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A todos,

Muito Obrigado

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Abstract

Excitotoxicity is thought to be one of the main mechanism behind neuronal death under several pathological conditions, like ischemia, epilepsy, trauma and neurodegenerative diseases. It is characterized by the release of high levels of excitatory amino acids, namely glutamate, in the synapse, causing excessive activation of glutamatergic receptors. Among those, NMDA receptors have a prominent role in mediating excitotoxicity. ATP is an important signaling molecule in the central nervous system, and P2 receptors (ATP receptors) are highly expressed both in neurons and in glia. P2 receptors can be divided into ionotropic P2X receptors and metabotropic P2Y receptors. During pathological events in the brain, ATP reaches high extracellular levels, and this molecule is thought to have an important role in mediating the outcome for neurons during this events. ATP may also modulate the activation of astrocytes and microglia, which may be related to the fate of neurons. The hippocampus is a brain structure, highly sensitive to pathological events, composed of three main glutamatergic regions, *Dentate Gyrus*, *Cornus Ammonis* 1 (CA1) and *Cornus Ammonis* 3 (CA3). The aims of this work was to evaluate the P2 antagonist PPADS (20μM) as a neuroprotector against neurotoxicity, in an *ex vivo* model of acutely dissociated hippocampal slices (400μm thickness), exposed to high levels of NMDA (100μM). Neuroprotection was assessed by trypan blue technique. NMDA caused a raise in the number of cells stained with trypan blue, both in CA1 and CA3 slices of the hippocampus. However, CA1 region showed higher vulnerability to NMDA. PPADS showed a slightly in decrease in the number of trypan blue stained cells in CA1 region, but not in the CA3 region. In slices treated with NMDA, astrogliosis occurred in the CA1 but not in the CA3 region, which can be related to the higher vulnerability of this area to NMDA. GFAP and CD11b immunoreactivity was tested to assess astrogliosis and microgliosis, respectively. Astrogliosis is characterized by cell proliferation, hypertrophy, and by an augmented immunoreactivity for GFAP. PPADS treated slices had no effect on astrocytes. On the other hand, microglia remained inactive even in the presence of NMDA, as they maintained their elongated and ramified morphology. In conclusion, PPADS may be neuroprotective against excitotoxic stimulus, and this effect is mainly due to direct neuronal modulation, since gliosis is not altered in the presence of PPADS.

Key Words: Excitotoxicity, P2 receptors, Neuroprotection, Reactive gliosis

Resumo

A excitotoxicidade é um dos principais mecanismos causadores de morte neuronal, associada a várias patologias cerebrais como isquemia, epilepsia, trauma e doenças neurodegenerativas. É caracterizada pela libertação de níveis elevados de aminoácidos excitatórios na sinapse nomeadamente, glutamato, levando a uma activação excessiva de receptores glutamatérgicos. Entre estes, os receptores de NMDA tem um papel proeminente na condução da excitotoxicidade. O ATP tem um papel importante no sistema nervoso central como molécula sinalizadora, e a expressão dos receptores P2 (receptores de ATP) nos neurónios e na glia é muito elevada. Os receptores P2 podem ser divididos em receptores P2X (ionotrópicos) e P2Y (metabotrópicos). O ATP pode chegar a níveis extracelulares elevados no seguimento de eventos patológicos, e esta molécula poderá ter um papel importante na morte ou sobrevivência das células neuronais perante aqueles eventos. A activação de astrócitos e da microglia pode ser regulada por ATP, podendo isto estar associado ao destino dos neurónios. O hipocampo é uma estrutura cerebral sensível a eventos patológicos, e pode ser dividida em três regiões, o *Giro Denteado*, *Cornus Ammonius* 1 (CA1) e *Cornus Ammonius* 3 (CA3). O objectivo do trabalho foi avaliar o papel neuroprotector do antagonista dos receptores P2, PPADS (20μM), a um evento excitotóxico, num modelo *ex vivo* de fatias de hipocampo (400μm de espessura) expostas a níveis elevados de NMDA (100μM). A técnica azul de tripan foi adoptada para avaliar a neuroprotecção. NMDA causou o aumento do número de células coradas com azul de tripan em ambas as regiões CA1 e CA3 das fatias de hipocampo. No entanto, a região CA1 demonstrou maior vulnerabilidade ao NMDA. O PPADS causou uma diminuição moderada no número de células coradas com azul de tripan na região CA1, mas não na região CA3. Para testar astrogliose e microgliose, foram usados os anticorpos contra GFAP e CD11b, respectivamente. Na região CA1 das fatias de hipocampo expostas a NMDA, astrogliose pôde ser observada, no entanto tal não se verificou na região CA3. Isto poderá estar relacionado com a maior vulnerabilidade ao NMDA observada na região CA1. A astrogliose foi caracterizada por uma proliferação e hipertrofia celular, e também por um aumento na immunoreactividade para o GFAP. Em fatias tratadas com o PPADS não se observou nenhuma alteração na população astrocitária nas regiões estudadas. A microglia, por sua vez, permaneceu inactiva mesmo na presença de NMDA, verificável pela sua morfologia ramificada e elongada. Em conclusão, o PPADS poderá ser neuroprotector contra o estímulo excitotóxico, e isto deverá ser

devido a um efeito directo na modulação neuronal, tendo em conta que o PPADS não altera o estado de activação das células gliais.

Palavras-chave: Excitotoxicidade, Receptores P2, Neuroprotecção, Gliose reactiva

Abbreviations

aCSF- Artificial cerebrospinal fluid

Ab- Antibody

ADP- Adenosine-5'-diphosphate

Ag- Antigen

AMPA- 2-amino-3-(3-hydroxy-5-methylisoxa-zol-4-yl) propionate

ATP- Adenosine-5'-triphosphate

CA- *Cornu ammonis*

CNS- Central nervous system

DAPI - 4',6-diamidino-2-phenylindole

DG- *Dentate gyrus*

E-NTPDases- ectonucleoside triphosphate diphosphohydrolases

E-NPPs- ectonucleotide pyrophosphatase/phosphodiesterases

ERK- Extracellular signal-regulate kinase

GABA- γ-aminobutyric acid

GFAP- Glial fibrillary acidic protein

JNK- c-Jun N-terminal kinase

MAPK- Mitogen-activated protein kinase

NHS- Normal horse serum

NMDA- N-methyl-D-aspartatic acid

PBS- Phosphate buffer solution

PI- Propidium iodide

PPADS- Pyridoxal-phosphate-6-azophenyl-2'-4'disulfonate

TB- Trypan blue

UDP- Uridine diphosphate

UTP- Uridine triphosphate

Introduction

Excitotoxicity is one of the main processes mediating neuronal death in different pathological conditions (ischemia, epilepsy, mechanical trauma and neurodegenerative diseases), and ionotropic N-Methyl-D-aspartatic acid (NMDA) receptors play a main role in this process (Choi 1988). In pathological conditions glutamate can be released alongside with adenosine triphosphate (ATP) to the extracellular medium and both can be mediating cell death (Melani et al 2005).

ATP is widely known for its role in the cell energetic metabolism, although ATP is also important in a variety of other biological functions. ATP can be released from almost all types of cells by different mechanisms, and can act as a signaling molecule. In the brain it can act as a fast neurotransmitter, but mainly as a neuromodulator (Cunha & Ribeiro 2000). Various purinergic-receptor subtypes have been shown to be widely distributed throughout the central nervous system (CNS), being present in neurons and glia (Burnstock 2007). In neurons ATP has potent long-term (trophic) roles in cell proliferation, growth and development and in disease and cytotoxicity, (Franke & Illes 2006; Zimmermann 2006b). In astrocytes, ATP leads initially to an increase of intracellular Ca^{2+} and subsequently to long-term changes including proliferation and apoptosis (Ciccarelli et al 2001). The functions of oligodendrocytes (Agresti et al 2005) and microglial cells (Inoue 2006) are also controlled by nucleotides.

Excitotoxicity

Excitotoxicity consists in neuronal cell death promoted by excessive levels of excitatory neurotransmitters, mainly glutamate, in the synapse, leading to a excessive Ca^{2+} levels in the cytoplasm due to deregulation of the mechanisms involved in calcium homeostasis. Excitatory amino acids mediated cell death is thought to be involved in acute brain damage under a variety of pathophysiological conditions like epilepsy, mechanical trauma, ischemia and neurodegenerative disease (figure 1) (Choi 1988). To date, virtually every glutamate receptor subtype has been implicated in mediating neurotoxicity, largely by a calcium dependent process (Arundine & Tymianski 2003). Of these, however, the ionotropic glutamate receptors remain recognized as playing key roles (Arundine & Tymianski 2003). The glutamate receptors are NMDA receptor, 2-amino-3-(3-hydroxy-5-methylisoxa-zol-4-yl) proprionate (AMPA) receptor, and kainate

receptor subtypes. AMPA receptors are widely distributed in the CNS and are expressed on many different types of neurons. They control a cation channel that is permeable to Na^+ and K^+ ions and slightly permeable to Ca^{2+} ions (Ascher & Nowak 1988). Activation of AMPA receptors results in the fast onset of an excitatory postsynaptic current with rapid desensitization (Mosbacher et al 1994). This current shapes the fast component of glutamatergic excitatory postsynaptic currents in CNS neurons (Hestrin et al 1990). NMDA receptor differs fundamentally from AMPA receptors in several ways. In contrast to AMPA receptors, NMDA receptors are significantly permeable to Ca^{2+} ions (MacDermott et al 1986), exhibit a high single channel conductance and desensitize much slower (Ascher & Nowak 2009). And the opening of the NMDA ligand-gated cation channel does not only depend on binding of agonist but is voltage-dependent, since the channel is blocked by Mg^{2+} ions at resting membrane potentials and a depolarization of the plasma membrane is required to relieve the Mg^{2+}-dependent block (Ascher & Nowak 2009).

NMDA receptors exist as either hetero-tetramer or -pentamer structures (Laube et al 1998). Five NMDA receptor subunits have been identified by molecular cloning studies, NR1 and NR2A–D (Hollmann & Heinemann 1994). Calcium-mediated neurotoxicity requires distinct signaling pathways to be triggered in cells, and such pathways are more efficiently triggered when Ca^{2+} ions enter neurons at specific entry points, particularly at Ca^{2+}-permeable glutamate receptors (Sattler et al 1998). This proposes the hypothesis that rate-limiting enzymes or substrates responsible for excitotoxicity must be co-localized with NMDA receptors. The role of PSD-95 (protein associated with receptors clustering in the pos-synaptic density) in mediating the excitotoxic response has been hypothesized (Sattler et al 1999).

Many enzymes are activated by transient or sustained Ca^{2+} elevation, so various effectors mechanisms may be implicated in Ca^{2+} mediated excitotoxic cell death. A variety of Ca^{2+} dependent hydrolytic enzymes, including lipases and proteases, have been suggested to be involved in excitotoxic neuronal damage (Arundine & Tymianski 2003). Activation of the Ca^{2+} dependent phospholipase A2 has been observed following NMDA receptor activation and the subsequent catabolism of released arachidonic acid by lipoxygenases and cyclooxygenases is associated with concomitant production of reactive oxygen species (Lazarewicz et al 1988). Among several Ca^{2+}-activated proteases, the activity of the Ca^{2+}-dependent cysteine protease calpain is increased following glutamate receptor-mediated Ca^{2+} loading (Lankiewicz et al 2000). Calpain

activation results in proteolysis of structural proteins and degradation of the neuronal cytoskeleton (Siman & Noszek 1988). Ca^{2+}-mediated activation of nitric oxide synthase may be another pathway involved in excitotoxic cell death since neurons constitutively express the isoenzyme called "neuronal nitric oxide synthase" (nNOS). nNOS is activated by glutamate receptor-mediated cytoplasm Ca^{2+} increases (Gunasekar et al 1995) and nNOS activation is linked to excitotoxic damage since inhibition of nitric oxide formation results in protection of neurons from glutamate receptor-mediated cell death (Gunasekar et al 1995). Besides the deleterious effect of Ca^{2+}, Na^{+} ion influx mediated by activation of NMDA type and non NMDA type glutamate receptors, followed by secondary influx of Cl^{-} and H_2O, leads to the swelling of neurons, an acute form of cell damage (Inglefield & Schwartz-Bloom 1998).

It was demonstrated a correlation between ATP receptors and glutamate evoked cell death (Volonte et al 1999). Both are co-released during pathological conditions in the brain. And the P2 antagonists pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS), Basilen Blue and 2,2' pyridylisatogen were effective in preventing glutamate evoked cell death in cultured rat cerebellar, cortical and hippocampal neurons (Lin et al 2005; Volonte et al 1999).

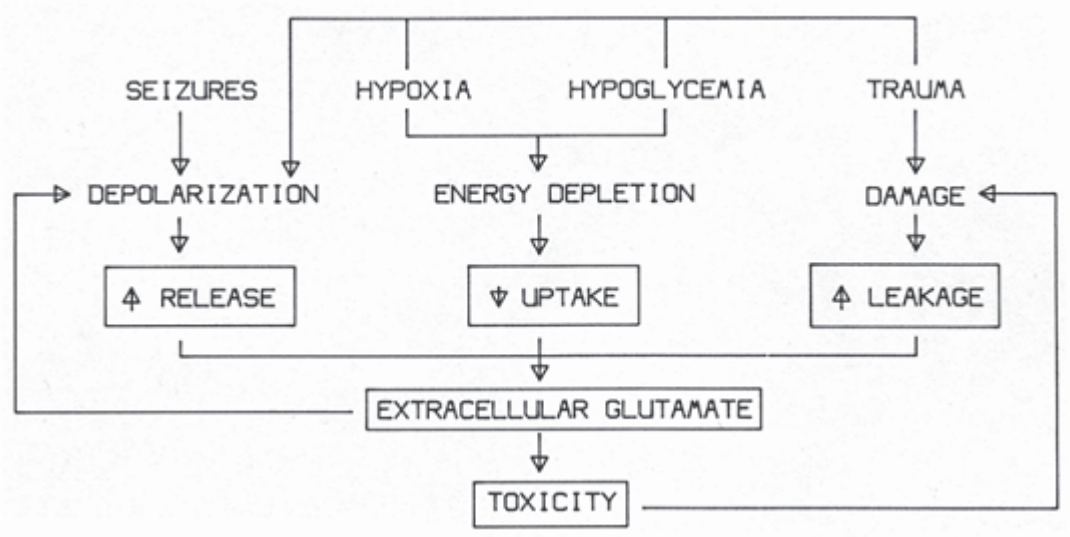

Figure 1 – The excitatory activity of the transmitter glutamate is linked to the toxic effects of seizures, hypoxia, hypoglycemia and trauma (Choi 1988).

P2 receptors

ATP acts through P2 receptors, metabotropic P2Y and ionotropic P2X. Seven distinct P2X subtypes have been cloned from mammalian species (Burnstock 2004). P2X receptors are classical cationic ligand-operated channels that open a pore permeable to Na^+, K^+ and Ca^{2+} upon ATP binding and are responsible for fast excitatory neurotransmission (Ralevic & Burnstock 1998). The P2X receptors are trimers (Nicke et al 1998), which assemble in homomeric or heteromeric fashion from seven distinct subunits encoded by seven distinct genes (designated $P2X_1$ to $P2X_7$) (Roberts et al 2006). All P2X subunits are expressed in neural cells, but this expression is heterogeneous in different brain regions and cell types. Peripheral neurons also express these receptors, which are implicated in temperature and pain perception (Khmyz et al 2008). $P2X_7$ were also identified in glial cells: in cortical astrocytes (Lalo et al 2008) and in spinal cord microglia (Tsuda et al 2003). Stimulation of P2X receptors triggers cytosolic Ca^{2+} signals in many CNS neurons (Pankratov et al 2009), and P2X-mediated presynaptic Ca^{2+} signals can regulate neurotransmitter release (Sperlagh et al 2007). Also, the involvement of the $P2X_7$ pore in apoptosis induction via sustained entry of Ca^{2+} into cells has been suggested (Ferrari et al 1999).

Up to now eight mammalian P2Y receptor subtypes ($P2Y_{1,2,4,6,11,12,13,14}$) have been characterized by molecular means (Abbracchio et al 2009). These receptors are activated not only by ATP, but also by other naturally occurring nucleotides or nucleotide-sugars, such as adenosine diphosphate (ADP), uridine triphosphate (UTP), uridine diphosphate (UDP), and UDP-glucose (Jacobson et al 2002). P2Y receptors are expressed very early in the embryonic CNS and are broadly distributed on both neurons and glia. Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) revealed that $P2Y_1$ and $P2Y_{11}$ mRNA was present in the human brain in large quantities when compared with other tissues, but only low to moderate levels of $P2Y_2$, $P2Y_4$, and $P2Y_6$ were detectable (Moore et al 2001). In immunohistochemical stainings of human brain slices, a striking neuronal localization of $P2Y_1$ receptors was confirmed (Moore et al 2000a). P2Y receptors might also generate homodimers or heterodimers with other P2Y receptors (Ecke et al 2008) or with other receptors (e.g. A_1 adenosine receptors) (Fischer & Krugel 2007). P2Y receptors belong to the superfamily of G protein-coupled receptors, and can be subdivided in two groups: receptors of the first subgroup principally use Gq/G11 to activate the phospholipase C/inositol triphosphate (IP3)

endoplasmic reticulum Ca^{2+} release pathway ($P2Y_{1,2,4,6,11}$) (Verkhratsky 2005), whereas receptors of the second subgroup almost exclusively couple to Gi/o, which inhibits adenylyl cyclase and modulate ion channels ($P2Y_{12,13,14}$) (Abbracchio et al 2006). Coupling of the same P2Y receptor to different G proteins is also possible, indicating agonist-specific signaling involving distinct active receptor conformations. For instance, activation of $P2Y_{11}$ receptors by ATP increases cAMP, IP3 and cytosolic Ca^{2+}, whereas activation by UTP produces Ca^{2+} mobilization without IP3 or cAMP increase (White et al 2003). In addition, P2Y receptors may activate phospholipases A2 and D, mitogen-activated protein kinases (MAPK), tyrosine kinase, and serine-threonine kinase

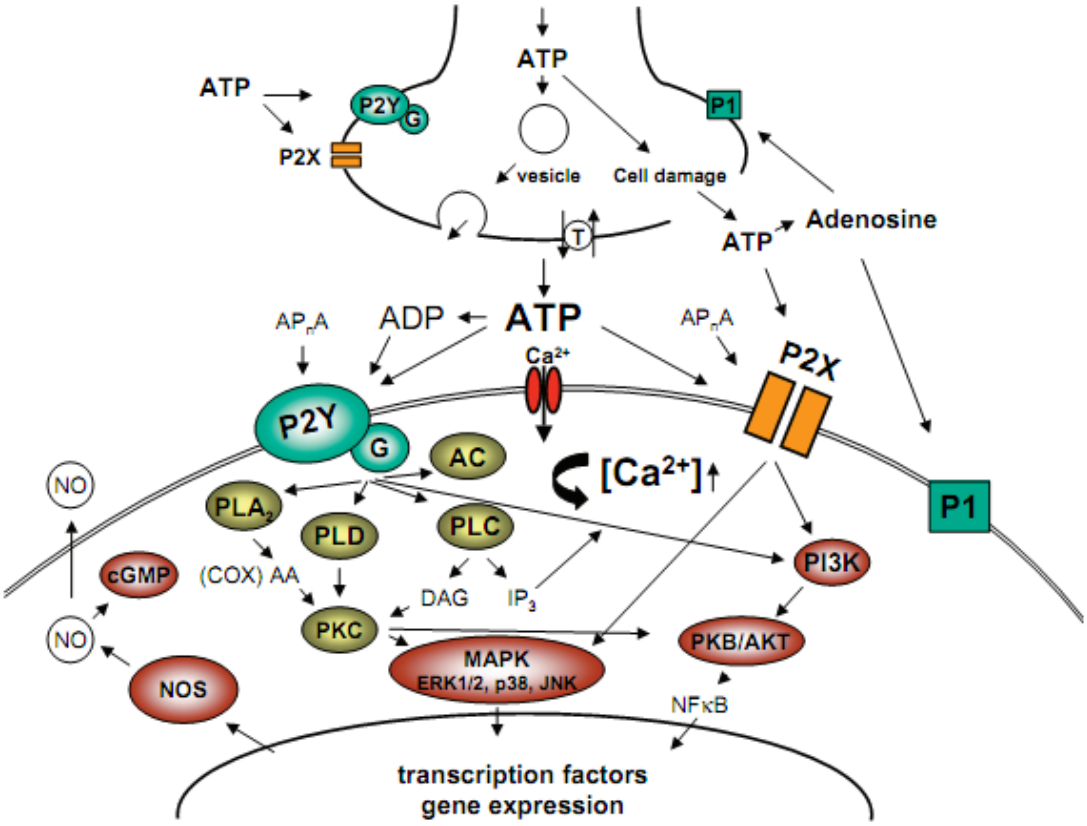

Figure 2 - Schematic diagram illustrating possible ATP actions: ATP release, metabolism, and interaction of second messenger systems following the activation of ionotropic P2X and G protein (G)-coupled P2Y receptors. The activation of P2 receptors usually leads to Ca2+ mobilization with and without Ca^{2+} entry from the extracellular space. Ca^{2+} may either enter the cell through the P2X receptor-channels themselves or through voltage-sensitive Ca^{2+} channels opened by the P2X receptor-evoked membrane depolarization. The functional responses may be regulated by a cross-talk of a great number of second-messenger pathways, e.g., via the activation of phospholipase A2, phospholipase D (PLD), protein kinase C (PKC), adenylate cyclase (AC), phospholipase C (PLC) stimulating diacylglycerol (DAG) and inositol-(1,4,5)-trisphosphate (IP3), nitric oxide synthase (NOS), phosphoinositide 3-kinase (PI3K)/serine-threonine kinase (AKT), mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinases (JNKs), and cyclic guanosine monophosphate (cGMP). Changes in the expression of e.g., arachidonic acid (AA), cyclooxygenase (COX), nitric oxide (NO), or nuclear factor kappa B (NFκB) mediated via P2 receptors are described (Franke & Illes 2006).

(figure 2). Classical members of the MAPK family, like extracellular signal-regulated kinases (ERKs), are implicated in cell growth, as well as differentiation, whereas stress-activated protein kinases and p38 appear to play a role in regulating the cell death machinery. Phosphoinositide 3-kinase/serine-threonine kinase pathways are associated with cell proliferation and differentiation (Neary et al 2004). P2Y receptor activation of the MAPK cascade, in particular ERK1/2 or c-Jun N-terminal kinases (JNKs), has been reported to be involved in caspase-3 activation and apoptosis (Neary et al 1999).

P2 receptors in glial cells

Glial cells are an abundant cell population present in the brain. According to their localization and their functional properties, glial cells are classified as Schwann cells (in the peripheral nervous system) and oligodendrocytes (in the central nervous system), which insulate axons and allow the fast and efficient propagation of the action potential; microglial cells, which serve an immune function in the brain; astrocytes, which represent the most abundant subclass of glial cells and which are responsible for extracellular K^+ homeostasis, metabolic support and regulation of the external chemical environment of neurons. All these types of glia express functional purinergic receptors (Verderio & Matteoli 2011). Stimulation of astrocytes by pharmacological means as well as by mechanical or osmotic stimulation may release ATP. Calcium waves in astrocytes were shown to be initiated and supported by the release of ATP (Cotrina et al 2000), leading to the stimulation of astrocytic $P2Y_1$ receptors (Fam et al 2003). These waves are defined as oscillations of intracellular free Ca^{2+} that propagate between neighboring astrocytes and are presently considered to constitute an information-processing system operating in parallel to neuronal circuits. The calcium waves in glial cells may also be initialized from ATP released from neighboring neurons (Verderio & Matteoli 2011). In addition to ATP, astrocytes were shown to release also excitatory glutamate and aspartate, and inhibitory γ-aminobutyric acid (GABA), amino acid transmitters (Bezzi & Volterra 2001; Nedergaard et al 2002). The stimulation of the $P2X_7$ receptor has been shown to mediate the release of glutamate and GABA from astrocytes (Duan et al 2003). ATP can also elicit cytokines and neurotrophins release from glial cells, contributing to synaptic plasticity (Verderio et al 2006; Zhang et al 2007). ATP released from astrocytes may also lead to neuron excitation or depression of transmission. In hypothalamic neurons, astrocyte derived ATP has been shown to enhance neuronal excitability through stimulation of postsynaptic P2X receptors,

present on neighboring neurons (Gordon et al 2005). On the other hand, it was observed that ATP released from glial cells inhibits the activity of sensor neurons responding to painful stimuli (Chen et al 2008). Hence, glia should be considered as an active partner at the synapse, dynamically regulating synaptic transmission (Newman 2003). ATP also controls and regulates many pathological reactions of glia, for example reactive astrogliosis, myelin disintegration and microglial motility and activation, which may be controlled by $P2Y_6$ and $P2Y_{12}$ receptors (figure 3) (Abbracchio & Ceruti 2006; Pocock & Kettenmann 2007).

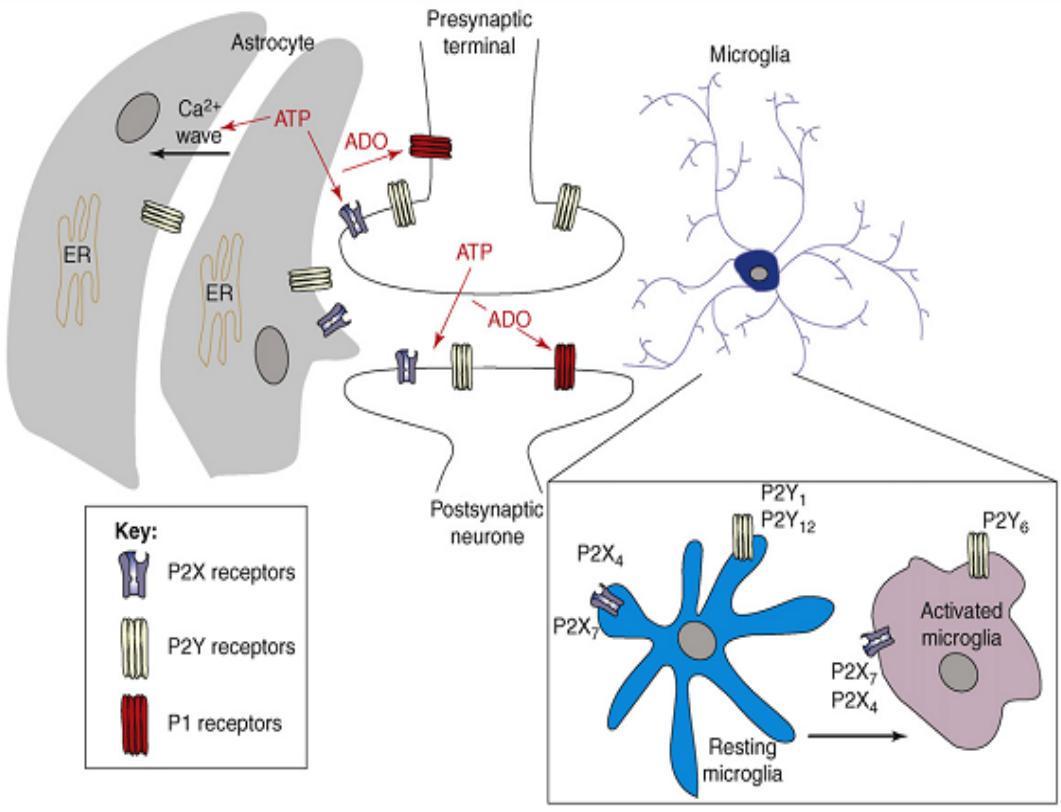

Figure 3 - Nucleotide-mediated transmission in the CNS. ATP released on its own or co-released with other transmitters during synaptic activity stimulates P2 receptors localized on postsynaptic and presynaptic membranes and in astroglia. Simultaneously degradation of ATP produces adenosine, which acts on P1 receptors. ATP can be also released from astrocytes, where it is instrumental for both initiation and propagation of glial Ca^{2+} waves and for glial–neuronal signalling, which can be mediated either by ATP or by adenosine. Purinoreceptors expressed in microglial cells control their activation and their expression might, in turn, be modified during this activation process (Abbracchio et al 2009).

ATP neuromodulation

ATP and other nucleotides are stored in secretory and synaptic vesicles. ATP is probably present in every synaptic and/or secretory vesicle, although at different concentrations, and can be co-stored and co-released with other neurotransmitters (e.g. GABA, noradrenaline or glutamate) (Pankratov et al 2006). There is compelling evidence for exocytotic neuronal vesicular release of ATP (Pankratov et al 2006). But there are additional mechanisms that may be involved in nucleotide release, including ATP-binding cassette transporters, connexin or pannexin hemichannels, plasmalemmal voltage-dependent anion channels and $P2X_7$ receptors (Abbracchio et al 2009). After release, ATP and other nucleotides undergo rapid enzymatic degradation by ectonucleotidases, which may be functionally relevant since they control the lifetime of ATP ligands, and ATP metabolites can act as physiological ligands for various purinergic receptors nucleosides (Zimmermann 2006a). P2Y receptors can be classified as preferring nucleoside triphosphates ($P2Y_2$, $P2Y_4$ and $P2Y_{11}$) or diphosphates ($P2Y_1$, $P2Y_6$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) (Abbracchio et al 2009) and ATP can be fully hydrolyzed to adenosine, a ligand of P1 receptors (Cunha et al 1998). All the identified ectonucleotidases are expressed in the brain: E-NTPDases (ectonucleoside triphosphate diphosphohydrolases), E-NPPs (ectonucleotide pyrophosphatase and/or phosphodiesterases), alkaline phosphatases and ecto-5'-nucleotidases. E-NTPDases and E-NPPs hydrolyze ATP and ADP to AMP, which is further hydrolyzed to adenosine by ecto-5'-nucleotidase (Zimmermann 2006a).

P2Y receptors have been found to control a large variety of neuronal ion channels including voltage-activated Ca^{2+} and K^+ channels as well as transmitter-gated ion channels. P2Y receptors were found to mainly inhibit voltage-activated Ca^{2+} channels in a pertussis toxin sensitive manner (G protein dependent) in neurons. These receptors can also inhibit KCNQ channels (which mediate M-type K^+ currents), the closure of these channels causes depolarization and leads to increased neuronal excitability. GIRK channels (G protein-coupled inwardly rectifying K^+ channels) can also be activated by P2Y receptors causing hyperpolarization of neurons. P2Y receptors can also regulate transmitter gated ion channels like the NMDA receptor, the vanilloid receptor 1 and the ATP-gated P2X receptors (Hussl & Boehm 2006).

Synaptic transmission requires the release of transmitters from presynaptic nerve terminals and the ensuing activation of postsynaptic receptors by the released

transmitters. Accordingly, synaptic transmission can be modulated either by changes in the presynaptic release or by changes in either the excitability of the postsynaptic membrane or in the signaling capabilities of the postsynaptic receptors. In both cases, the regulation of synaptic transmission via P2Y receptors relies most commonly on the modulation of ion channels. Voltage-activated Ca^{2+} channels, in particular N- and P/Q-type channels, are located at presynaptic nerve terminals and link invading action potentials to transmembrane Ca^{2+} influx and concomitant vesicle exocytosis. As a consequence, the modulation of these ion channels via GPCRs (G-protein coupled receptors) leads to changes in transmitter release (Stevens 2004). The control of GIRKs and KCNQ channels will rather cause changes in the postsynaptic excitability. And the modulation of transmitter-gated ion channels will mostly cause alterations in the signaling of postsynaptic receptors. In fact, P2Y receptors were found to mediate nucleotide-dependent changes in synaptic transmission via both, pre- and postsynaptic effects (Hussl & Boehm 2006).

The release-enhancing effects of nucleotides appeared to be mostly mediated by receptors of the P2X family. The inhibitory effects, in contrast, rather involved P2Y receptors as suggested for the release of noradrenaline, serotonin, and dopamine (figure 4) (Cunha & Ribeiro 2000). In hippocampal neurons, presynaptic P2Y receptors were reported to mediate an inhibition of glutamate release, whereas P2X receptors mediate a facilitatory effect (Rodrigues et al 2005). Nucleotides were also reported to either inhibit or enhance synaptic transmission, acting on postsynaptic sites. In the prefrontal and parietal cortex, for instance, activation of a $P2Y_1$-like receptor reduced glutamatergic transmission, but only the component involving NMDA receptors (Luthardt et al 2003).

P2X receptors also have a role in modulating synaptic plasticity. Application of ATP triggers substantial Ca^{2+} signals in central neurons, which can be mediated by Ca^{2+} entry through P2X receptors (Kirischuk et al 1996), and contrary to NMDA receptors or voltage-gated calcium channels, Ca^{2+} influx via P2X receptors does not require membrane depolarization and therefore can contribute to generation of intracellular calcium signals at resting membrane potentials (Pankratov et al 2009). The cytoplasmic Ca^{2+} signals are important for synaptic depression or potentiation (Debanne et al 2003). A further important property of P2X receptors is their ability to interact with other neurotransmitter receptors. P2X receptors can interact with nicotinic ACh receptors and

GABA receptors, and activation of P2X receptors caused Ca^{2+}-dependent inactivation of NMDA receptors in hippocampal pyramidal neurons (Pankratov et al 2009).

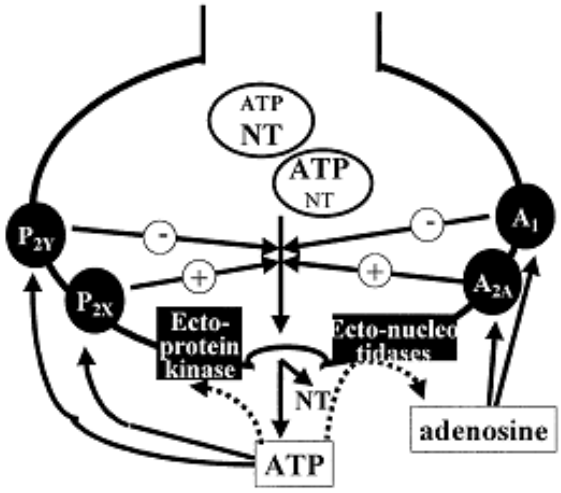

Figure 4 - Purinergic presynaptic modulation of neurotransmitter release (Cunha & Ribeiro 2000).

ATP-mediated death and survival in the CNS

Extracellular ATP, *per se*, has been found to be toxic for a variety of mature differentiated neurons, including cerebellar, striatal, and hippocampal neurons. The nucleotide was reported to induce both, apoptotic and necrotic features of degeneration after only a few minutes of exposure and with a time lag of at most 2 hours (Amadio et al 2002). These findings have led to the hypothesis that extracellular ATP can be an important mediator of neuropathological events of brain injury. ATP can rise to high extracellular levels after different kinds of injuries in the CNS (ischemia, hypoxia, traumatic insult or epilepsy) (Dale & Frenguelli 2009; Melani et al 2005) and through the activation of P2 receptors can modulate release of neurotransmitters and induce functional and morphological responses like necrosis, apoptosis or regenerative processes (Franke & Illes 2006) (figure 5).

ATP in pathological conditions

Under pathological conditions of hypoxia or ischemia, extracellular purine nucleotides leak from damaged cells and thereby may reach high concentrations in the

extracellular space (Phillis et al 1993). An up-regulation of the $P2X_7$ receptor expression (on astrocytes, microglia, and neurons) appears to contribute to the mechanisms of cell death caused by in vivo and in vitro ischemia (Cavaliere et al 2004; Franke et al 2004a). $P2Y_1$ receptors are intensely expressed in Purkinje cells in deep layers of the cerebral cortex and in ischemia-sensitive areas of the hippocampus (Moran-Jimenez & Matute 2000). In conclusion, extensive evidence demonstrates a post-ischemic time and region-dependent up-regulation of P2X ($P2X_2$, $P2X_4$, $P2X_7$) and P2Y ($P2Y_1$) receptor subtypes on neurons and glial cells and suggests a direct role of this receptor family in the pathophysiology of cerebral ischemia in vitro and in vivo (Franke & Illes 2006).

ATP also has a role in epilepsy. The intraventricular injection of high doses of ATP in rats evoked severe clonic-tonic convulsions. Unilateral microinjection of nucleotides into the rat prepiriform cortex caused a convulsant response antagonized by suramin (P2 receptors antagonist) (Weisman et al 1996). When perfused with a zero magnesium medium containing 4-aminopyridine hippocampal slices generate epileptiform bursts of an interictal nature, whereas ATP and adenosine depressed this epileptiform activity (Ross et al 1998). In seizure prone gerbils $P2X_2$ and $P2X_4$ receptor expression is significantly reduced when compared with normal gerbils (Kang et al 2003).

Alzheimer's disease involves the progressive extracellular deposition of amyloid β-peptide, which can damage neurons, leading to their dysfunction and death (Haughey & Mattson 2003). ATP has been shown to increase the vulnerability of neurons to amyloid β-peptide induced death (Haughey & Mattson 2003). Extracellular ATP, acting through the $P2X_7$ receptor, can alter β-amyloid peptide-induced cytokine secretion from human macrophages and microglia and, thus, may be an important modulator of neuroinflammation in this disease (Rampe et al 2004). $P2Y_1$ receptor was localized at a number of characteristic Alzheimer's disease structures such as neurofibrillary tangles, neuritic plaques, and neuropil threads in the hippocampus and entorhinal cortex. The intense $P2Y_1$ staining observed over pathological Alzheimer's disease structures might imply that this receptor is involved either directly or indirectly in signaling events mediating pyramidal cell neurodegeneration (Moore et al 2000b).

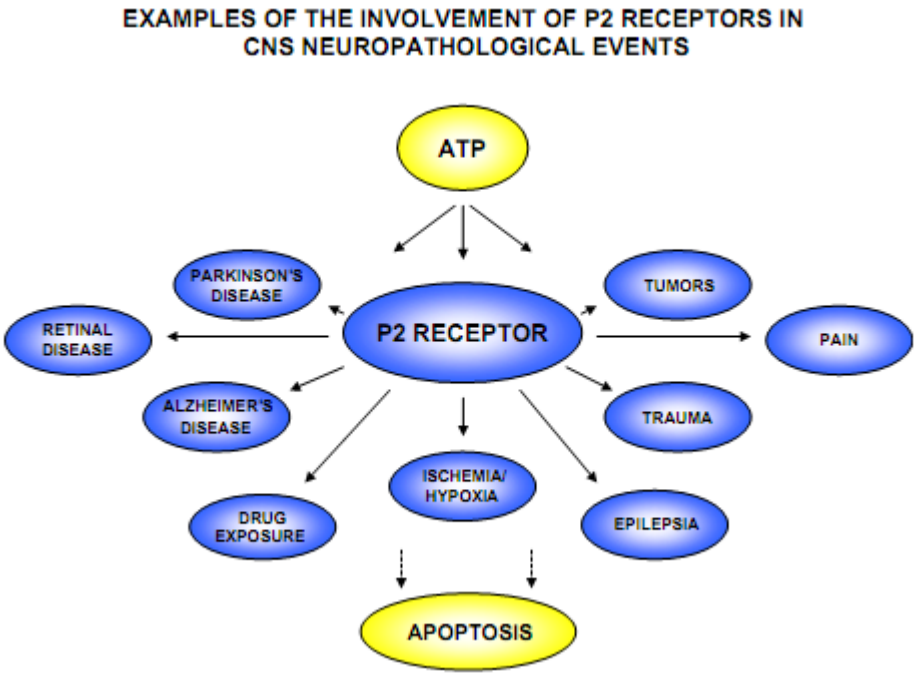

Figure 5 - Schematic diagram illustrating examples of the diversity of P2 receptor involvement in CNS neuropathological events. Extracellular ATP mediates, via P2 receptors, various kinds of ''acute'' and ''chronic'' pathological conditions, in some case, resulting in apoptosis (Franke & Illes 2006).

P2 receptor mediated neuroprotection

Several P2 receptor antagonists appear to be beneficial in the treatment of various examples of P2 receptor-mediated neurodegeneration.

In vitro, several P2 antagonists showed a protective effect against excessive glutamate exposure on primary neurons (Volonte & Merlo 1996), and to hypoglycemia and/or chemical ischemia in primary neuronal dissociated and organotypic CNS cultures (Cavaliere et al 2004; Cavaliere et al 2001).

The application of wide-range P2 receptor antagonists, namely, reactive blue 2, PPADS, or suramin, diminished lesions caused by intrastriatally injected ATP (Ryu et al 2002), intra-accumbal injection of P2 receptor agonists, and middle cerebral artery occlusion (which causes ischemia) (Kharlamov et al 2002). It was also demonstrated that an antibody raised against $P2Y_1$ receptors decreased the ADPβS-induced proliferation of astrocytes (Franke et al 2004b).

$P2Y_1$ receptor antagonist MRS2179 prevents the damage caused by Aβ1-42 peptide and glutamate in hippocampal neurons in culture (Porciúncula et al 2006). Which suggest a proeminent role of the tonic activation of $P2Y_1$ receptors on the expression of neuronal damage.

ATP as a trophic factor

Purines may induce neurite outgrowth during de novo neuritogenesis (D'Ambrosi et al 2000; Gysbers & Rathbone 1996), regeneration (D'Ambrosi et al 2001), proliferation (Rathbone et al 1999; Sanches et al 2002), and survival after serum or growth factor deprivation (D'Ambrosi et al 2001; Fujita et al 2000). P2 receptor agonists induce fiber outgrowth in organotypic slice cocultures of the dopaminergic and hippocampal systems, which could be inhibited by PPADS, suggesting the involvement of P2 receptors (Franke & Illes 2006).

Extracellular ATP can act in combination with growth factors to stimulate astrocyte proliferation and contribute to the process of reactive gliosis, a hypertrophic/hyperplastic reaction that is frequently seen in brain trauma, stroke/ischemia, epilepsy, and various neurodegenerative disorders. Astrogliosis has been interpreted as a beneficial response, which enables the injured brain to restore its damaged functions. Otherwise, the glial matrix forms a physical barrier preventing regeneration and remyelination (Ciccarelli et al 2001; James & Butt 2002). In cultured rat cortical or striatal astrocytes, extracellular ATP and other P2 receptor agonists induced stellation, an increase in the GFAP immunoreactivity and content, elongation, stimulation of DNA synthesis, and increase in cell number (Abbracchio et al 1999; Neary et al 1996; Rathbone et al 1999). ATP induces the release of arachidonic acid metabolites and can cause trophic factor synthesis in astrocytes and neurons (Brambilla et al 2002). A synergistic interaction between basic fibroblast growth factor and ATP has been reported on DNA synthesis in primary cultures of rat cortical astrocytes to enhance the glial effects (Neary et al 1996; Neary et al 1994).

Objectives

- Evaluate the neuroprotective role on pyramidal neurons mediated by the unselective P2 receptor antagonist PPADS, in an *ex-vivo* model of acutely dissociated hippocampal slices exposed to high levels of the excitatory amino-acid NMDA.

- Analyze if the glial cells, astrocytes and microglia, have a role in mediating the neuroprotective effects of PPADS on pyramidal neurons.

Material and Methods

Acute hippocampal slices prepared from young adult (8-12 weeks) male Wistar rats was the model used in the experiments of this work, allowing an *ex-vivo* study.

Hippocampus:

The hippocampus is a bilateral, incurved seahorse shaped structure, located beneath the neocortex. It is involved in physiological processes like learning and memory, and in pathological processes like Alzheimer's disease and epilepsy. The hippocampus can be divided in two regions, the *dentate gyrus* (DG) and *Cornu Ammonis* (CA). The DG and CA are each composed by layers of the cell bodies of two different type of neurons, the granule and pyramidal neurons, respectively (figure 6). The axons of the granule cells, the mossy fibers, project to the pyramidal cells of the CA3 region. The pyramidal cells of CA3 project their axons, the collaterals of Schaffer, to the pyramidal cells of CA1. In the DG and CA regions the interneurons, basket and mossy cells, establish symmetrical synaptic contacts with granular and pyramidal cells. Symmetrical contacts are histological criteria indicative of inhibitory synapses. The DG and CA circuits are mainly glutamatergic excitatory, whereas interneurons neurotransmitter is GABA, the main inhibitory neurotransmitter of the nervous system (Taupin 2007).

Methods:

Animals:

Male Wistar rats, aged 8-12 weeks old (young adults) obtained from Charles River (Barcelona, Spain) were used. All animal procedures were conducted according to the European Community Guidelines for Animal Care. Animals were housed four per cage unless otherwise stated and maintained under a controlled environment: 23±2ºC of temperature, 55±5% humidity, 12h light/dark cycle. Animals were offered free access to food and water.

Slice preparation:

Animals were anaesthetized under halothane atmosphere, decapitated and their hippocampi were rapidly dissected and placed in ice-cold oxygenated (95% O_2 and 5%

CO_2) artificial cerebrospinal fluid (aCSF). Hippocampi were sliced transversally (400 μm thick) using a McIlwain tissue chopper and kept in a resting chamber, immersed in oxygenated aCSF, at room temperature for at least 1 hour prior to usage (Pugliese et al 2009).

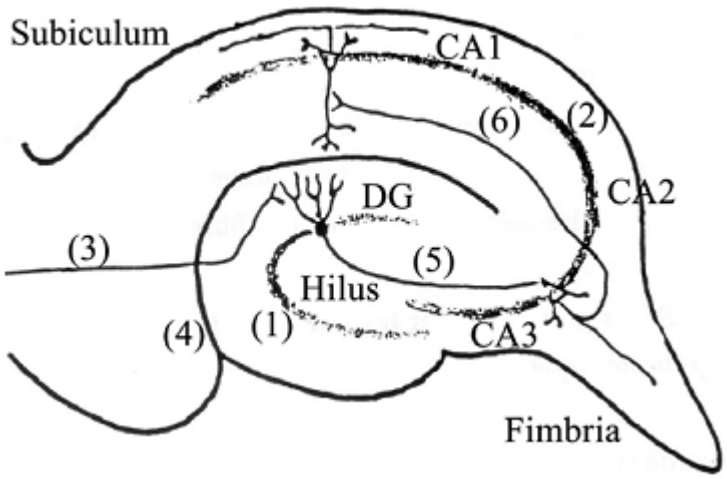

Figure 6 - The granule cell layer is the main layer of the DG (1). The pyramidal cell layer is the main layer of the CA (2). The perforant fibers (axons from the entorhinal cortex) (3), project to the dentate granule cells through the hippocampal fissure. The hippocampal fissure is a cell-free natural division that separates the DG from the CA1 region (4). The granule cells project their axons, the mossy fibers (5), to the dendrites of the pyramidal cells of CA3. The pyramidal cells of CA3 send collaterals, the collaterals of Schaeffer (6), to the pyramidal cells of CA1 forming the tri-synaptic circuit 8 (Taupin 2007).

Slice superfusion:

After the resting period of 1 hour three slices of the dorsal hippocampus were placed in small chambers (0,1 mL) completely submerged, surrounded by two nylon meshes, and superfused with oxygenated aCSF (31–32°C) at a constant flow rate of 1.5–1.8 $mL.min^{-1}$, achieved by the use of a peristaltic pump (figure 7). Slices were left 30 minutes in the chambers before the application of the drugs and the excitotoxic stimulus (NMDA), so that the slices could adapt to the new environment. Changes in superfusing solutions (NMDA and PPADS) reached the preparation in 5 minutes and this delay was taken into account in the calculations. In these experiments four chambers were used, each chamber for a different experimental condition:

- Chamber 1 – Control;
- Chamber 2 – NMDA;
- Chamber 3 – PPADS;
- Chamber 4 – PPADS + NMDA.

After the adaptation period of 30 minutes (with constant superfusion with oxygenated aCSF), PPADS (20 μM in aCSF) and NMDA (100μM in aCSF) were applied to the preparation. NMDA was applied during 1 hour in chambers 2 and 4, after which slices where allowed for a recovery time of 3 hours. PPADS was applied in chamber and 3 and 4. In chamber 4 PPADS was applied 15 minutes before and during NMDA application, and for the 3 hours after ceasing NMDA application. In chamber 1, slices were continuously superfused with oxygenated aCSF. All the solutions used in the experiments were under constant oxygenation.

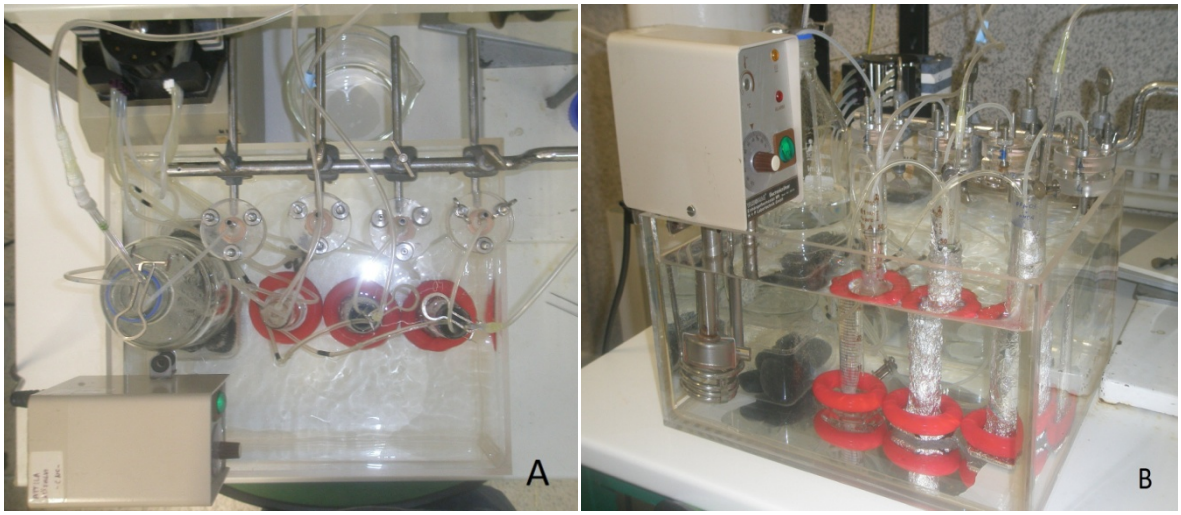

Figure 7 - Setup of the superfusion system used in these experiments, view from the top (A) and from the side (B).

Cell viability:

Immediately after the superfusion, slices were placed in a chamber with oxygenated aCSF solution containing 0.05% trypan blue (previously filtrated with a filter of 0,2μm) for 30 minutes. Trypan blue (TB) readily penetrates dead and dying cells and stains them blue while leaving living cells unstained (DeRenzis & Schechtman 1973). After the staining, slices were washed for 30 minutes, and then were fixed in 4% paraformaldehyde at 4ºC overnight. To allow observation in microscope, slices needed to be cut to a lower thickness, so the next day slices were extensively washed with phosphate buffer solution (PBS) and transferred to a solution containing 25% sucrose for at least 24 hours, to cryoprotect the slices and allow the cut in the cryostat. In the cryostat, slices were cut once again to a thinner section of 40μm, and only the inner

slices were used and stored in PBS at 4ºC, the more superficial slices (the first 120 µm) were discarded as they contain more dead cells due to the trauma of the initial cutting and incubation. The slices obtained were then mounted in gelatin-coated slides with dako fluorescent mounting medium (Dakocytomation, Inc.). The slices were examined under transmitted light under a Zeiss Microscope (Carl Zeiss, Inc.) equipped with digital camera, at a magnification of 20x. Pictures were taken, using Stereo Investigator software (MicroBrightField, Inc.) of the CA1 and CA3 regions of the hippocampus. The TB stained cells were then counted using the Image-J software.

Immunohistochemistry:

Immediately after the end of the superfusion, slices were fixed in 4% paraformaldehyde at 4ºC overnight. The next day, slices were extensively washed in PBS and transferred to a solution containing 25% sucrose for at least 24 hours. To allow immunohistochemistry assays, slices were cut further to a thickness of 30µm using the cryostat. Only the inner slices were saved and stored in PBS at 4ºC.

The immunohistochemistry technique consists in demonstrate the presence of an antigen (Ag) in a tissue, taking advantage of the specific bonding between an antibody (Ab) and the Ag. To rise the sensitivity of the technique, a second Ab (specific to the primary Ab), may be used (figure 8). To make these linkages visible in microscopy, a tag has to be bound to the antibody. The stain can be a metal, an enzyme, a fluorescent tag, among others (Ramos-Vara 2005).

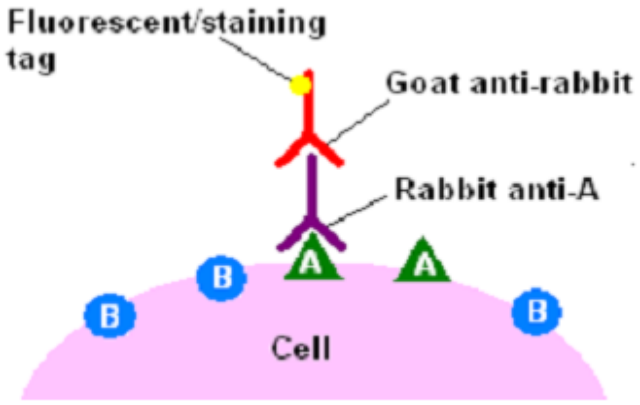

Figure 8 – Schematic diagram illustrating the immunohistochemistry technique. A secondary antibody (goat anti-rabbit) bound to the tag, is linked to the primary antibody (rabbit anti-A), which is himself, linked to the antigen.

In this project, we tested antibodies for the glial markers GFAP (glial fibrillary acid protein) and CD11b, markers for astrocytes (Pekny & Nilsson 2005) and microglia (Jensen et al 1997) respectively (table1). In the protocol used slices were treated initially with citric acid solution for 20 minutes to unmask the

epitopes. Then they were permeabilized and blocked for 1 hour with a blocking solution containing 5% normal horse serum (NHS) and 0,25% Triton X-100 in PBS. After slices were incubated over-night at room temperature with antibodies (prepared in the blocking solution) against CD11b, GFAP and SNAP-25. The incubation with the antibody for synaptophysin was only for 2 hours. Slices were then washed three times in PBS and subsequently incubated with secondary antibody conjugated with the fluorophore (Alexa Fluor, prepared in a solution containing 2% NHS and 0,25% Triton X-100 in PBS) for 2 hours at room temperature. In the following step, slices were washed again 3 times in PBS and incubated 15 minutes with 4',6-diamidino-2-phenylindole (DAPI) prepared in PBS to mark the nuclei of cells. Finally slices were mounted on slides using dako fluorescent mounting medium.

The slices were then examined under a Zeiss Microscope (equipped with digital camera) with reflected light and adequate light filter, at a magnification of 20x. Pictures were taken, using the Stereo Investigator software of the CA1 and CA3 regions of the hippocampus.

The intensity of binding between Ab and Ag was then analyzed: Densitometric analysis was conducted measuring the average gray value in each region of interest using Image-J software (NIH, http://rsb.info.nih.gov/ij/). Data were normalized to the average value found in control slices in each experiment.

Table 1 – Primary and Secondary antibodies used in this work

Antibody	Supplier	Host	Type	Dilution
GFAP	Dako	Rabbit	Polyclonal	1:4000
CD11b	ABD Serotec	Mouse	Monoclonal	1:100
Anti-Rabbit Alexa Fluor 594	Invitrogen	Donkey	IgG(H+L)	1:400
Anti-Mouse Alexa Fluor 488	Invitrogen	Donkey	IgG(H+L)	1:400

Statistical analysis:

Data were analysed using Prism 3.02 software (Graphpad Software). All numerical data are expressed as the mean ± SEM. Data were tested for statistical significance with the two-way ANOVA test or by analysis with Newman-Keuls post-hoc test, as appropriate. A value of $P < 0.05$ was considered significant.

Solutions:

- aCSF (mM) - 124 NaCl; 3 KCl; 1,25 NaH_2PO_4; 10 Glucose; 26 $NaHCO_3$; 1 $MgSO_4$; 2 $CaCl_2$; pH= 7.4
- NMDA - 100μM in aCSF
- PPADS tetrasodium salt - 20μM in aCSF
- Trypan Blue – 0,5% TB in aCSF
- Paraformaldeide (PAF)– 4% PAF; 0,9% NaCl; 4% Sucrose
- Sacarose solution – 0,25% NaCl; 25% Sucrose
- PBS (mM) – 140 NaCl; 3 KCl; 20 NaH_2PO_4; 15 KH_2PO_4; pH= 7.4
- Citric Acid – 10mM in PBS
- Blocking solution 1 – 0,25% Triton X-100; 5% NHS in PBS
- Blocking solution 2 – 0,25% Triton X-100; 2% NHS in PBS
- DAPI – 0,2 μg/mL in PBS

Results

Neuroprotection afforded by PPADS

Trypan blue was used to test the viability of pyramidal neurons, in the CA3 and CA1 region of hippocampal slices exposed to NMDA (100μM) and/or PPADS (20 μM). NMDA increased the number of pyramidal cells that incorporate trypan blue both in the CA1 (Two-way ANOVA, $p<0.01$, n=4-7) and CA3 (two-way ANOVA, $p<0.01$, n=4-7) regions of hippocampal slices, when compared to untreated slices. However, in the CA1 region the number of cells stained with trypan blue was higher (176±23.8% of control, $p<0.01$, Newman-Keuls post-hoc test) than in the CA3 region (156.2±19.8% of control, $p<0.05$, Newman-Keuls post-hoc test). PPADS had no statistically significant effect on the number of cells stained with trypan blue, both in the CA1 (two-way ANOVA, $p=0.093$, n=4-7) and CA3 (two-way ANOVA, $p=0.198$, n=4-7) regions. However, in the CA1 region, PPADS+NMDA showed a tendency (135.6±20.1% of control, $p=0.099$, Newman-Keuls post-hoc test) to decreasing in the number of stained neurons compared with neurons of slices treated with NMDA alone (figure 9). Which was not considered for the CA3 region (124.6±7.2%, $p=0.109$, Newman-Keuls post-hoc test). These results suggest a neuroprotective role for PPADS in the CA1 region.

Evaluation of glial response to NMDA and PPADS

Responses of microglia and astrocytes were evaluated by imunoreactivity for CD11b and GFAP, respectively. Microglia remained in a resting state for all conditions tested, as observable by their elongated and ramified morphology (figure 11). Activated microglia usually shows ameboid morphology (Wollmer et al 2001). Control slices demonstrate a resting state for astrocytes, although in CA1 region of NMDA (100μM) treated slices, astrocytes undergo astrogliosis characterized by cell proliferation and hypertrophy and an augmentation of imunoreactivity for GFAP. So, NMDA increased GFAP imunoreactivity in comparison with slices not treated with NMDA, independently of the presence of PPADS (two-way ANOVA, $p<0.001$, n=2-5) in CA1 region (figure 10). PPADS had no effect on GFAP immunoreactivity (two-way ANOVA, $p=0.47$, n=2-5). Taking in account these results, PPADS was not able to reverse the induction of astrogliosis by NMDA. In the CA3 region, astrogliosis could not be detected (figure 10).

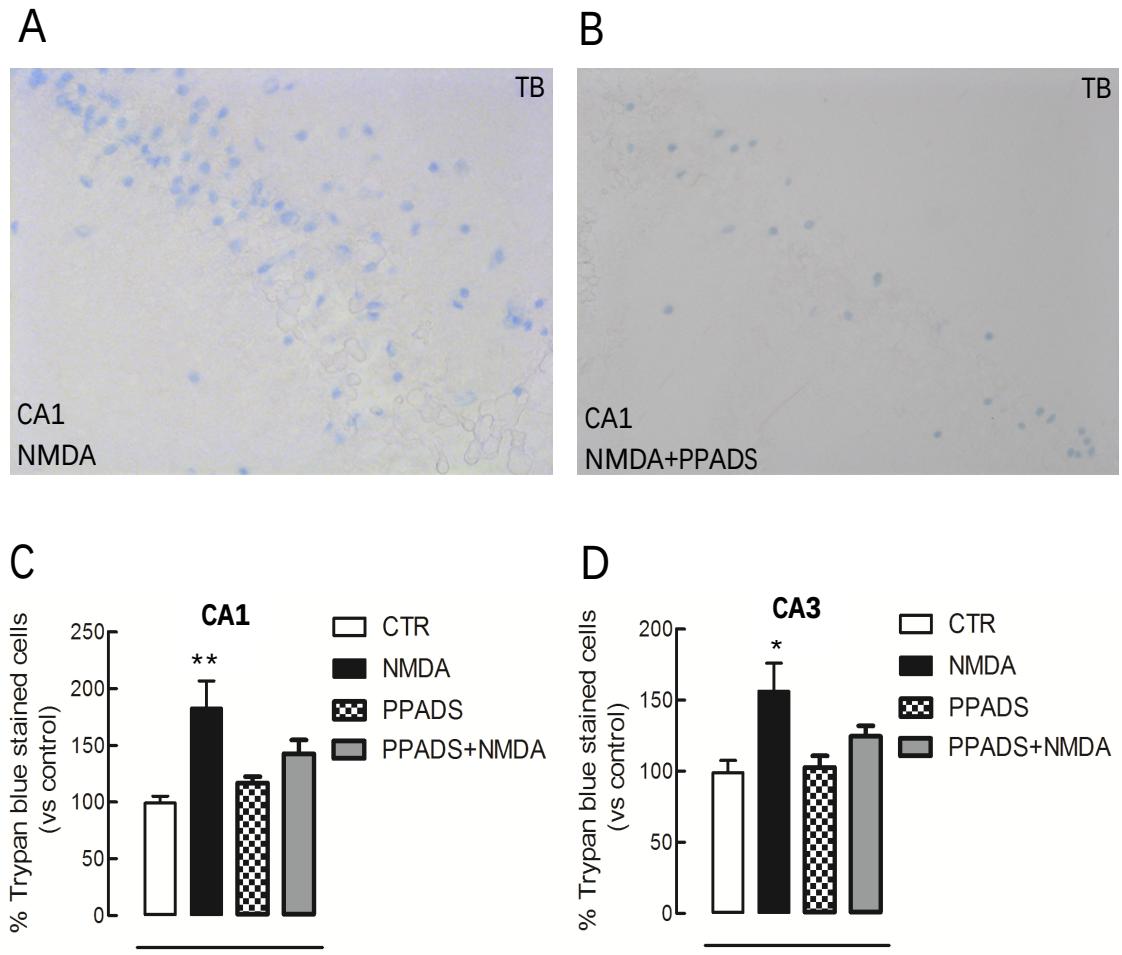

Figure 9- Analysis of cell viability in hippocampal slices treated with NMDA (100µM) and PPADS (20µM) in the CA1 region; (A) Representative image of trypan blue staining in a NMDA treated slice. (B) Representative image of trypan blue staining in NMDA+PPADS treated slices. NMDA+PPADS decreased the number of cells stained by TB compared with NMDA alone treated slices. (C) Histogram representing the % of cells stained with TB in the different conditions when compared with control slices in the CA1 region. NMDA increased the number of trypan blue stained cells compared to non-treated slices (Two-way ANOVA, $p<0.01$, n=4-7). NMDA+PPADS decreased the number of stained cells, although results were not significant (two way ANOVA, $p=0.0930$, n=4-7). (D) Histogram representing the % of cells stained with TB in the different conditions when compared with control slices in the CA3 region. NMDA increased TB staining compared to non-treated slices (Two-way ANOVA, $p<0.01$, n=4-7). PPADS had no effect on TB staining (two-way ANOVA, $p=0.198$, n=4-7). *$p<0.05$, **$p<0.01$, Newman-Keuls post-hoc test in comparison to control.

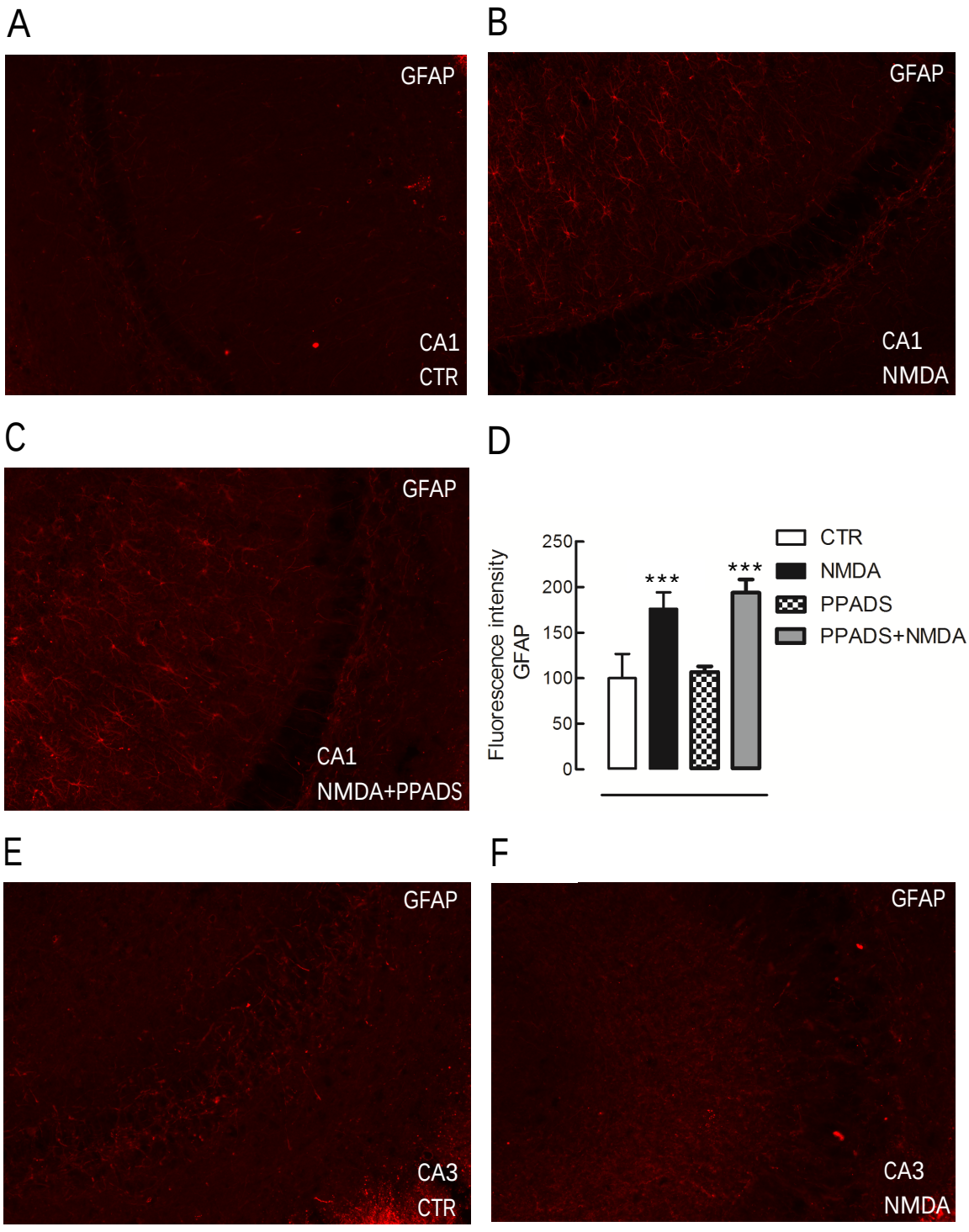

Figure 10 – Analysis of immunoreactivity for GFAP of the CA1 and CA3 region of hippocampal slices. In the CA1 region astrocytes of control slices remain inactive (A). NMDA evoked activation of astrocytes in slices, as observed by astrocyte proliferation and hypertrophy (B). PPADS did not reverse this effect (C). (D) Histogram representing GFAP immunoreactivity in the different conditions compared with control. NMDA augmented immunoreactivity for GFAP in slices where this compound was present (two-way ANOVA, $p<0.001$, n=2-5). PPADS (20μM) had no effect on GFAP immunoreactivity (two-way ANOVA, $p=0.47$, n=2-5). In the CA3 region astrocytes remained inactive, even in the presence of NMDA (E,F).***$p<0.001$, two-way ANOVA, NMDA effect.

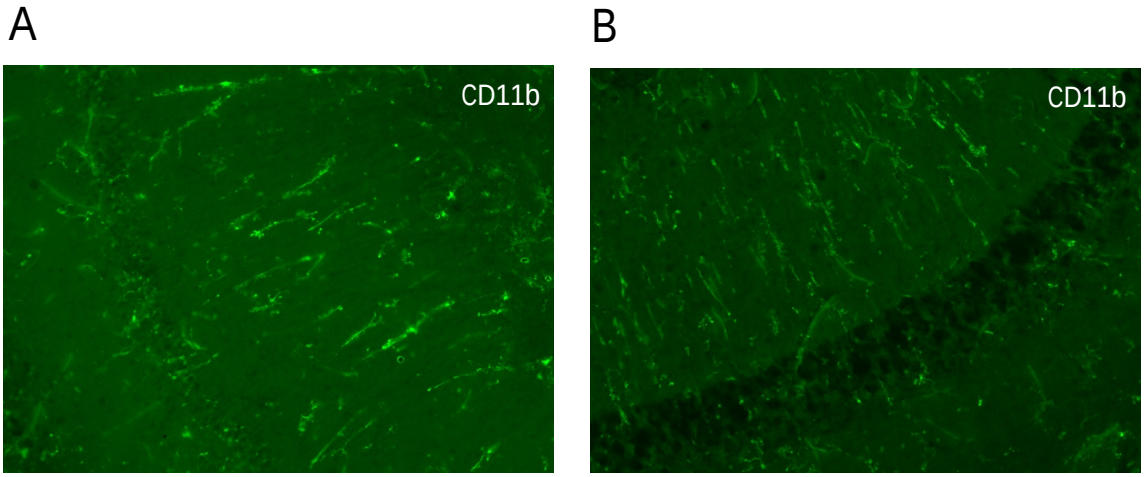

Figure 11 - Analysis of immunoreactivity for CD11b of the CA1 region of hippocampal slices. Microglia remains in a resting state (in an elongated and ramified morphology) both in the control (A) and NMDA (100μM) (B) treated slices of the CA1 region of hippocampus.

Discussion

Excitatory amino acids are known to be released in excessive levels during several pathological conditions, causing cell death and neurodegeneration (Choi 1988). One of the main player mediating excitotoxic events is the glutamate receptor NMDA, since it is highly permeable to Ca^{2+} ions (MacDermott et al 1986), which are known to mediate cell death and to the widely described neuroprotective effect of Mk-801 (selective NMDA antagonist) on glutamate mediated excitotoxicity and ischemic events (Volonte et al 1999). Over activation of NMDA receptor leads to a deregulation of intracellular calcium homeostasis, and is the main mechanism behind neuronal cell death in excitotoxic events (Hartley et al 1993). Excessive elevations in intracellular Ca^{2+} may over activate proteases, lipases, phosphatases, and endonucleases that either directly damage cell structure or induce the formation of oxidative free radicals that mediate cell death (Arundine & Tymianski 2003). Besides that, Na^{+} ion influx mediated by activation of NMDA receptors leads to a secondary influx of Cl^{-} and H_2O and causes the swelling of neurons, an acute form of cell damage (Inglefield & Schwartz-Bloom 1998). In my work hippocampal slices exposed to a high concentration of NMDA (100μM) was used, to reproduce a model of excitotoxicity, as used by other works in literature (Lin et al 2005; Volonte et al 1999). Hippocampus has been noted to be particularly vulnerable to many different types of excitotoxic insults, in concert with the high density of glutamatergic receptors in this brain area (Cater et al 2007; Prendergast et al 2004). Hippocampal slices are a useful model of study, as they retain a three-dimensional structure, preserve most of the synaptic and anatomical organization of the neuronal circuitry, have functional characteristics similar to those observed in vivo (Legradi et al 2011), express functional properties such as neurotransmitter release and synaptic transmission, and this model also allows at the same time to study gliotic population responses, thereby providing a useful model to explore responses to pathophysiological events (Holopainen 2005). In this work NMDA clearly caused the degeneration of the pyramidal cells of the CA1 and CA3 regions, as expected (Borsello et al 2003), and this was seen by an increased number of cells that incorporated trypan blue compared to untreated slices. Trypan blue enters degenerating cells, due to the loss of their membrane integrity and this technique was utilized based in previous studies in hippocampal slices (Legradi et al 2011). Propidium iodide (PI) staining technique (fluorescent compound that enters degenerating cells) based on previous work with this

technique in the same biological model (Ebrahimi et al 2010; Pugliese et al 2009) was also used. However, the differences observed, in PI fluorescence intensity, between control and NMDA treated slices was not satisfactory. Which is in agreement with Ebrahimi et al (2010) study, that suggests that it may be due to high intracellular concentration of short RNAs that also binds PI. The region that showed higher cell degeneration was CA1 compared to CA3, which is in accordance with other studies where excitotoxic and ischemic damage in hippocampal slices was tested (Bernaudin et al 1998; Butler et al 2010), this may occur as a consequence of higher density of NMDA receptor, and specific NMDA subunits (Butler et al 2010) in the CA1 region (selective vulnerability). The DG granular cells response to NMDA toxicity was also tested, although no differences was seen, mainly due to difficulty in maintaining this structure intact during the manipulation of the material and also due to the reduced size and high number of granular neurons in this region, making difficult to count with the trypan blue technique. Also, a higher density of glutamatergic receptors is described to be in the CA1 and CA3 regions of the hippocampus (Monaghan & Cotman 1985).

ATP rise to high extracellular levels during pathological events, as it can be co-released with excitatory amino acids or as a consequence of loss of membrane integrity by dying cells. This ATP may be acting on P2X and P2Y and exacerbate neural death. P2Y receptors are known to regulate Ca^{2+} channels (Hussl & Boehm 2006), and the $P2X_7$ receptor is himself highly permeable to Ca^{2+} channels and activated at high levels of ATP (Rassendren et al 1997). $P2X_7$ upregulation has been observed in a number of pathological models, including energy deprivation (Cavaliere et al 2004), in vivo ischemia (Franke et al 2004a), epilepsy (Vianna et al 2002) and mechanical injury (Franke et al 2006), so it has been described as a death receptor (Di Virgilio et al 1998). This receptor induce a massive depletion of intracellular K^+, and lead to the activation of multiple caspases (caspase 3 and caspase 9), degradation of nuclear laminin, DNA fragmentation, nuclear condensation, and apoptotic body formation (Morelli et al 2003). P2Y receptor activation of the MAPK cascade, in particular ERK1/2 or JNKs, has been reported to be involved in caspase-3 activation and apoptosis (Neary et al 1999). A direct cross-talk between NMDA and purinergic receptor has also been proposed (Luthardt et al 2003; Wirkner et al 2002). So, ATP and excitatory amino acids may act in synergism to mediate neuronal degeneration following acute and chronic insults. ATP antagonist (basilen blue, suramin, oxATP) have been described as neuroprotective in excitotoxicity and in several pathological conditions indirectly associated with

excitotoxicity, like ischemia, trauma and neurodegenerative diseases *in vitro* (cerebelar, cortical, and hippocampal cultured neurons and hippocampal organotypical cultures) (Amadio et al 2002; Cavaliere et al 2004; Cavaliere et al 2001; Runden-Pran et al 2005; Volonte et al 1999) and *in vivo* (Cavaliere et al 2003; Kim et al 2006). However no studies on the neuroprotective effect of P2 modulators in acutely dissociated hippocampal slices were found. In this work PPADS was able to reduce trypan blue staining in CA1 region of the hippocampus, however this results were less significant in the CA3 region maybe due to the lesser amount of neural death observable in this region as explained before. PPADS is a non-selective P2 receptors antagonist, acting both on metabotropic P2Y and ionotropic P2X receptors (Lambrecht et al 1992). Previous work in our lab (unpublished results) and in literature, demonstrate the neuroprotective effect of this compound on cultured hippocampal and cerebelar neuronal cells (Amadio et al 2002; Cavaliere et al 2001; Lin et al 2005) and in hippocampal organotypical cultures (Cavaliere et al 2004; Runden-Pran et al 2005) under ischemia and exposition to extracellular ATP and NMDA. However, Volonté et al (1999) were not able to see a beneficial effect of PPADS on cerebelar granule neurons exposed to glutamate, as opposed with Lin et al (2005) which show neuroprotection of this compound in the same biological model exposed to glutamate and NMDA. But in Lin et al (2005) work, PPADS was present before, during and after the excitotoxic stimulus, whereas in the first, PPADS was present only during the stimulus. This may highlight the importance of the timeline in which the drug is present. In this work PPADS was present 15 minutes before, during and 3 hours after the excitotoxic stimulus.

ATP is known to participate in both short-term calcium signaling events and in long-term proliferation, differentiation, and death of glia (Cotrina et al 2000). In astrocytes, ATP initiate calcium waves (Cotrina et al 2000), by stimulation of astrocytic $P2Y_1$ and $P2X_7$ receptors (Fumagalli et al 2003) (Fam et al 2003). In response to receptor activation and Ca^{2+} elevations, astrocytes can release both ATP and glutamate, mediate inflammation and pathological processes (Burnstock 2007). Under pathological events ATP also induces astrogliosis in the brain, and it is characterized by cell proliferation, astrocytes hypertrophy, increased expression of the astroglial specific marker GFAP and elongation of GFAP-positive processes (Neary et al 1996). Astrocyte responses to injury are aimed at both protecting the nervous system by scavenging extracellular glutamate, storing energy, supplying neighboring cells with energetic compounds, neutralizing toxic substances, and at sealing off the damaged area to preserve the less

affected tissue (Buffo et al 2010). But this process may lead to predominant reparative or destructive outcomes depending on the context in which they occur and time point after damage (Buffo et al 2010). Excessive release of the inflammation mediators, cytokines by astrocytes and microglia can have a deleterious role in the neurons survival (Marchetti & Abbracchio 2005), and $P2X_7$ receptor play a major role in releasing this agents (Di Virgilio 2007). Besides inflammatory mediators, reactive astrocytes can also produce and release arachidonic acid metabolites, nitric oxide and reactive oxygen species, which may enhance neuronal degeneration (Marchetti & Abbracchio 2005). Astrocyte activation can also form a glial scar, beneficial in isolating the damage area from the undamaged area, however it can also have negative effect in creating a barrier for neural regeneration (Liuzzi & Lasek 1987). Also, intermediate filament induction of astrocytes has a positive role shortly after damage, where it helps reduce the toxic effects of extracellular glutamate (Li et al 2008). Though, astrocytes can play a dual role either beneficial or detrimental to neurons.

So, the next step of this project would be to study if the glial response to injury may be playing a role in the neuroprotective effect shown by PPADS. GFAP immunoreactivity was used in this work to evaluate astrogliosis. The results obtained show that astrocytes are activated in the CA1 region of hippocampal slices superfused with NMDA, this is in agreement with a previous study, also in acute dissociated hippocampal slices, which undergo oxygen and glucose deprivation and in a timeline similar to the one I used (Pugliese et al 2009). In the CA3 region a lower number of astrocytes were activated. This is in agreement with a previous work in hippocampal slices culture exposed to NMDA (Kunkler & Kraig 1997), which suggests that astrogliosis occur as a response to a stressful situation for the neurons, caused by excessive levels of the excitatory amino acid, and the extent of the astrocytic activation may be proportional to the gravity of the neural damage in the area (Kunkler & Kraig 1997). In accordance, my results demonstrated a higher CA1 pyramidal cell vulnerability to NMDA compared to CA3 pyramidal cells. PPADS was not able to reverse astrogliosis, i.e., in slices treated with the drug, astrocytes remained in an active state. This suggests that astrocytes are not playing a major role in mediating either protective or deleterious effect on CA1 pyramidal neurons. The potential dual role (beneficial and detrimental) of astrocytes may not be significant. The results also suggest a prominent role of NMDA receptors on astrocyte activation, since the

antagonizing effect of PPADS on P2 receptors was not strong enough to inhibit astrogliosis (Palygin et al 2010).

Microglial cells are the major cellular elements with immune function in the CNS and play important roles in orchestrating inflammatory brain responses to pathological events (Farber & Kettenmann 2006). Activated microglia can proliferate, actively migrate to the site of injury, phagocyte cellular debris following injury and release a variety of factors, such as cytokines, chemokines, nitric oxide, and growth factors, thereby affecting the pathologic process (Farber & Kettenmann 2006). Activation of both P2X and P2Y receptors in microglia leads to an increase in intracellular calcium concentration and activate K^+ currents (Moller et al 2000; Walz et al 1993). These receptors are involved in controlling microglial movement under both physiological and pathophysiological conditions and ATP induce chemotactic response of the microglial processes (Davalos et al 2005). In ischemic lesion, microglial cells up-regulate the expression of the $P2X_7$ receptor (Franke et al 2004a), and this receptor is functionally linked to the release of several substances which are thought to influence the pathologic process in microglia (Farber & Kettenmann 2006). This receptor is predominantly activated at high ATP concentrations. Thus, only high ATP levels may be causing neuronal damage mediated by microglia. Low concentration of ATP lead to rapid release of plasminogen from microglia, which is considered to be neuroprotective (Inoue 2002). Taking in account the deleterious effect in neurons mediated by microglia, following excessive extracellular ATP, microglial activation was evaluated in different conditions using an antibody for CD11b, a marker of microglia. Microglia was not in an active state in slices treated with NMDA, which suggests that they are not playing a role in mediating the survival of the pyramidal cells. In a study on organotypical hippocampal slices, microgliosis was present in the CA1 region immediately after ischemic insult (Bernaudin et al 1998). However in a work with acute hippocampal slices, microglia remained in a resting state following oxygen-glucose deprivation (Pugliese et al 2009), which is in agreement with the results obtained in this work. This may be explained by a higher plasticity of cells in culture (Kunkler1997) in compare to *in vivo* systems, suggesting that acute hippocampal slices may by more representative of the processes *in vivo*.

The results obtained from this work suggest a prominent neural role for the neuroprotective effect mediated by PPADS in detriment of the gliotic role, in the first hours following excitotoxic events. Molecular studies on synaptic and gliotic markers

may clarify the specific glial/neural role on neuroprotection. Also, studies on organotypic hippocampal cultures (that allows a longer viability of the biological material) would permit to study modulations on a longer timescale. Also, following studies on specific P2 receptors are required for a better understanding of the mechanisms behind this neuroprotection. Among those, the P2Y1 and P2X7 may be interesting targets for future studies (Franke et al 2006).

Conclusions

An *ex vivo* model of acute hippocampal slices was used in this project to study the neuroprotective effect of the P2 antagonist, PPADS, on an excitotoxic event mediated by NMDA, using trypan blue to evaluate cell viability.

NMDA caused neurodegeneration both on CA1 and CA3 region of hippocampus with a higher extent on the first region. Which indicated that CA1 region is more vulnerable to excitotoxicity in compare with CA3 region. PPADS caused a tendency in diminishing trypan blue staining in CA1 region. Although in CA3 region, such result was not as clear, which does not permit to conclude that the same effect occured in CA3 region.

The glial response was also tested in this work, using GFAP and CD11b immunoreactivity to assess astroglial and microglial behavior respectively. Astrocytes were in an active state in slices exposed to NMDA in the CA1 region but not in the CA3 region, which may reflect the higher extent of damage occurring in this area. However, microglia showed no response, which may suggest that the timeline of the experiment or the extent of injury occurring is not enough to lead microglia to an active state, or it may be due to the biological model employed in this work. PPADS had no effect neither in astrocytes nor microglia, suggesting that these populations are not playing a major role mediating neuroprotection.

So, in this model of excitotoxicity, the neuroprotective effect seen with PPADS may be due to a direct modulation of neurons, since the glial cells, astrocytes and microglia, did not respond to the drug application.

Future studies using different biological models and techniques, and studying more specific types of P2 receptors are required to fully understand the mechanisms behind neuroprotection afforded by ATP receptors.

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