11b (microglial marker) – red. Cell nuclei were counterstained with DAPI (blue fluorescence). Scale bar, 10 μm.

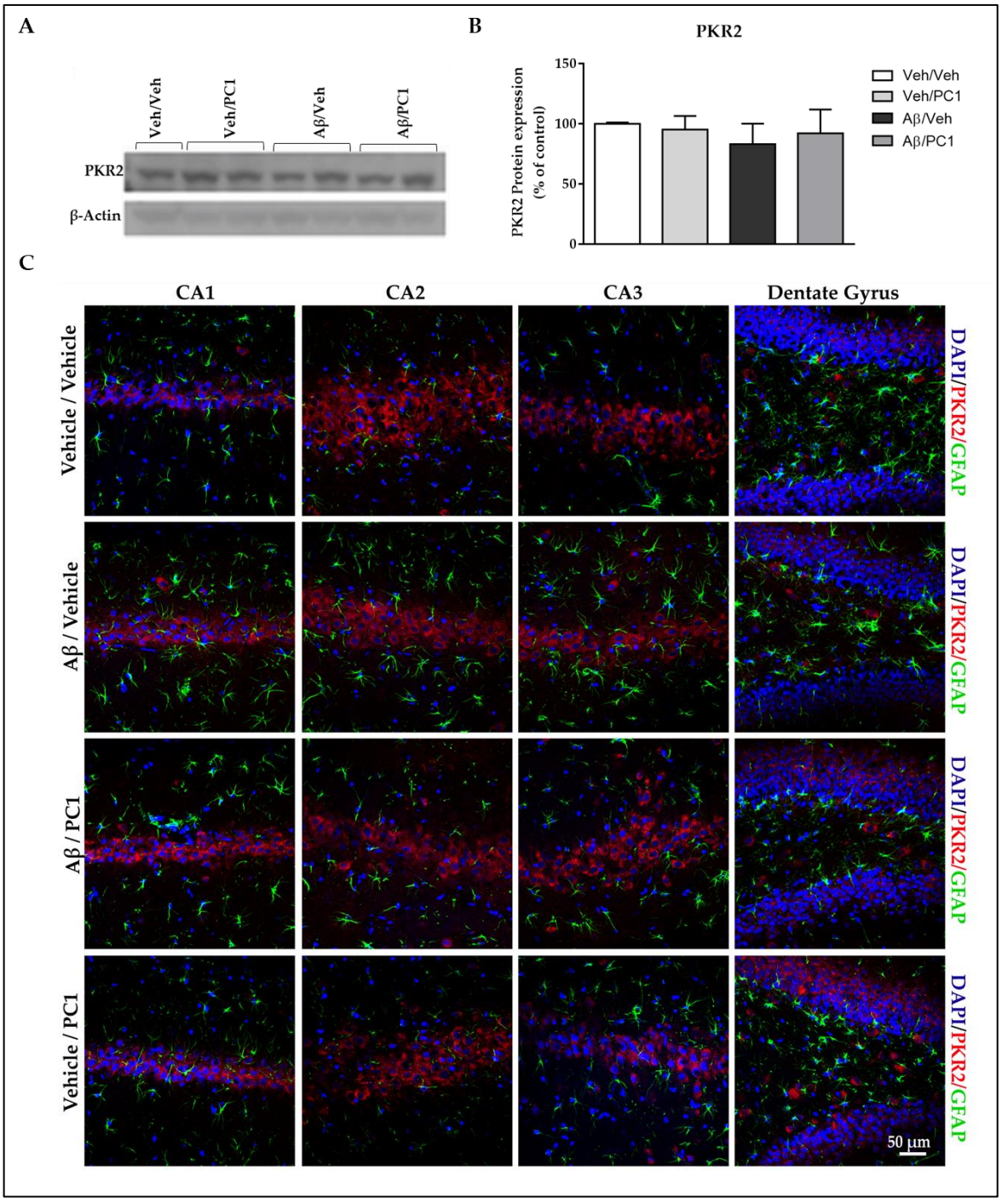


Figure 28. PKR2 protein expression in hippocampus of Vehicle/Vehicle, Aβ1-42/Vehicle, Aβ1-42/PC1 and Vehicle/PC1 rats.

(**A**) Representative WB analysis showing PKR2 protein amount in hippocampus. (**B**) Optical density (OD) of corresponding WB bands expressed as the ratio of PKR1 and β-actin signal. Data are express as mean ± SEM. (**C**) Immunofluorescence double-staining of PKR2 - red - with GFAP (astrocytic marker) – green - in CA1, CA2, CA3 and DG. Cell nuclei were counterstained with DAPI (blue fluorescence). Scale bar, 50 μm.

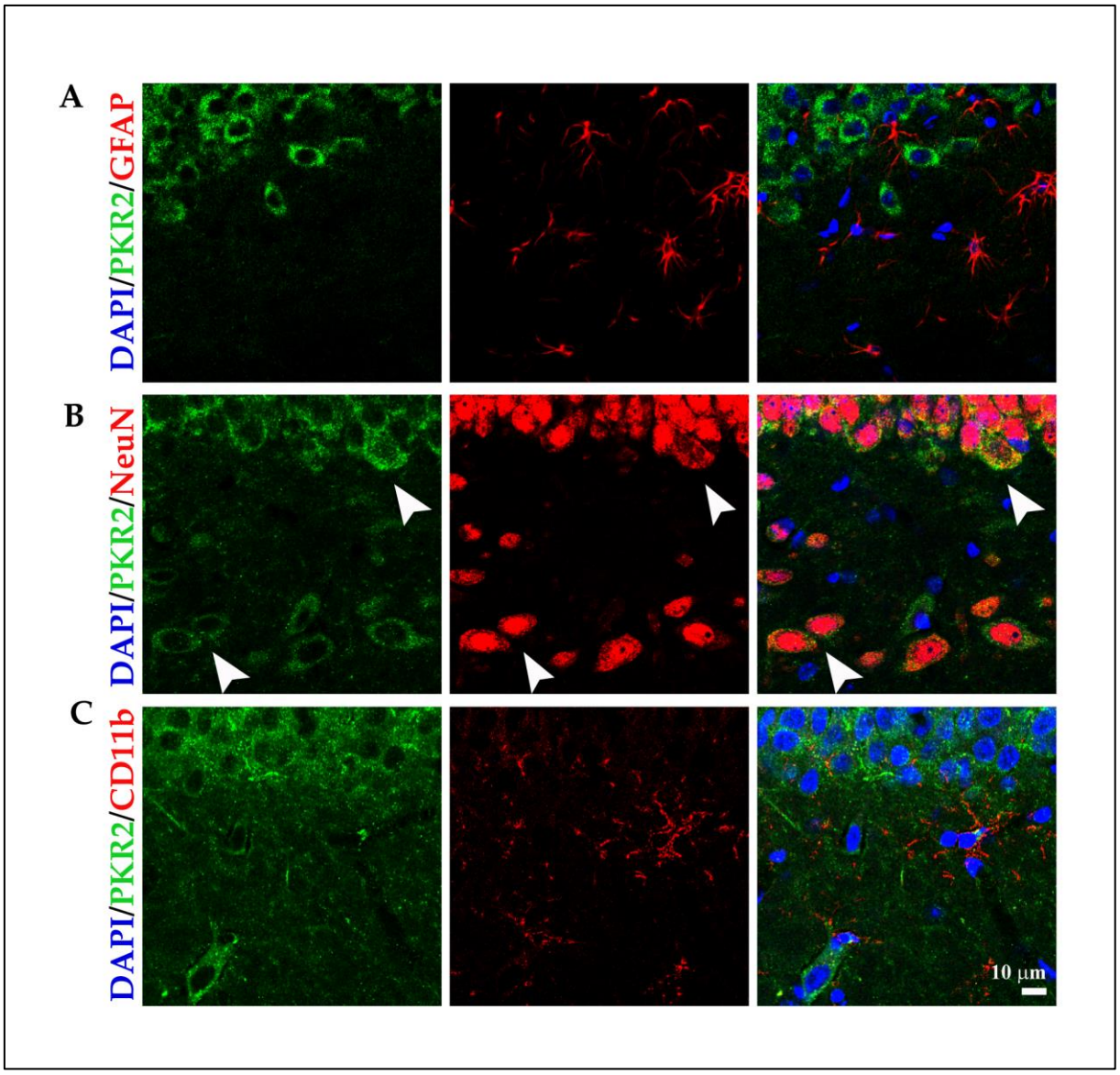


Figure 29. PKR2 cellular localization in the hippocampus. Immunofluorescence double-staining of PKR2 – green - and (**A**) GFAP (astrocytic marker), (**B**) NeuN (neuronal marker) and (**C**) CD11b (microglial marker) – red. Cell nuclei were counterstained with DAPI (blue fluorescence). Scale bar, 10 μm.

III.4. Aβ1-42 i.c.v. infusion induces neuroinflammation, glia activation and neuronal death

In hippocampus of Aβ1-42 injected rats, iNOS and phospho-NF-κB p65 (Ser536) protein levels (a marker of activation of NF-κB, a transcriptional factor activated by inflammatory stimuli) were statistically increased compared to Vehicle injected rats (Figure 30).

PC1 treatment was able to restore the physiological levels of these proteins.

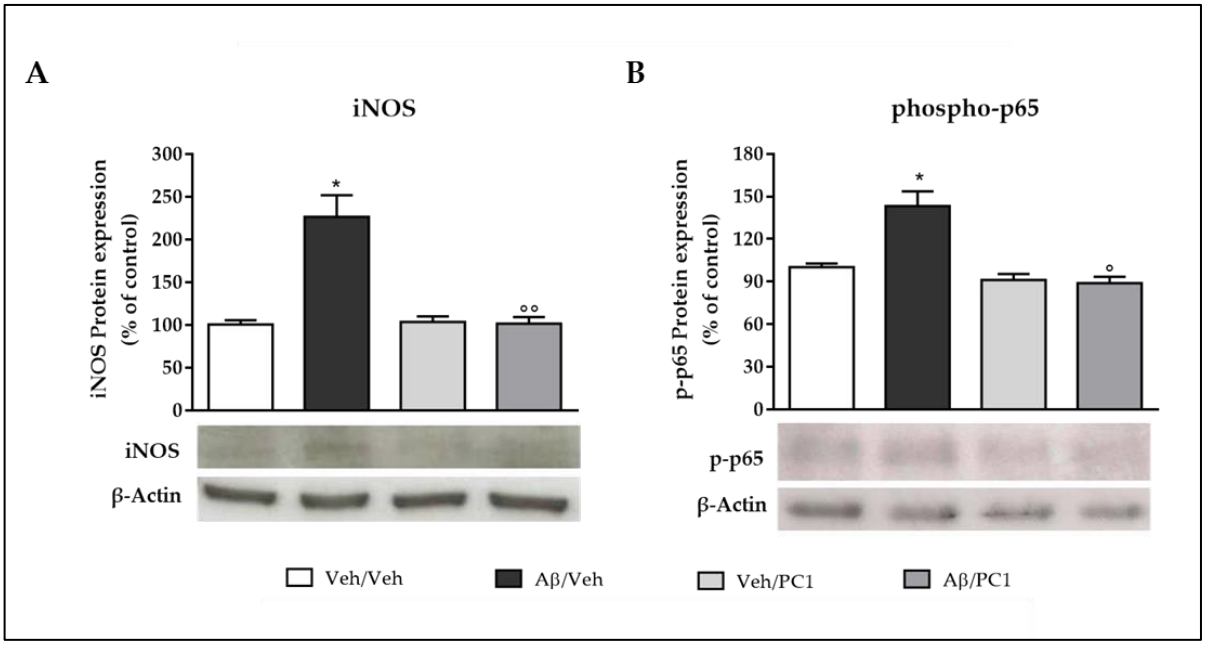


Figure 30. Neuroinflammatory markers in hippocampus. Representative WB analysis showing iNOS (**A**) and phospho-p65 (**B**) proteins amount in hippocampus. In graphs are showed the Optical Density (OD) of corresponding WB bands expressed as the ratio of iNOS or phospho-p65 and β-actin signal. Data are express as mean ± SEM.

GFAP (astrocytic marker) and Iba-1 (microglial marker) protein levels were statistically increased in hippocampus of Aβ1-42 injected rats compared to Vehicle injected rats (Figure 31); these data were according to IF assay where the immunofluorescence signal of GFAP and CD11b (astrocytic and microglia markers,

respectively) were increased. PC1 treatment was able to reduce the activation of astrocytes and microglia in $A\beta_{1-42}$ injected rats.

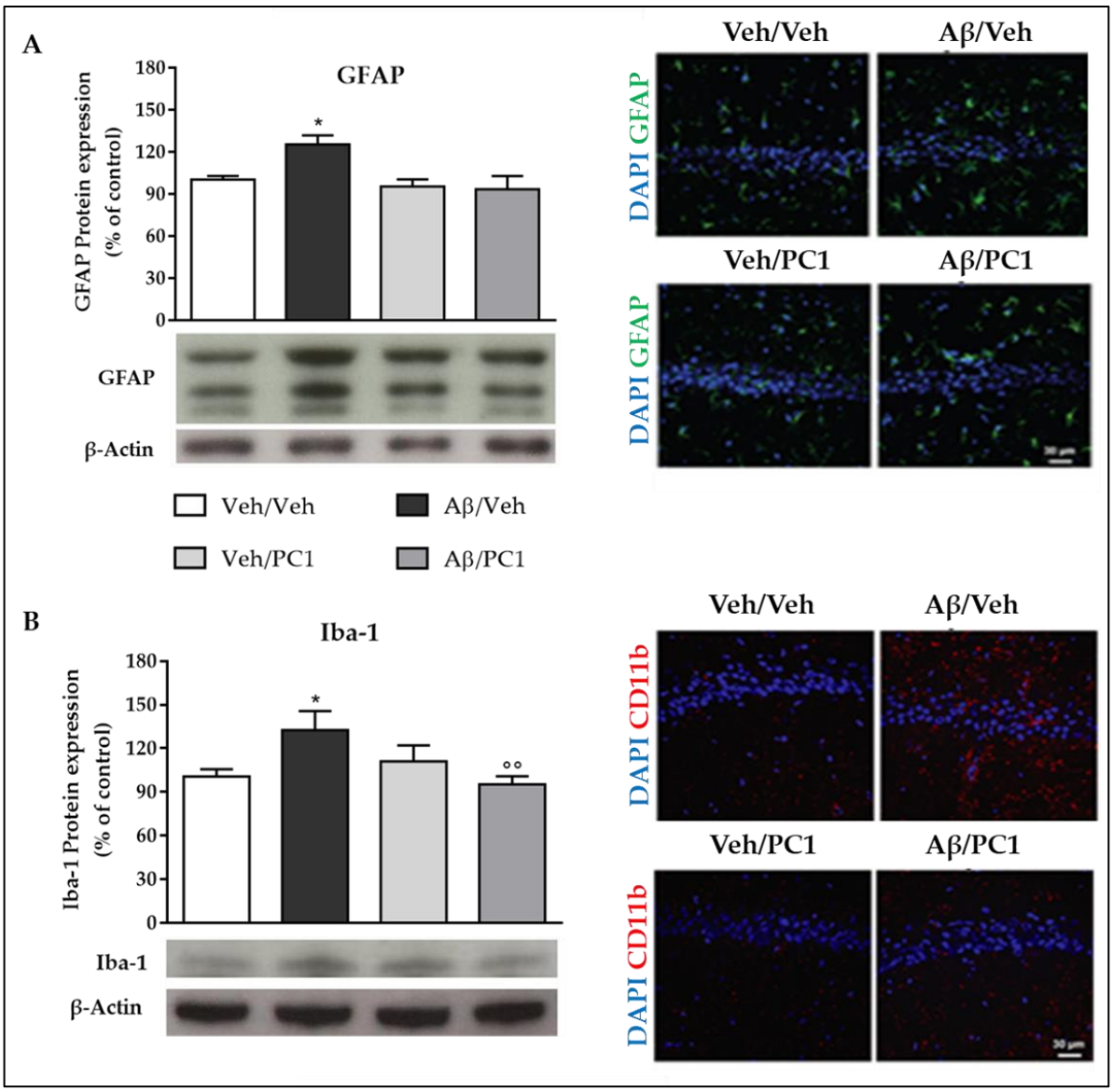


Figure 31. Activation of microglia and astrocytes in hippocampus. Representative WB analysis showing GFAP (**A, left**) and Iba-1 (**B, left**) proteins amount in hippocampus. In graphs are shown the Optical Density (OD) of corresponding WB bands expressed as the ratio of GFAP or Iba-1 and β-actin signal. Data are express as mean ± SEM. Immunofluorescence staining of GFAP (astrocytic marker) – green - (**A, right**) and CD11b (microglial marker) – red (**B, right**). Cell nuclei were counterstained with DAPI (blue fluorescence). Scale bar, 30 μm.

Aβ1-42 infusion induced neuronal death in rat hippocampus, as demonstrated by a decrease protein levels of MAP2 compared to Vehicle injected rats (Figure 32 A).

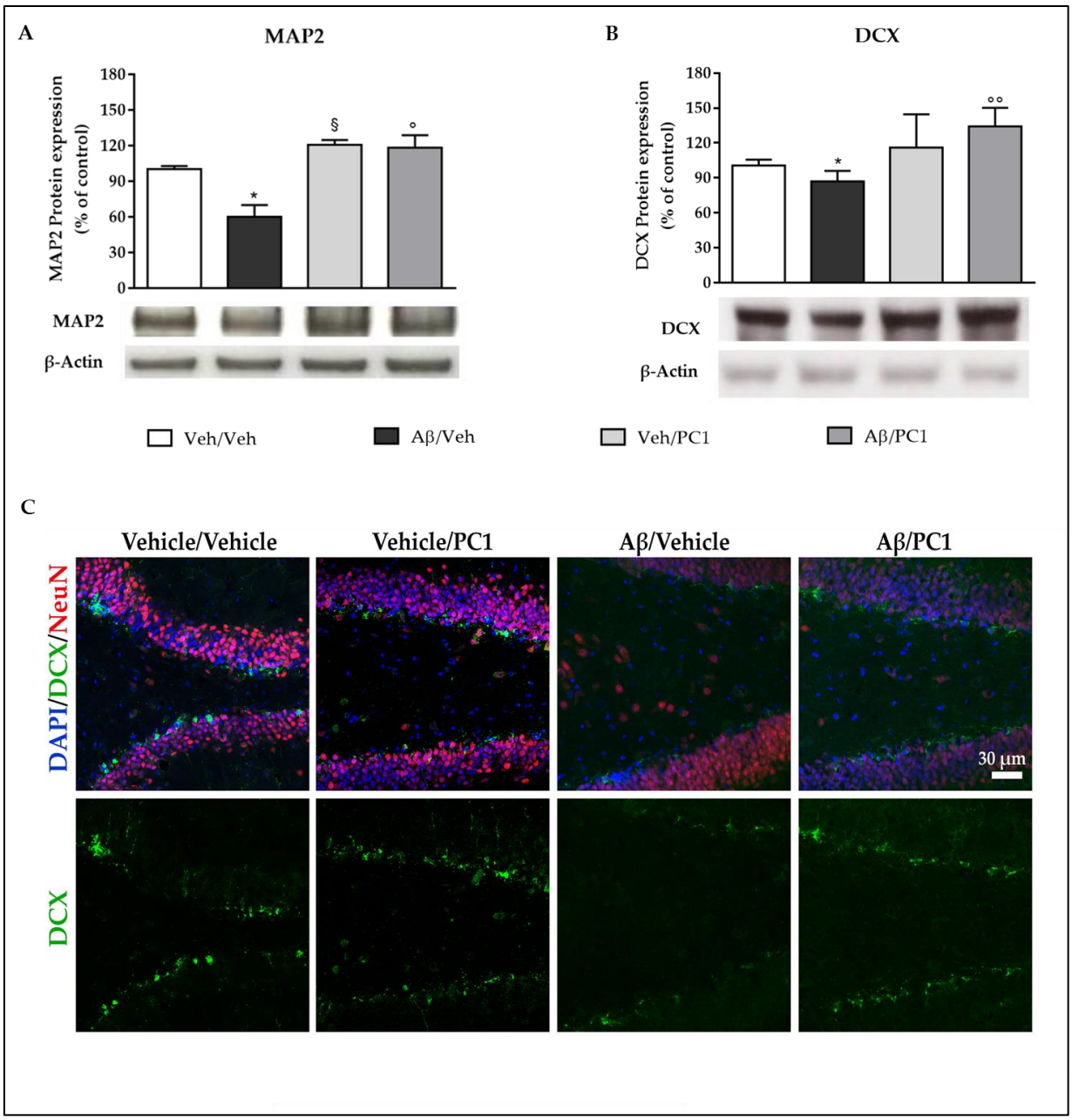


Figure 32. Neuronal markers in hippocampus. Representative WB analysis showing MAP2 (**A**) and Doublecortin (DCX) (**B**) proteins amount in hippocampus. In graphs are shown the Optical Density (OD) of corresponding WB bands expressed as the ratio of MAP2 or DCX and β-actin signal. Data are express as mean ± SEM. Immunofluorescence (**C**) double staining of DCX – green - and NeuN – red (neuronal marker). Cell nuclei were counterstained with DAPI (blue fluorescence). Scale bar, 30 µm.

PC1 treatment diminished neuronal death, as demonstrated by the increase of WB signal. WB analysis showed a significant decrease of DCX (Doublecortin, a neurogenesis marker) protein levels in the hippocampus of $A\beta_{1-42}$ injected rats compared to control group (Figure 32 B) and PC1 treatment was able to restore protein levels. These data were in accordance to IF assay that showed a decrease of DCX immunofluorescence signal in DG compare to Vehicle injected rats. PC1 treatment was able to counteract Aβ-induced DCX reduction and restored the hippocampal neurogenesis.

III.5. Discussion

Alzheimer's disease (AD) is a chronic progressive neurodegenerative disorder and is the principal cause of dementia in the elderly, representing a big issue for human health worldwide.

AD patients manifest cognitive decline and deficits in social competence and the causes are not completely understood. Compared to previous years, steps forward have been made and neuroinflammation seems to play a key role: indeed, the abnormal production of pro-inflammatory cytokines and chemokines in AD brains drive to microglia and astrocytes activation that lead to neuronal damage (*Lyman M. et al.,2014*).

Neuropathological hallmarks of AD correlate with the alteration in the production or clearance of β amyloid (Aβ) peptides and neurofibrillary tangles formation. (*Cleary J. P. et al., 2010*). Aβ has been so far one of the most important targets for the development of drugs in the AD treatment (*Hardy J. and Selkoe, D. J., 2002*). Nowadays, drugs used in therapy can only slow down the progression of cognitive and behavioural impairment (*McNaull B.B. et al., 2010*).

In a recent study, Severini and co-workers demonstrated that PK system is modulated in response to Aβ injury *in vitro*. Indeed, in primary cortical cultures (CNs), $A\beta_{1-42}$

peptide increases the mRNA and protein levels of PK2/PKRs and the PKRs antagonist, PC1, dose-dependently protects CNs against $A\beta_{1-42}$-induced neurotoxicity by reducing the PK2 up-regulation (*Severini C. et al., 2015*).

Here, we have analysed the involvement of the PK system and the possible role of the PKRs antagonist PC1 *in vivo*, in a non-transgenic animal model of Alzheimer's disease, induced by intracerebroventricular (i.c.v.) infusion of $A\beta_{1-42}$ peptide.

According to previous *in vitro* data, PK2 mRNA is modulated already at day 1 after $A\beta_{1-42}$ i.c.v. infusion in both prefrontal cortex (PFC) and hippocampus, suggesting that PK2 increase is an early response to Aβ injury. Accordingly, PK2 protein level is increased in response to $A\beta_{1-42}$ peptide both in neurons and astrocytes and its expression is reduced by PC1 administration.

The PKRs mRNA and protein expression levels are also increased by $A\beta_{1-42}$ i.c.v. administration but only in the PFC. PKR1 and PKR2 immunoreactivity signals are localized in prefrontal neurons. PC1 treatment has no effect on PKRs expression levels. In hippocampus of Aβ infused rats, PKR1 shows a tendency to increase and the treatment with PC1, the PKRs antagonist, induces an additional increase of its expression even if it is not statistically significant. Immunofluorescence (IF) assay confirms its increase, mainly in neurons of CA2 and CA1 areas.

Recently, new neuronal circuits were found in hippocampus, such as DG-CA2-CA1 circuit: new-born neurons in DG project to pyramidal neurons in CA2 area and then to the neurons in CA1, this circuit provides an information transfer in the dorsal and ventral directions of the hippocampus and seems to be involved in declarative learning (*Dudek S.M. et al., 2016*).

We suppose that PKR1 is probably involved in learning processes. The functional involvement of PKR1 in learning processes is sustained by the ability of PC1, which is a PKR1 preferring antagonist, to increase the DG neurogenesis and to improve the cognitive behavioural tasks in $A\beta_{1-42}$ infused rats (Morris Water Maze test, data not show).

As far as concerned PKR2, its mRNA is increased after Aβ infusion, but neither Western Blot (WB) or IF assays show any modulation of the protein. PKR2 is usually augmented both as mRNA and as protein in other animal models of inflammation or neuropathy (*Maftei D. et al., 2014*; *Kisliouk T. et al., 2005*) and, probably, this discrepancy between PKR2 mRNA levels and protein expression is due to absence of a linear correlation between mRNA abundance and protein, because it is a fine-regulated process by several cellular mechanisms. (*Liu Y. et al., 2016*).

The resident immune cells of the CNS, microglia and astrocytes, are considered the primary cell types involved in the innate immune response in the brain. Biochemical analysis of hippocampal tissues display activation of astrocytes and microglia, as showed by GFAP and Iba-1/CD11b increase. This process occurs both in acute injury and in chronic neurodegenerative disorders. Probably, as suggested by numerous studies, astrocytes can form a protective barrier between Aβ deposits and neurons but, in response to chronic stress (such as pro-inflammatory cytokines), they could overexpress BACE1 enzyme, becoming a source of Aβ peptides themselves (*Roßner S. et al., 2005*).

Furthermore, microglia activation can drive to the synthesis of cytokines, chemokines and neurotoxic factors as demonstrated by activation of NF-κB (here showed by phospho-p65 protein increase). These molecules amplify neuroinflammation which lead to neuronal loss (*Walter S. et al., 2007*) as proved by the decrease of MAP-2 protein levels.

The functional involvement of the PK system in Aβ-induced toxicity is further demonstrated by the ability of PC1 to exert simultaneously anti-inflammatory and neuroprotective effects. Indeed, PC1, not only decreases the up-regulation of PK2, but is able to reduce the glial activation, as demonstrated by the decrease of GFAP and Iba-1/CD11b signals. Moreover, the use of the PKRs antagonist impairs the activation of NF-κB, reducing the transcription of its target genes, such as cytokines and

chemokines. Our results further show that PC1 restores the physiological levels of MAP-2, suggesting the reduction of Aβ-induced neuronal death.

Data from literature report that, in addition to glial activation and massive release of pro-inflammatory molecules, other mechanisms are involved in neuronal damage. Among these mechanisms there are impaired hippocampal neurogenesis and imbalance between GABAergic and glutamatergic neurotransmission.

Neurogenesis occurs in dentate gyrus and persists through adulthood in rodents and humans. It regulates hippocampal plasticity and is critical for memory and learning processes (*James B. A. et al., 2014*). It is tightly regulated by neuronal activity and, in particular, by the balance between GABAergic and glutamatergic inputs (*Zhao et al., 2008*). Aβ peptide is able to affect adult neurogenesis by altering the balance between excitatory and inhibitory inputs onto neurons (*Sun B. et al., 2009*). As demonstrated by Caioli and colleagues, Bv8 (the amphibian homologue of PK2), as well as the $A\beta_{1-42}$ insult, is able to alter glutamatergic transmission, augmenting the AMPA currents in neurons and this effect is blocked by PC1 treatment (*Caioli S. et al., 2017*). According to the literature, in this study, we observe impaired neurogenesis in $A\beta_{1-42}$ infused rats and PC1 is able to restore the physiological neurogenesis. Probably, this effect is due to its ability to block the up-modulation of glutamatergic neurotransmission, as mentioned above.

Taken together, these results indicate that PK system plays a role in Aβ-induced toxicity *in vivo*, representing an innovative approach for the study of AD etiopathology. Moreover, the pharmacological block of PKRs seems to be a good strategy for a potential AD treatment.

5. CONCLUSIONS

Nowadays, it is accepted that the Prokineticin system has a great relevance in a variety of physiological and pathological states.

The data that I presented in this work, demonstrate a clear involvement of the PK system in inflammatory/neuroinflammatory processes employed in colitis and in neurodegenerative pathology of Alzheimer's disease.

Blocking the Prokineticin system may be a good potential strategy for the pharmacological treatment of these diseases.

6. MATERIALS AND METHODS

6.1. Animals

All experiments were carried out in:

Rats:

- Female Wistar rats (Charles River, Calco, Italy) weighing 280–320 g were mated and their offspring were used for experimental procedures.

-Male Sprague-Dawley rats (Charles River, Calco, Italy) weighing 280-320g at the time of surgical procedure. Animals were housed two for cage.

Mice:

-Male C57BL/6J wild type (WT), PKR1- or PKR2-null mice, generated by Lexicon Genetics (The Woodlands, TX, USA) weighing 25-30 g. Animals were kept in a temperature and humidity-controlled room (22 ± 2°C, 50–60%), with access to water and food *ad libitum*.

Each protocol was approved by the Animal Care and Use Committee of the Italian Ministry of Health according to European Commission directives and all efforts were made to minimize the number of animals and their suffering.

6.2. Procedures

6.2.1. CORT-nursed model and colitis induction (TNBS intracolonic infusion)

This procedure was performed by Dr. Casolini's research group.

Female Wistar rats, were mated and then housed individually. After the birth, mothers and their offspring had *ad libitum* access to a solution of 0.2 mg/ml hemisuccinate corticosterone (CORT-nursed rats) or tap water (control rats) until weaning (day 21 after birth) and then housed three per cage.

Three-month-old male CORT-nursed rats and their controls were used for further experiments. At the beginning of the experiment, male rats were housed one per cage until their sacrifice.

Colitis was induced by instillation of TNBS (2,4,6-trinitrobenzenesulphonic acid) (Sigma Aldrich, St Louis, MO, USA) at the dose of 30 mg/kg in 0.3 ml of 50% ethanol (vol/vol). Animals were anesthetised by intraperitoneal (i.p.) injection of xylazine (0.6 mg/kg) and ketamine (120 mg/kg) and were randomized into two groups: 1) healthy rats (both control and CORT-nursed rats) infused with 0.3 ml of vehicle and 2) colitic rats (both control and CORT-nursed rats) infused with TNBS. The infusion was performed through a silicone catheter (with an external diameter of 2 mm) introduced into the distal colon, 6 cm proximal to the anus and then, rats were held in a head-down position for 2–3 min, to distribute the agents within the entire colon. (*Morris G.P. et al., 1989*). On the fourth day after TNBS or vehicle instillation, all animals were euthanized by CO_2 inhalation and, for each experimental group, colonic tissue was collected and stored at -80° C until use. In this study, the total number of rats analysed was 17 divided as follows: control healthy (n = 4), control colitic (n = 5), CORT-nursed healthy (n = 4) and CORT-nursed colitic (n = 4).

6.2.2. Non-transgenic animal model of Alzheimer's disease: $A\beta_{1-42}$ i.c.v. infusion

$A\beta_{1-42}$ peptide was purchased from Abcam (Abcam, Cambridge, UK) and stock solutions (1 mg/ml) were prepared in Saline (0.9% NaCl) and stored to − 20 °C. Aliquots of Aβ peptides were allowed aggregating by incubation at 37 °C for 72 h before in vivo infusion. Rats were anesthetized with a solution of ketamine-xylazine (60 + 10 mg/kg, intraperitoneal, i.p.). The skull was positioned in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA), an incision was made along the midline, the scalp was retracted and the area surrounding bregma was cleaned and dried. Infusions into the left lateral ventricle (coordinates; AP=20.80 mm, ML= +2.0 mm, DV=24.5 mm

with respect to bregma) were performed by using 30-gauge injection needles connected to 50-μL Hamilton microsyringes by polyethylene (PE-20) tubing. 5 μL of $A\beta_{1-42}$ (1 mg/ml) or its vehicle (saline) were infused at the rate of 1 μL/min by an automated syringe pump (KD Scientific, Holliston, MA, USA). The injection needles were retained within the target area for 60 seconds following infusion to maximize diffusion.

6.3. Drugs injection

PK2β and PK2 (synthesized by Dr. Rossella Miele) were injected topically into the plantar region of mice hindpaws (ipl), in a volume of 20 μl. PK2β and PK2 were diluted in sterile saline.

PC1 was injected subcutaneously (s.c.) into the flank region in a volume of 200 μL/ 100g body weight. PC1 was dissolved in sterile saline + 0.007% DMSO (dimethyl sulfoxide) and administrated at the dose of 150 μg/kg s.c. twice a day, for two weeks starting from the day of $A\beta_{1-42}$ i.c.v. infusion.

6.4. Behavioural tests

6.4.1. Measurement of thermal nociception

Thermal hyperalgesia was evaluated using Hot Plate test (Ugo Basile, Verona, Italy). Mice were placed on a surface heated to 48°C surrounded by a plexiglass cylinder. The latency to various behavioural responses, including jumping, kicking, shaking of foot, holding the feet tightly against the body and licking the forepaw, the hind paw or both has been used as a measure of pain sensitivity (*Hunskaar S. et al., 1986*). On day of the experiment, the nociceptive threshold to thermal stimuli was measured before and at established time points after drug administration. A cut-off time of 15 s was assigned prior to the experiments to avoid damage to the animals.

The effect of drug was calculated as the percentage change in nociceptive threshold from the baseline threshold (% ΔNT) according to the following equation: (% ΔNT) = 100 x (NTTS – NTB)/ NTB where NTTS is the nociceptive threshold at that time in the presence of the test solution and NTB is the baseline nociceptive threshold.

6.4.2. Measurement of tactile allodynia

Behavioural experiments were carried out in a reserved quiet temperature-controlled room. The tactile allodynia was assessed by calibrated Von Frey filaments (2 Biological Instruments, Italy). Animals were placed in individual Plexiglas boxes on a raised metal mesh surface and allowed to acclimatize for 30-60 min before the test. The filaments are manually held, and they can be adjusted according to the paw posture. Testing was initiated with a medium-sized filament, which was applied for 3 to 6 s until the filament bent slightly. If the animal withdrew the paw, the response was considered positive and a filament one size smaller was tried. Conversely, if no response was observed, a filament one size larger was tried. The protocol was repeated until five changes in behaviour had been observed. The 50% withdrawal threshold was determined according to the following equation: Xf+kD where Xf is the value of the last von Frey filament used, k is the Dixon value for the positive/negative pattern, and D is the logarithmic difference between stimuli (*Dixon W. J., 1980*).

6.5. Biochemical assay

6.5.1. RNA extraction and qPCR

Total RNA was extracted from each sample using the TRIzol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA yield and purity were determined by spectrophotometric absorption at 260 and 280 nm. 1 μg of mRNA was used to perform reverse transcription (Reverse Transcriptase, Promega) to obtain cDNA. 25 ng of cDNA was amplified by real-time PCR (iCycler; Bio-Rad) by

SensiMix™ SYBR (Bioline, Rome, IT) using 0.3 μM of each primer, in a total volume of 25 μl. All the measures were performed in triplicate. Cycling conditions were: 95°C for 10 min (for polymerase activation), followed by 40 cycles at 95°C for 15 min, 55±5°C (temperature depends on the Tm of the primers) for 15 s and 72°C for 15 s. The reaction mixture without cDNA was used as negative control. The Ct (threshold cycle) value of the specific gene of interest was normalised to the Ct value of the endogenous control, (β-actin or Glyceraldehyde 3-phosphate dehydrogenase, GAPDH), and the comparative Ct method (2-ΔΔCt) was then applied using control healthy rats as the reference samples.

The sequences for primer used are:

β-actin = Forward: 5'-AGATGACCCAGATCATGTTTG-3' Reverse: 5'-TAGATGGGCACAGTGTGG-3'

GAPDH = Forward: 5'- TGGCCACCAGTAACATGCAA -3' Reverse: 5'-CTCGATGTCCAGGGCTAGCT -3'

PK2 = Forward: 5'-TCATCACCGGGGCTTGCG-3' Reverse: 5'-TAACTTTCCGAGTCAGGG-3'

PK2L = Forward: 5'-AGGAAAGAAGAAGGGCGAAG-3' Reverse: 5'-TCCTTAAACATGCCAAACCTG-3'

PKR1 = Forward: 5'-CGCACCGTCTCCCTCTAC -3' Reverse: 5'-GTTTGACACTTCATCCGCG-3'

PKR2 = Forward: 5'-CTCCGTCAACTACCTTCGTA -3' Reverse: 5'-GAGGCGGTCTGGTAATTCA-3'

6.5.2. Western Blot assay

Tissues (whole colon, hippocampus and organotypic cultured of DRG) were homogenized in ice-cold lysis buffer (Tris HCl pH 7.5 50 mM, NaCl 150 mM, ethylenediaminetetraacetic acid (EDTA) 1 mM and 1% Triton X) or RIPA buffer (Sigma Aldrich, St. Louis, MO, USA) containing protease cocktail inhibitors (1% v/v) (Sigma Aldrich St. Louis, MO, USA) and centrifuged at 12000 r.p.m. for 10 minutes at 4°C. Protein concentrations were determined using the Bradford protein assay. Thirty or forty micrograms of protein were resuspended in Laemmli sample buffer (Sigma Aldrich St. Louis, MO, USA), boiled for five minutes and separated on 8%, 10 % or 15% SDS-polyacrylamide gels.

After electrophoresis (Bio-Rad, Mini Protean Tetra-Cell, Hercules, CA, USA), the proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) which were blocked for 1 hour at room temperature with: 1% (w/v) non-fat milk and 1% (w/v) bovine serum albumin (BSA) in Tris-buffered saline-Tween 0,1% (TBS-T) for PK2, PKR1, PKR2, STAT3, pSTAT3 and β-actin (loading control) or 5% w/v no-fat dry milk powder in TBS-T for GFAP, COX-2 and iNOS or a solution containing 5% (w/v) BSA in TBS-T for S100β, Iba-1, p65 and MAP2.

After blocking, these were incubated overnight at 4°C with one of the primary antibodies (Table 3).

The next day, membranes were broadly washed in TBS-T 0.5%, and then incubated with the proper secondary horseradish peroxidase-conjugated antibodies for 1 hour at room temperature (for more details refer to the table).

Immunocomplex were visualized with an enhanced chemiluminescence (ECL) system (Amersham, Bucks, UK) and digitized images of immunoreactive bands were acquired and the area of each immunoreactive band was measured using the NIH ImageJ medical imaging software.

A ratio of target to loading control were then determined and these values were compared for statistical analyses.

Primary Antibodies	Company	Host	Blocking Solution	Concentration	Secondary Antibodies
PK2	Abcam	Rabbit	1%Milk 1%BSA	1:500 in Milk 1% BSA 1%	1:2000 in 1%Milk 1%BSA
PKR1	Santa Cruz Biotechnology	Goat	1%Milk 1%BSA	1:500 in Milk 1% BSA 1%	1:8000 in 1%Milk 1%BSA
PKR2	Santa Cruz Biotechnology	Goat	1%Milk 1%BSA	1:500 in Milk 1% BSA 1%	1:8000 in 1%Milk 1%BSA
β-Actin	Sigma Aldrich	Mouse	1%Milk 1%BSA	1:5000 in Milk 1% BSA 1%	1:10000 in 1%Milk 1%BSA
GFAP	Abcam	Rabbit	Milk 5%	1: 50'000 in Milk 5%	1: 10000 in Milk 5%
S100β	Epitomics	Rabbit	BSA 5%	1: 1000 in BSA 5%	1:10000 in BSA 5%
Iba-1	Abcam	Goat	BSA 1%	1:1000 BSA 1%	1:10000 in BSA 1%
COX-2	Cell Signaling Technology	Rabbit	Milk 5%	1: 10'000 in Milk 5%	1:10000 in Milk 5%
iNOS	Sigma Aldrich	Rabbit	Milk 5%	1: 9000 in BSA 1%	1:10000 BSA 1%
p65	Cell Signaling Technology	Rabbit	BSA 5%	1:2000 in BSA 5%	1:10000 in Milk 5%
MAP-2	Novus Biologicals	Mouse	BSA 5%	1:250 in BSA 5%	1:10000 in BSA 5%
STAT-3	Cell Signaling Technology	Mouse	1%Milk 1%BSA	1:1000 in Milk 1% BSA 1%	1:2000 in 1%Milk 1%BSA
pSTAT3	Cell Signaling Technology	Mouse	1%Milk 1%BSA	1:1000 in Milk 1% BSA 1%	1:2000 in 1%Milk 1%BSA

Table 3. Primary and secondary antibodies and relative WB blocking conditions and buffers.

6.5.3. Immunofluorescence assay

Rats were anaesthetized with mixture of ketamine-xylazine (60 + 10 mg/kg, i.p.) and intracardially perfused with a phosphate buffer saline (PBS) solution followed by ice-cold 4% paraformaldehyde solution in PBS. Brains, were post-fixed for 24 hours, transferred in 30% sucrose/PBS solution at 4°C until they sank and coronary sectioned using a cryostat (Leica), in 40 μm thick slices. Serial free-floating brain sections were incubated at 4°C for 48 hours with the following primary antibodies diluted in PBS-0.3% Triton X-100: 1/200 rabbit polyclonal anti-PK2 (Abcam, Cambridge, UK), 1/200 rabbit polyclonal anti-PKR1 and PKR2 (Alomone Labs, Jerusalem, Israel), 1/500 mouse monoclonal anti-neuronal nuclei (NeuN), 1/400 mouse polyclonal antiglial fibrillary acidic protein (GFAP) (Immunological Sciences, Rome, Italy), 1/200 MAP2 (Novus Biologicals, Littleton, CO, USA), 1/200 doublecortin (DCX, Cell Signaling Technology, Danvers, USA). After three washes, sections were then incubated for 2 hours at room temperature in 1:200 anti-species IgG antibodies coupled to Alexa Fluor®-488 or 555 (Immunological Sciences, Rome, Italy). Nuclei were stained with DAPI 1/500 (Sigma Aldrich). After that, sections were mounted on slides, air dried and coverslipped using Fluoromount (Immunological Sciences, Rome, Italy). The stained sections were examined by confocal laser scanning microscope (Leica SP5, Leica Microsystems, Wetzlar, Germany). Confocal acquisition settings were identical among the different experimental cases. For Figure production, brightness and contrast of images were adjusted by applying the same values, and by taking care to leave a tissue fluorescence background for visual appreciation of the all fluorescence intensity features and to help comparison between the different experimental groups. Final Figures were assembled by using Adobe Photoshop 7 and Adobe Illustrator 10. Boundaries and subdivisions of cortical and hippocampal structures were identified on the base of the DAPI histofluorescence using a rat brain atlas (Paxinos). Image acquisitions were performed on prefrontal cortex and CA1, CA2, CA3 and Dentate Gyrus (DG) hippocampal region.

6.6. Statistical Analysis

Results are expressed as mean ± SEM. When appropriate, one-way ANOVA with Tukey's test for multiple comparisons or two-way repeated measures ANOVA with Bonferroni's post-tests, was performed using GraphPad Prism 5 for Windows version 5.4. Results are expressed as mean ± SEM. Statistical significance was set at $p<0.05$. All analysis were performed using GraphPad Prism 5 for Windows version 5.4

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