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MODULATION OF GLUTAMATE UPTAKE FROM CULTURED ASTROGLIAL CELLS: ROLE OF P2 RECEPTORS

(Diploma thesis)

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1. ABSTRACT

Glutamate is the major excitatory neurotransmitter in the central nervous system, and there is emerging evidence that its release is subject to presynaptic regulation by P2 receptors. Activation of P2X receptors elicited glutamate release from terminal of dorsal horn neurons of spinal cord and hippocampus whereas activation of P2Y receptors has been shown to inhibit glutamate release in the hippocampus. Glial cells express several subtypes of P2Y receptors and there is growing evidence that these cells are active elements at synapses, they release ATP upon activation of glutamate receptors and participate in the regulation of glutamatergic transmission.

The aim of the present study was to investigate the role of P2Y receptors in the regulation of glutamatergic transmission in rat brain cortex; whether uptake of glutamate by glial cells is influenced by P2Y receptor-activation and to identify the subtypes of P2Y receptors involved. Primary cultures of cortical astrocytes obtained from brain hemispheres of newborn rats were used as a model to study the influence of P2Y receptors on the uptake of glutamate.

We clarified the role of several P2Y agonists and we identified the subtypes of P2Y receptor involved in the regulation of synaptic concentration of glutamate which may provide the knowledge for the development of selective drugs and a new approach for drug intervention in pathologies that have been associated with an excessive activity of glutamatergic transmission such as ischemic neuronal cell death, apoptosis, epilepsy or naturopathic pain.

2. ABBREVIATIONS

ADP	adenosine- 5'- diphosphate
ADPβS	adenosine- β- thiodiphosphate
AMPA	α- amino- 3- hydroxy- 5- methylisoxazole- 4- propionate
ATP	adenosine- 5'- triphosphate
ATPγS	adenosine- γ- thiotriphosphate
DL- TBOA	DL- threo- β- benzyolxyaspartate
DMEM	Dulbecco's modified eagle medium
FCS	foetal calf serum
GABA	γ- aminobutyric acid
HEPES	N- [2- hydroxyethyl] piperazine- N'- [2- ethanosulfonic acid]
LPS	lipopolysaccharide
NMDA	N- methyl- D- aspartate
PMA	phorbol- 12- myristate- 13- acetate
PBS	phosphate- buffered saline
PKC	protein kinase C
UTP	uridinetriphosphate
3- Phe- UDP	3- phenacyl UDP disodium salt

3. INTRODUCTION

3.1. Glial cells

Glial cells include the astrocytes, oligodendrocytes, Schwann cells and microglia. They have several of roles in the nervous system. Some of their roles place the glial cells in a subservient position, supporting the physiology of associated neurons: to surround neurons and hold them in place, to supply nutrients and oxygen to neurons, to insulate one neuron from another, and to destroy and remove the dead cells.

However, recent experimental evidence suggests that some glial cells also interact closely with neurons and participate in the regulation of synaptic neurotransmission. Their role in the uptake of neurotransmitters is well-known and recent experimental work provided evidence that stimuli producing an elevation in Ca^{2+} levels in glial cells lead to the release of messengers, which in turn regulates neuronal Ca^{2+} levels and synaptic neurotransmission, proving arguments that have forced a reconsideration of the role of glial cells in the nervous system.

As astrocytes and perisynaptic Schwann cells are intimately associated with the synapse, they receive signals from the presynaptic neuron and respond by releasing feedback signals, and therefore it has been proposed that glial cells should be considered as an integral modulatory component of the synapse. Actually, it is generaly accepted that glial cells are active partners in the synapse and that synapses are tripartite, consisting of synaptically associated glia as well as the presynaptic and postsynaptic nerve terminals (Araque et al., 1999).

3.1.1 Astrocytes

Astrocytes are large stellate cells with numerous cytoplasmic processes that radiate outward. They are the most abundant of the glial cells in the CNS, constituting up to 90% of the nervous tissue in some areas in the brain. Astrocytes have processes, that terminate in end-feet surrounding the capillaries of the CNS and the entire surface of these capillaries is covered by the astrocyte end-feet. The astrocyte end-feet surrounding blood capillaries of the CNS are rich in glucose transport carriers. These carriers help to transport glucose from the blood into astrocytes, where it is converted into lactic acid. The lactic acid is then released and taken into the neurons, where it is metabolized aerobically into CO_2 and H_2O for ATP energy (Bender, 1997).

In addition, astrocytes have other extensions adjacent to the synapses between the axon terminal of one neuron and the dendrite of cell body of another neuron. The astrocytes are thus ideally situated to influence the interactions between neurons and between neurons and the blood.

Astrocytes are known to take up K^+ from the extracellular fluid. Since K^+ is released from active neurons during the production of nerve impulses, this action of astrocytes may be important in maintaining the proper ionic environment for neurons. They also take up some neurotransmitters released from the axon terminals of neurons. The neurotransmitter glutamate is taken into the astrocytes and then transformed into glutamine. The glutamine is then released from the astrocytes and made available to the neurons, which use it to resyntetize the neurotransmitter (Keyser et al., 2007).

For much of the past century, astrocytes have been considered passive bystanders that merely provided support to neuronal networks. Research that has been carried out over the past decade changed this view, and astrocytes are now sharing the limelight with neurons because these cells have been shown to have important roles in the regulation of synaptic transmission. Astrocytes detect neuronal activity and can release chemical transmitters, which in turn control synaptic activity. This new understanding has led to the idea that astrocytes are intimately involved in the regulation of neuronal network function *in vivo* and, thus, are crucial determinants of higher brain functions and, consequently, of behavior (Halassa et al., 2007).

3.1.2 Oligodendrocytes and Schwann Cells

Oligodendrocytes and Schwann cells are small cells with relatively few processes. These cells insulate axons by forming a myelin sheath, which greatly enhances the conduction of electrical signals. They form this sheath by wrapping their membranous processes concentrically around the axon in a tight spiral.

Oligodendrocytes are myelin-forming cells in the CNS (Ndubaku and Bellard, 2007), but there are also oligodendrocytes that may not be directly connected to the myelin shealth. Satellite oligodendrocytes are perineuronal and may serve to regulate the microenvironment around neurons. Oligodendrocytes develop from bipotential progenitor cell, often referred to as an oligodendrocyte progenitor cell (OPC). Both OPCs and oligodendrocytes die in a variety of CNS diseases: hypoxia, ischemia, multiple sclerosis, or after CNS trauma, such as brain and spinal cord injury. This delayed oligodendrocyte death may reduce the effectiveness

of neural conduction in the spared axons that often exist after spinal cord injury. (Miller et al., 2007)

Schwann cells provide supportive role in the peripheral nervous system (Eguchi et al., 2003). They enswathe the axon and play an important part in axonal growth and regeneration, myelinization, and normal electrophysiological conductivity. Therefore, damage to Schwann cells is likely to have a direct effect on the conductivity on the axon and cause pathologic changes. Some pathologic conditions, such as diabetic peripheral neuropaty, have been associated with degenerative changes in Schwann cells (Park et al, 2005).

3.1.3 Microglia

Microglia is a type of glial cell that acts as the first and main form of active immune defense in the central nervous system (CNS). Microglia constitutes 20% of the total glial cell population within the brain. Unlike astrocytes, individual microglia is distributed in large non-overlapping regions throughout the brain and spine (Kreutzberg, 1995). Microglia is constantly moving and analyzing the CNS for damaged neurons, plaques, and infectious agents (Gehrmann et al., 1995). The brain and spinal cord are considered “immune privileged” organs in that they are separated from the rest of the body by a series of endothelial cells known as the blood-brain barrier, which prevents most infections from reaching the vulnerable nervous tissue. In the case where infectious agents are directly introduced to the brain or cross the blood-brain barrier, microglial cells must react quickly to destroy the infectious agents before they damage the neural tissue. Due to the unavailability of antibodies from the rest of the body (antibodies are too large to cross the blood-brain barrier), microglia must be able to recognize foreign bodies, swallow them, and act as antigen-presenting cells activating T-cells. Since this process must be done quickly to prevent potentially fatal damage, microglia is extremely sensitive to even small pathological changes in the CNS (Dissing-Olesen et al., 2007). They achieve this sensitivity in part by having unique potassium channels that respond to even small changes in extracellular potassium (Gehrmann et al., 1995).

3.2. Communication between neurons and glial cells

The most notable feature of nerve cells in the brain is their ability to communicate with one another and transmit information over great distances along specific pathways. The main form of communication is represented by chemical synaptic transmission occurring at the synapse, the site where presynaptic terminals come into apposition with a specialised structure of the postsynaptic neuronal membrane. A better understanding of the rules governing interactions between neurones is believed to be crucidal for elucidating the principles that underlie brain function.

In astrocytes, the other major cell population in the brain, gap junctions, i.e. channels that allow the passage of ions and small molecules between coupled cells, provide an alternative pathway for direct, intercellular communication. Astrocytes are, in fact, equipped with various types of the same ion channels and membrane bound receptors for neurotransmitters which support communication between neurones. A series of recent observations suggest the existence of a form of bidirectional communication between neurones and astrocytes (Carmignoto, 2000)

Astrocytes are coupled to one another by gap junctions, thereby forming a large syncitium. The numerous fine processes of the astrocyte can make contact with the vasculature and with neuronal synapses. This structural relationship provides the opportunity for bidirectional signalling between neurons and the vasculature. Thus, the astrocyte is thought to have important roles in metabolic support and in conveying the level of neuronal activity to the vasculature to regulate local blood flow. At synapses, astrocytes express high densities of neurotransmitter transporters that are responsible for the clearance of transmitters such as glutamate and GABA. The recent identification of their Ca^{2+} signalling activity and ability to release gliotransmitters suggested that astrocytes are intimately involved in the control of neuronal activity and synaptic transmission (Halassa et al., 2007).

3.2.1. Role of Ca^{2+} in neuron-astroglia signalling

In excitable cells, such as neurones, the increase in intracellular Ca^{2+} plays a well established role in the regulation of various important functions, such as neurotransmitter release, synaptic plasticity, enzymes activation and gene expression. In non-excitable cells, such as astrocytes, neurotransmitter-activated $[Ca^{2+}]_i$ changes also have substantial functional significance. Indeed, a transient rise in cytosolic Ca^{2+} can produce in astrocytes several intermediate and long-term structural and functional changes. Calcium activated protein kinase C and calcium-calmodulin dependent kinases, translate transient calcium changes into long-lasting modulations through phosphorilation of ion channels, enzymes, transporters and cytoskeleton proteins. Calcium increases may quickly mobilise arachidonic acid and produce long-lasting changes by modulating gene expression which affects glia proliferation and differentiation (Carmignoto, 2000).

It is now known that Ca^{2+} signals in astrocytes induce the release of chemical transmitters. The release of these gliotransmitters – in a process termed gliotransmission – can lead to paracrine actions on astrocytes, supporting inter-astrocytic Ca^{2+} signals, in addition to signalling to neurons to regulate neuronal excitability and synaptic transmission. Astrocytes release several transmitters, including glutamate and, D-serine, ATP, homocysteic acid, taurine, and larger molecules such as atrial natriuretic factor (ANF) and tumor necrosis factor-α (TNF-α; see Table 1).

Table 1. Gliotransmission: the release of various chemical transmitters from astrocytes*

Transmitter	**Ca^{2+} dependence**	**Receptor**	**Actions**
Glutamate	yes	NMDA	Synchronous depolarization
		Kainate	Increases miniature postsynaptic current frequency
		mGlu	Increases probability of release and AMPA-receptor-dependent miniature postsynaptic current frequency
ATP	unclear	P2X	Insertion of AMPA receptors into postsynaptic site
		P2Y	Paracrine actions in astrocytic Ca^{2+} waves in cultures
		A_1	When degraded to adenosine results in suppression of synaptic transmission
D-Serine	yes	NMDA	Increases NMDA-dependent synaptic transmission, important for the induction of LTP in many brain regions

* Halassa et al., 2007

Astrocytes surround synaptic junctions and help maintain the extracelular environment by providing physical integrity, and regulating the extracelular ion and neurotransmitter concentration. Ca^{2+} imaging, molecular, and electrophysiological methods show that, under appropriate conditions, oligodendrocyte precursor cells (OPC), astrocytes and Schwann cells can detect the vesicular release of neurotransmitters. The stimulated glia can subsequently regulate synaptic strength by releasing neurotransmitters, such as glutamate, into the synaptic cleft.

For decades, it has been known that, following injury to the nervous system or in conditions such as Parkinson's disease, Alzheimer's disease, epilepsy, schizophrenia and depression, the structure and protein expression of the astrocyte are altered. However, it was not known whether these structural changes represent a reaction to injury, in which the astrocyte is performing a supportive function in an attempt to prevent further injury, or whether the astrocyte is providing detrimental signals that contribute to the disorder. Because it has been assumed that many disorders of the nervous system are rooted in alterations of synaptic transmission and it is now understood that gliotransmission regulates synaptic transmission, it becomes important to ask whether astrocytes contribute to synaptic abnormalities and, thus, to the pathophysiology of such disorders (Halassa et al., 2007).

3.2.2. Role of glutamate in neuron-astroglia signalling

L-glutamate is the major excitatory neurotransmitter in the mammalian CNS that contributes not only to fast synaptic neurotransmission, but also to complex physiological processes like memory, learning, plasticity and neuronal cell death.

Glutamate is synthesized in the cytoplasm and stored in synaptic vesicles by an uptake system that depends on the proton electrochemical gradient, the vesicular glutamate transporters (VGLUTs). Following its exocytotic release, glutamate activates ionotropic glutamate receptors for fast excitatory neurotransmission and metabotropic receptors for slower modulatory affects on transmission. To terminate the action of glutamate and maintain its intracellular concentration below excitotoxic levels, Na^+-dependent high affinity glutamate transporters (excitatory amino acid transporters: EAATs) located on the plasma membrane of neurons and glial cells rapidly remove glutamate from the extacellular space (Shigeri et al., 2004) because high concentration of glutamate in the synaptic cleft can cause neuronal injury through a multifaceted process termed excitotoxicity. Transporters for glutamate functionally play a role in preserving the local integrity of excitatory synaptic transmission. Two types of EAATs: glial and neuronal, have been distinguished (Hertz, 2006). The majority of synapses in the CNS are in close apposition with glia, and glial EAATs are responsible for the bulk of glutamate uptake, whereas neuronal EAATs appear to have more specialised roles. (Beart, O'Shea, 2007)

Glutamate is thought to be released not only synaptically but also extrasynaptically by exocytosis, cystine-glutamate antiporter and volume-regulated anion channels. However, most of the glutamate is released synaptically and transits through the glutamate-glutamine cycle before being repackaged into synaptic vesicles. Glutamate that is taken up by glial cells is metabolized to glutamine, which is then transported back into neurons, converted to glutamate and sequestered into synaptic vesicles by the VGLUTs (Shigeri et al., 2004).

Astrocytes indirectly control glutamate transmission through the efficient and specific uptake of this excitatory neurotransmitter. Ensuring the physiological clearance of glutamate from the synaptic cleft they protect neurones against excessive excitatory stimulation. Indeed, high concentration of amino acid elicits neuronal damage and related excitotoxic events are suggested to participate in the progression of several insults of the CNS (Vermeiren et al., 2005).

Dysfunctional astrocytes might underlie certain neurological disorders and psychiatric states. In particular, the case of epilepsy and schizophrenia because astrocytes are known to control

synchronized neuronal activity and *N*-methyl-D-aspartic acid (NMDA)-receptor function, two processes that are related to these disorders (Halassa et al., 2007).

3.2.2.1. Glutamate receptors on glial cells

Glutamate receptors are the predominant mediators of excitatory neuronal transmission in the CNS. These receptors have traditionally been categorised into two major groups termed ionotropic (iGluR) and metabotropic (mGluR) receptors according to their pharmacological, electrophysiological and biochemical characteristics. iGluR are multimeric proteins directly coupled to cation-specific ion channels which can be subdivided into AMPA, kainate and NMDA receptors. mGluR are coupled to G proteins and modulate the production of second messengers. AMPA receptors are usually coexpressed with NMDA receptors at synapses (Carmignoto ,2000).

Ionotropic glutamate receptors (NMDA, AMPA and kainate receptors)

AMPA receptors

The AMPA receptors mediate fast synaptic transmission (Dev et al., 1999) and are composed of homo or heteromeric complex of four subunits (GluR 1-4) (Sager et al., 2008). As a differences in subunit expression, posttranscriptional changes, and different splicing modifications provide a wide range of functional diversity and complexity to these types of receptors, though effects on desensitisation time and receptor trafficking. Activation of AMPA receptors opens the pore of the channel permitting the inward flow of sodium, resulting in the depolarisation of neuronal membrane. This change in the intracellular charge relieves Mg^{2+}-blockade of the NMDA receptor channel, permitting passage of Ca^{2+} through the pore. During periods of glutamatergic excitation, AMPA receptor mediated depolarisation of the postsynaptic membrane makes it possible for activation of NMDA receptors, which initiate Ca^{2+}-dependent intracellular signalling pathways that modulate the surface presence of AMPA receptors. A modification in AMPA receptors, at the postsynaptic membrane, cause changes in synaptic strength and regulates various forms of synaptic plasticity (Zarate and Manji, 2008).

When agonist occupy the binding domain of individual receptor subunits, this domain closes, triggering rearrangements that couple agonist binding to channel opening. Glutamate and

other agonists (AMPA, quisqualate, 2-Me- Tet- AMPA) bind at a base of a deep cleft between two globular domains, causing the translation and rotation of domain 2 such that the cleft closes.(Zhang et al., 2003)

Kainate receptors

Kainate receptors are a family of glutamate receptors that particiate in normal synaptic transmission and in some forms of long- term potentiation, and their aberrant function may underlie neuropathological states, including epilepsy. Kainate receptors consist of five subunits (GluR5-7 and KA1, KA2) (Fisahn, 2005) and they can form homomeric or heteromeric receptors. To form a functional kainate receptor, the GluR5, GluR6 or GluR7 subunits must be present. The subunit composition of kainate receptor modulates the pharmacological and physiological properties of the receptor, such as sensitivity to antagonists, rectification properties, desensitization and calcium permeability (Hegarty, 2007).

NMDA receptors

N- methyl- D- aspartate receptor is highly permeable to calcium ions and plays a key role in the plasticity of synapses, which is believed to underlie memory and learning, as well as the development of the nervous system. Abnormal activation has been suggested to lead to neuronal cell death observed in many acute and chronic disorders such as ischemia, stroke, Alzheimer's disease, and Huntingdon's disease.

The NMDA receptor forms a heterodimer between NR1 and NR2 subunits. Other subunits, namely the NR3A and NR3B subunits, have an inhibitory effect on receptor activity. Multiple receptor isoforms with distinct brain distributions and functional properties arise by selective splicing of the NR1 transcript and differential expression of the NR2 subunits (Stephenson, 2006). There are eight variants of NR1 subunits and various isoforms of NR2 subunits (GRIN2A, GRIN2B, GRIN2C and GRIN2D). They contain the binding site for the neurotransmitter glutamate. Unlike NR1 subunits, NR2 subunits are expressed differentially across various cell types and control the electrophysiological properties of the NMDA receptor. One particular subunit, the NR2B, is mainly present in immature neurons and in extrasynaptic locations, and contains the binding-site for the selective inhibitor ifenprodil (Liu et al., 2004). Activation of NMDA receptors requires binding of glutamate or aspartate. In addition, NMDA receptors also require the binding of the coagonist glycine for the efficient

opening of the ion channel, which is a part of this receptor. NMDA receptor antagonists such as memantine, amantadine, dextromorphan, dextrophan, ibogaine (Popik et al., 1994), ketamine (Khanna et al.,2002), nitrous oxide, phencyclidine and tramadol are used as anesthetics for animals and sometimes humans, and are often used as a recreational drugs because of their hallucinogenic properties.

Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluRs) are a novel family of recently cloned G protein-coupled receptors. These receptors are heterogenous and coupled to multiple second messenger systems that include increases in phosphoinositide hydrolysis, activation of phospholipase D, decreases in cAMP formation, increases in cAMP formation, and changes in ion channel function (Schoepp, 1993).

Eight mGluR subtypes have been cloned which are subdivided into three groups based on their sequence homology, pharmacology and coupling to second messenger systems. The group I mGluRs (mGluR1 and R5) couple to phospholipase C and inositol-1, 2, 5-triphosphate turnover, while group II (mGluR2 and R3) and group III (mGluR4, R6, R7, R8) negatively couple to 3, 5-cyclic monophosphate turnover. The role of mGluRs in synaptic transmission and plasticity depends on their coupling activity and their pre- and postsynaptic localisation. Group II and III mGluRs are presynaptic autoreceptors controlling glutamate release, while group I mGluRs play a role in regulating neuronal excitability and in facilitating the long- term potentiation and depression at both pre- and postsynaptic levels (Grassi et al., 2002).

3.2.2.2. Glutamate release from glial cells

Although transmitter glutamate is often supposed to exert all of its effect on neuronal receptors, it should not be forgotten that astrocytes also express glutamate receptors (especially AMPA receptors and metabotropic receptors). The ability of glutamate to activate receptors on astrocytes is not different from that of many classical transmitters. It is unique that stimulation of glutamatergic receptors on astrocytes themselves can reinforce ongoing synaptic neuronal activity and even involve previously resting neurons in such activity (Hertz, 2006). Four release mechanisms have been postulated: a) vesicular release dependent on exeternal calcium or Ca^{2+} released from intracellular stores, b) an indometacin-sensitive

process in astrocytes, c) reversed operation of glutamate transporters and d) release through swelling- activated anion channels (Rossi et al., 2000). ATP triggers transient cell swelling, which activates volume-sensitive channels, allowing glutamate and other substances, such as aspartate and taurine, to be released (Takano, 2005). Because astrocytic glutamate transport is electrogenic and uses the transmembrane electrochemical gradients for Na^+, K^+, and H^+, severe disruption of these gradients or membrane depolarisation under conditions of energy failure (ischaemia and hypoglycemia) can lead to glutamate release from astrocytes by reversal glutamate transport (Ye et al., 1999).

3.2.2.3. Glutamate uptake systems in glial cells

Glutamate neurotransmission is terminated by cellular uptake of the neurotransmitter (Waagepetersen et al., 2005). After release of glutamate to the synaptic cleft it is re-accumulated into adjacent cells by Na^+-dependent glutamate transporters. Uptake of glutamate into astrocytes is mediated by the two glutamate transporters GLAST and GLT1 (or EAAT1 and 2) and into neurons by EAAC1 (or EAAT3), EAAT4, and EAAT5 (Hertz, 2006). EAAT1 is present in glial cells throughout the CNS being highly expressed in Bergmann glia of the cerebellum. EAAT2 is almost exclusively glial and is widespread and highly abundant throughout the CNS. The transporters EAAT3 and EAAT4 are present predominantly in neurons. EAAT5 is present in rod photoreceptor and bipollar cells of the retina (Beart, O'Shea, 2007). Although both neurons and glia contain glutamate transporters, it is generally accepted, that the uptake capacity of astrocytes is much higher than that of neurons (Hoogland, 2005).

High affinity transporters for glutamate (excitatory amino- acid transporters (EAATs)) represent a unique family of proteins that display considerable homology (50- 60% at the amino- acid level).

Glial EAATs also serve another vital role in the CNS by supplying glutamate for metabolic processes such as the glutamate- glutamine cycle. Thus, a range of evidence suggests that alterations in glutamate metabolism or glial viability resulting from impaired glutamate transport may also contribute to neuronal damage. As well as reducing the potentially toxic build- up of extracellular glutamate, it is likely that glutamate uptake by glial EAATs also signals the energy needs or nearby neurons via activation of the Na^+/ K^+ ATPase and glucose transporters, and changes in the levels of ATP and lactate. This growing body of evidence suggests an interactive role between glial EAATs and intermediary metabolism that has both physiological and pathological relevance.

Transport of glutamate by EAATs is well documented to involve the co- transport of three Na^+ and one H^+, and the counter transport of one K^+ enabling EAATs to maintain a large concentration gradient across the cell membrane. There now seems a general consensus from several laboratories that EAATs likely to subserve at least dual functions both as a transporter and ion channel, whereby residues in the carboxyl- terminal and amino- terminal portions, respectively, are the molecular determinants critical for these different functions (Beart and O'Shea, 2007).

3.2.3. Role of ATP in neuron-astroglia signalling

Multiple mechanisms for ATP release and the widespread distribution of purinergic receptors throughout the nervous system indicate that ATP is an important messenger in neuron-glial signalling and is associated with a variety of functions. Diffusion of ATP beyond the synaptic cleft can activate P2 receptors on presynaptic astrocytes and terminal Schwann cells; and changes in extracellular ion concentration accompanying action potential firing in extrasynaptic regions, such as the nodes of Ranvier, can cause Ca^{2+} transiens in paranodal Schwann cells. ATP can also act as a neuron- glial signalling molecule expanding the functional significance of activity dependent neuron-glial communication beyond process associated with homeostasis of the extracellular environment surrounding neurons (Fields, Stevens, 2000).

Many properties of ATP make it an ideal molecule for cell-cell signalling: it is small, rapidly difusing molecule, highly unstable and not abundant in the extracellular environment. Recently, ATP release by the mechanically stimulated astrocytes has been identified as a key signalling molecule in astrocytic Ca^{2+} waves, resolving a controversy that has persisted since the waves were first observed in response to glutamate stimulation. Ca^{2+} diffusion through gap junctions and extracellular signalling molecules has been proposed as key signalling molecules for astrocytic Ca^{2+} waves and it appears that both could contribute. Ca^{2+} wave propagation in dorsal spinal cord astrocytes is mediated by P2Y receptors, and in the mammalian retina ATP generates intracellular Ca^{2+} waves that propagate through network of glial cells (Fields, Stevens, 2000).

3.2.3.1. Release of ATP from glial cells

ATP fulfils multiple intracellular functions, as the central cellular energy currency, ATP supports the synthesis of biological compounds, energizes transport and generates mechanical force and movement. As a phosphate donor it is a co mediator of numerous regulatory

functions. ATP and also acetylcholine serve as extracellular signalling substance in the nervous system and in other tissues.

The release pathways of cellular ATP are more complex than for other signalling molecules. ATP can be released from cells via different mechanisms, involving both constitutive and regulated release. The latter includes exocytosis, whereby ATP is co released with other messenger substances from e.g. nerve terminals, chromaffin cells or platelets. ATP may also be stored in and release from vesicular organelles in astrocytes.

Activation of glutamatergic neurons causes ATP release from astrocytes, which in turn induces both homo-and heterosynaptic suppression through presynaptic P2Y receptors (Zhang et al.). ATP release is stimulated by depolarisation, which is dependent on extracellular Ca^{2+}, activation of L and P/Q type voltage sensitive Ca^{2+} channels, and the synaptic protein SNAP-25. This results in extracellular accumulation of adenosine, an effect that is partly antagonized by inhibiting ectonucleotidase activity.

After release, ATP is rapidly hydrolyzed to adenosine by ectonucleotidases. Similar to other neurotransmitters, removal of ATP helps to terminate its response, but, in addition, adenosine then acts on presynaptic adenosine receptors to inhibit neurotransmitter release and depress synaptic transmission at the neuromuscular junction (Fields and Stevens, 2000).

Additional potential release mechanisms for ATP and other nucleotides such as UTP and UDP include release via connexin or pannexin hemichannels, the release via anion channels or constitutive release via secretory pathway. ATP can also be released in a retrograde manner from the activated postsynaptic site (Zimmermann, 2008).

3.2.3.2. P2 receptors on glial cells

A large family of receptors, called purinergic receptors, mediates the various cellular effects of extracellular ATP. It is known, that all types of glia (i.e. microglia, oligodendrocytes, astrocytes and Schwann cells) have membrane receptors for extracellular ATP (purinergic receptors). These receptors are coupled to G proteins (P2Y receptors) or are intrinsic transmembrane channels (P2X receptors) permeable to Na^{+} and Ca^{2+} ions. Within the central and peripheral nervous system both types of receptors display a widespread distribution and subserve a variety of functions in both, glial and neuronal cells.

Astrocytes, for example, express some P2X and a larger number of P2Y receptors, activation of which first triggers increases in intracellular Ca^{2+} and then leads to long- term changes such as proliferation or cell death. The functions of oligodendrocytes and microglial cells are also controlled by nucleotides acting at P2X and P2Y receptors.

Likewise, neuron express both classes of P2 receptors: P2X receptors are mainly involved in fast synaptic transmission, whereas P2Y receptors rather mediate slow changes of synaptic transmission and neuronal excitability. In addition, P2 receptors may mediate long-term trophic or toxic effects of nucleotides, such as differentiation, neurite growth, survival or cell death.

While ionotrophic receptors are most commonly involved in fast neurotransmission, metabotrophic receptors are rather involved in neuromodulation. Typically, they elicit changes in neuronal excitability via heterotrimeric G proteins.

Accordingly, neuronal P2X receptors frequently mediate synaptic transmission in response to adenosine triphosphate (ATP) released from presynaptic nerve terminals by vesicle exocytosis. P2Y receptors, however, may be 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. Hence, these latter receptors are not only the direct target of ATP, the nucleotide stored in vesicles at the highest concentrations, but also of other nucleotides and their metabolites. Therefore, the activation and functions of neuronal P2Y receptors depend not only on the release of ATP, but also on the presence of ATP degrading enzymes. Whether activated by endogenously released nucleotides or exogenous agonists, neuronal P2Y receptors mediate neuromodulatory changes of neuronal excitability and/or synaptic transmission and control neurodegeneration and regeneration (Hussel and Boehm, 2006).

P2Y receptors

$P2Y_1$ subtype

In most species, ADP is a more potent agonist than ATP and their 2- methylthio derivatives are more potent than the parent compounds. UTP, UDP, CTP, and GTP are inactive. At present, the most potent agonist known is the N-methanocarba analogue of 2-MeSADP, MRS2365. ATP is, in fact, a partial agonist at the $P2Y_1$ receptor and so at low levels of receptor expression will act as an antagonist. Extracellular acidification and alkalinization do not appear to modify the activity of ATP. Activation of this receptor evokes an increase in intracellular IP_3 levels and the release of intracellular Ca^{2+} stores, in a PTX- sensitive manner. Nothern blotting and RT-PCR revealed $P2Y_1$ receptor mRNA in most human tissues, including the brain, heart, placenta, lungs, liver, skeletal muscle, kidneys, pancreas and various blood cells.

$P2Y_2$ subtype

$P2Y_2$ receptors are fully activated by equivalent concentrations of ATP and UTP, whereas ADP and UDP are less effective agonists. UTPγS has been shown to be and hydrolysis resistant agonist of $P2Y_2$ receptors. Suramin acts as a competitive antagonist of human and rat $P2Y_2$ receptors. $P2Y_2$ receptors can directly couple to $PLC\beta_1$ via $G\alpha_{q/11}$ protein to mediate the production of IP_3 and diacylglycerol (DAG), second messenger for calcium release from intercellular stores and PKC activation, respectively.

Expression of $P2Y_2$ receptor mRNA has been detected in human skeletal muscle, heart, brain, spleen, lymphocytes, macrophages, bone marrow, and lung, with lower expression levels detected in liver, stomach and pancreas.

$P2Y_4$ subtype

UTP is the most potent activator of the recombinant human $P2Y_4$ receptor. ATP behaves as the competitive antagonist. At recombinant rat and mouse $P2Y_4$ receptors ATP and UTP are equipotent and ITP, GTP and CTP have a lower potency. No selective antagonists are available. Extracellular acidification enhanced the potency of ATP and UTP at rat $P2Y_4$, but not at rat $P2Y_2$. Zn^{2+} inhibited the ATP response at the rat $P2Y_4$ receptor but had no effect on rat $P2Y_2$. $P2Y_4$ receptor couples mainly to a $G_{q/11}$ protein and accessorily to a $G_{i/o}$ protein.

Among human organs, $P2Y_4$ mRNA was detected in placenta by Northern blotting and was not abundant in the intestine. RT- PCR revealed the presence of $P2Y_4$ message in human umbilical vein endothelial cells, peripheral blood leukocytes, foetal cardiomyocytes, and various cell lines derived from the human lung. In the rat the expression of $P2Y_4$ message in heart and brain was much higher in neonates than in adults.

$P2Y_6$ subtype

$P2Y_6$ receptors are UDP receptors. At the human $P2Y_6$ receptor, the rank order of potency of various nucleotides is as follows: UDP>UTP>ADP>2-MeSATP>ATP. Adenine dinucleotides have little effect on $P2Y_6$ receptor, whereas uridine diphosphate is a selective agonist of $P2Y_6$. No selective competitive antagonist is available.

In the absence of reconstitution data, there is no hard molecular evidence of $P2Y_6$ interaction with specific G proteins. The IP_3 response to UDP of the recombinant $P2Y_6$ receptor is insensitive to PTX inhibition, suggesting a coupling to $G_{q/11}$. A $P2Y_6$-mediated increase in cAMP has been reported, but it is probably an indirect effect mediated by prostaglandins,

since it was at least partially inhibited by indomethacin. Northern blotting has revealed a rather wide tissue distribution of $P2Y_6$ mRNA. In particular, the $P2Y_6$ transcript has been found in human spleen, thymus, placenta, intestine, and blood leukocytes, and in rat lung, spleen, stomach, intestine, and aorta. Consistent with those initial observations, the expression and potential role of the $P2Y_6$ receptor has been documented in placenta, vascular smooth muscle, epithelia, and immune cells. $P2Y_6$ message is present in smooth muscle cells cultured from the rat aorta.

$P2Y_{12}$ subtype

ADP is a natural agonist of this receptor, whereas conflicting results were reported concerning the effects of ATP and its triphosphate analogs. For diphosphates, the rank order of agonist potency in all cases reported is 2-MeSADP>ADP>ADPβS. Concerning ATP and its analogs, they were found to be agonists either in native $P2Y_{12}$-expressing cells or in some heterologously transfected cells. The $P2Y_{12}$ receptor is mostly expressed in the megakaryocyte/platelet lineage. Glial cells, brain capillary endothelial cells, smooth muscle cells, and chromaffin cells also express $P2Y_{12}$ receptors. The precise role of this receptor in these locations is still under study. The platelet $P2Y_{12}$ receptor is coupled to $G\alpha_{i2}$.

$P2Y_{13}$ subtype

ADP and Ap_3A are naturally occurring agonists of $P2Y_{13}$ receptor. The activity of ATP may vary according to the level of expression of the $P2Y_{13}$ receptor in different recombinant systems. The relative potency of ADP differed according to assay used. $P2Y_{13}$ receptor is primarily coupled to a $G_{i/o}$ protein. The only exception was the increased cAMP formation observed at high ADP concentrations, and that presumably results from promiscuous coupling to G_s, a phenomenon observed with other recombinant $G_{i/o}$- coupled receptors, such as the α_2-adrenergic receptor. $P2Y_{13}$ mRNA was amplified in several human organs (spleen, brain, placenta liver, bone marrow, lung, heart, peripheral leukocyte).

$P2Y_{14}$ subtype

The $P2Y_{14}$ receptor is activated by UDP-glucose as well as UDP-galactose, UDP- glucuronic acid, and UDP-N-glucosamine but not by uridine or adenine nucleotides. No selective antagonists are available. The $P2Y_{14}$ receptor couples to the $G_{i/o}$ family of G proteins.

$P2Y_{14}$ mRNA is widely distributed in the human body with moderate to high levels observed in placenta, adipose tissue, stomach, intestine, selected brain regions, spleen, lung, heart, bone marrow and thymus (Abbracchio et al., 2006).

4. EXPERIMENTAL PROCEDURES

4.1. Preparation of cell cultures

Primary astroglial cultures were prepared from cerebral hemispheres of newborn rats. The brains were placed in ice- cold phosphate- buffered saline (PBS) containing 0,2% glucose and hemispheres were freed from meninges and blood vessels. After washing twice with ice- cold PBS, the hemispheres were cut into small pieces in „culture medium“, i.e. Dulbecco's Modified Eagle Medium containing 1 g/l N- acetyl-alanyl-L-glutamine and supplemented with 10% foetal calf serum, 50 U/ml penicilin, 50μg/ml streptomycin and 10 ng/ml LPS. Tissue from 2 to 4 hemispheres was then dissociated by trituration in 10 ml of culture medium. The cell suspension was passed through a 40-μm pore nylon mesh and centrifuged at 200 g for 5 min. The pellet was resuspended in medium and the suspension was centrifuged again at 200 g for 5 min. Resuspension and centrifugation was repeated twice, and the final pellet suspended in the final medium. About 500 microliters of the cell suspension was seeded in each well of 24 well plates, at density 2-3 hemispheres per plate. The cultures were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was replaced one day after the preparation and subsequently twice a week and 20-day-old cultures were used in the experiments.

4.2. Glutamate uptake experiments

The culture medium was removed and astrocytes were washed with warm HEPES-buffered saline solution (HBSS) containing (mM): 135 NaCl, 5.0 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 10 sodium pyruvate, 10 HEPES and 10 glucose. After rising cells with buffer, they were incubated in buffer or with drugs for 30 minutes. After the incubation period astrocyte cultures were further incubated for 10 min at 37°C in buffer containing 10.0 nmol/L of [^{3}H]-glutamate and several concentrations of unlabeled glutamate in the range of 10 μM- 1 mM. Uptake was terminated by removing the medium and washing the cells three times with ice-cold HBSS. This was immediately followed by cell lysis in 0.2 M NaOH. Aliquots were taken for liquid scintillation counting (with Optiphase „Hisafe“ coctail) and for Brandford's protein assay using bovine serum albumin as the protein standard. Radioactivity was analysed by scintillation counting in a Beckman LS 6500 scintillation counter. Effects of drugs on

glutamate uptake were evaluated using [^{3}H]-glutamate uptake as an indicator of the total glutamate taken up by the cells.

Data are expressed as means ± standard errors of the mean (S.E.M.) from n number of tissue preparations. Statistical analysis of the effect of drugs on basal tritium outflow and evoked tritium overflow was carried out using the unpaired Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. P values lower than 0.05 were considered to indicate significant differences.

4.3. Materials

The following materials were used: adenosine-β-thiodiphosphate (ADPβS), adenosine-γ-thiotriphosphate (ATPγS), uridinetriphosphate (UTP) and phorbol-12- myristate-13-acetate (PMA), DL-threo-β-benzyloxyaspartate (DL- TBOA), lipopolysaccharide from Salmonella typhimurium (LPS), penicilin G and streptomycin (Sigma-Aldrich, Portugal); 3-phenacyl UDP disodium salt (3- Phe-UDP) (Tocris, UK); foetal calf serum (FCS), Dulbecco's Modified Eagle Medium (Biochrom, Germany); optifase "Hisafe" 3 coctail (Perkin Elmer, USA), Bio-Rad Protein Assay Standart II (Bio- Rad Laboratories, Portugal) and L-[G-^{3}] glutamic acid (specific activity 49 Ci/mmol; Amersham, UK).

5. RESULTS

The influence of culture conditions on basal glutamate uptake was evaluated in cultures with 20 DIV (20 days) that were synchronized before the uptake experiments. Synchronized cultures were deprived of FCS for 48 hours before the uptake experiments. In culture, serum provides a wide variety of macromolecular proteins, low molecular weight nutrients, carrier proteins for water-insoluble components, and other compounds necessary for in vitro growth of cells. Serum also adds buffering capacity to the medium and binds or neutralizes toxic components and under these conditions cells are growing in different phases of cell cycle. When FCS is removed from the medium, the cells will stop the proliferation and synchronize their cell cycle in the G_0 phase with consequent changes on the expression of membrane proteins, i.e. receptors, channels and transporters, which regulate their physiology. The influence of the cell cycle phase on the ability of astrocytes to take up glutamate from the medium is shown in **Figure 1**.

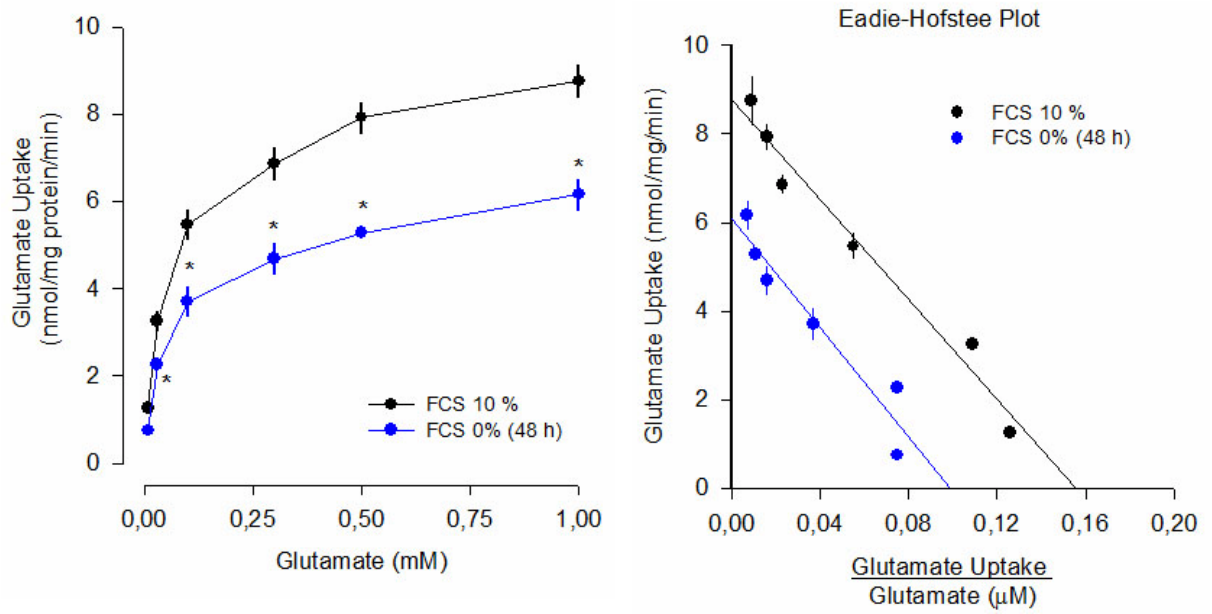

Figure 1. Glutamate uptake from 20 DIV astrocyte cultures. In synchronized cultures cells were deprived of FCS 48 h before uptake experiments. Cell cultures were incubated with buffer for 30 min and then with [^{3}H]-Glutamate (10 nM) plus unlabelled glutamate (10-1000 µM) for further 10 min. Effects of drugs on glutamate uptake were evaluated using [^{3}H] - glutamate uptake as an indicator of the total glutamate taken up by the cells. Values are means ± S.E.M. for (*n*) experiments. Significant differences from solvent: * $P < 0.05$.

Calculation of Km and Vmax from the Eadie-Hofstee Plot representation indicates that there was no change in the affinity of glutamate transporters in both types of cultures; the Km was 62,11± 4,38 μM (n=4) and 54,47 ± 5,12 μM (n=9) in synchronized and non-synchronized cultures, respectively. However, the capacity of synchronized cells to take up glutamate was significantly reduced: the Vmax was 6,13 ± 0,21 nmol/mg protein/min (n=4) in synchronized cultures and 8,58 ± 0,36 nmol/mg protein/min (n=4; P<0.05) in non-syncronized cultures. All subsequent experiments were performed with proliferating cultures containing 10 % FCS.

In order to better characterize the glutamate uptake systems in our culture conditions and to test whether our model was suitable for evaluating the effects of drugs that influence the uptake of glutamate from astrocytes, we evaluated the effect of compounds that have previously shown to increase and inhibit the uptake of glutamate: PMA (1 and 10 μM), an activator of protein kinase C that has been previously shown to increase the uptake of glutamate (Hyun Lee et al., 2003) and DL-TBOA which is so far, the most potent competitive non-transportable blocker of glutamate uptake (Shimamoto et al., 1998).
As expected, PMA increased glutamate uptake in a concentration dependent manner (**Figure 2**) whereas DL-TBOA (300 μM) attenuated glutamate uptake (**Figure 2**). Km and Vmax values of glutamate uptake obtained in control cultures and cultures treated with PMA and DL-TBOA are shown in **Table 1**.

Table 1. Glutamate uptake from 20 DIV astrocyte cultures.

Drugs present	Km (μM)	Vmax (nmol/mg protein/min)
Solvent	50,26 ± 5,14 (6)	8,58 ± 0,30 (6)
PMA (1 μM)	47,05 ± 7,22 (4)	10,86 ± 0,63 (4)*
PMA (10 μM)	46,11 ± 2,84 (4)	13,05 ± 0,56 (4)*
DL-TBOA (300 mM)	62,61 ± 3,93 (4)	7,54 ± 0,19 (4)*

Cell cultures were incubated with solvent or drugs for 30 min and then [^{3}H]-Glutamate (10 nM) plus unlabelled glutamate (10-1000 μM) for further 10 min. Effects of drugs on glutamate uptake were evaluated using [^{3}H]- glutamate uptake as an indicator of the total glutamate taken up by the cells. Values are means ± S.E.M. for (*n*) experiments. Significant differences from solvent: * $P < 0.05$.

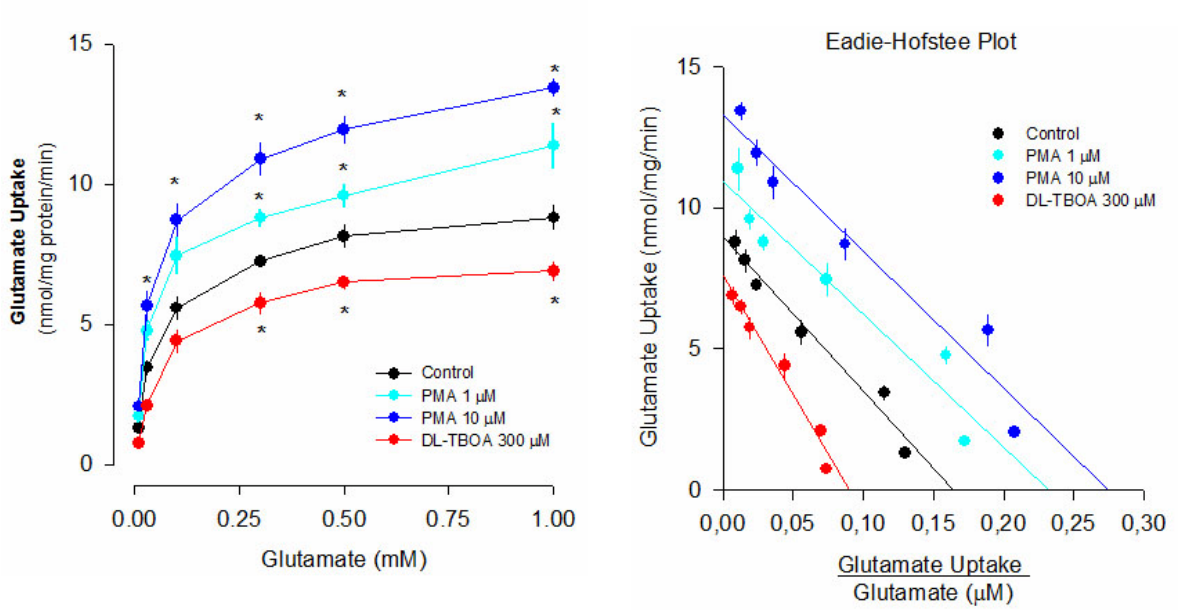

Figure 2. Glutamate uptake from 20 DIV astrocyte cultures: effect of PMA and DL-TBOA. Cell cultures were incubated with solvent or drugs for 30 min and then with [^{3}H]-Glutamate (10 nM) plus unlabelled glutamate (10-1000 µM) for further 10 min. Effects of drugs on glutamate uptake were evaluated using [^{3}H]- glutamate uptake as an indicator of the total glutamate taken up by the cells. Values are means ± S.E.M. for (*n*) experiments. Significant differences from solvent: * $P < 0.05$.

The Km value for glutamate uptake was very similar for both concentrations of PMA tested and was not significantly different from the control, indicating that activation of PKC does not influence the affinity of the glutamate transporters; but it increases maximal capacity of glutamate transporters as indicated by the increase in the Vmax for both concentrations tested (Table1). The uptake inhibitor DL-TBOA also caused no change on the transporters affinity but decreased the maximal capacity of transport.

The influence of agonists of P2Y receptors on glutamate uptake was then tested. The effect of agonists that activate $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor-subtypes is shown in **Figure 3**. UTP is an agonist of $P2Y_2$, $P2Y_4$, receptors, but this compound is not stable being enzymatically decomposed into UDP, which is agonist of $P2Y_6$ receptor. 3-Phenacydil-UDP (3-Phe-UDP) is a selective $P2Y_6$ receptor-agonist (Abbracchio, 2006).

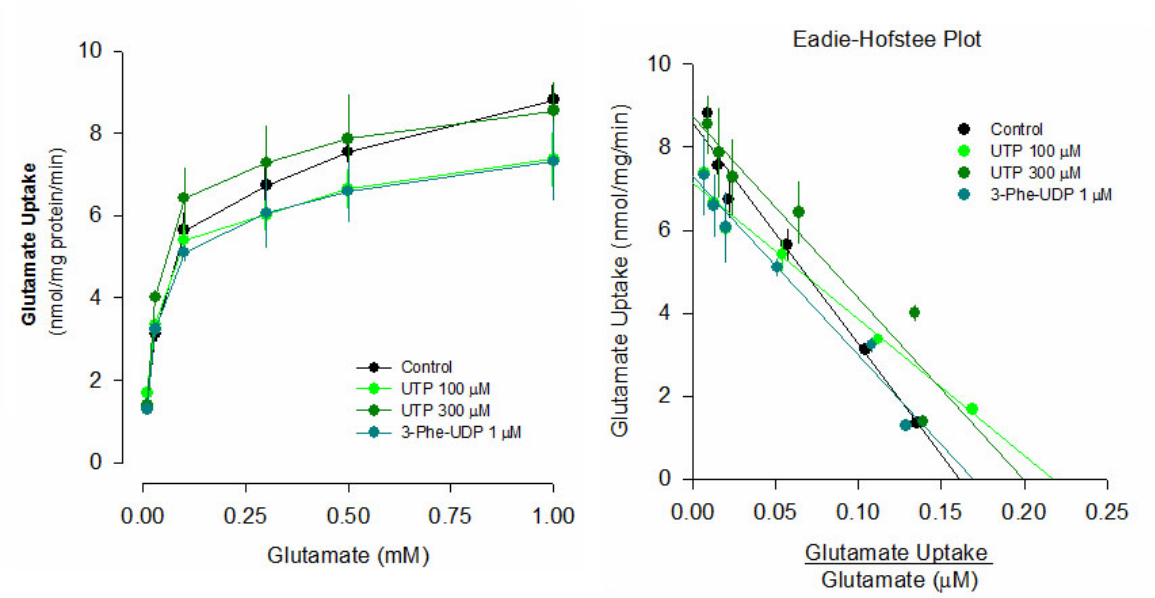

Figure 3. Glutamate uptake from 20 DIV astrocyte cultures: effect of UTP and 3-Phe-UDP. Cell cultures were incubated with solvent or drugs for 30 min and then with [^{3}H]-Glutamate (10 nM) plus unlabelled glutamate (10-1000 µM) for further 10 min. Effects of drugs on glutamate uptake were evaluated using [^{3}H]-glutamate uptake as an indicator of the total glutamate taken up by the cells. Values are means ± S.E.M. for (*n*) experiments.

As it is shown in **Table 2** (below), these UTP, UDP and 3-Phen-UDP had no effect on glutamate uptake from astrocytes. Both Km and Vmax were similar in treated and untreated cultures, indicating no changes in the affinity and maximal transport capacity of glutamate transporters.

Table 2. Glutamate uptake from 20 DIV astrocyte cultures.

Drugs present	Km (μM)	Vmax (nmol/mg protein/min)
Solvent	52,82 ± 5,85 (7)	8,51 ± 0,44 (7)
UTP 100 μM	32,07 ± 2,09 (4)	7,10 ± 0,41 (4)
UTP 300 μM	43,02 ± 7,79 (4)	8,67 ± 1,09 (4)
3-Phe-UDP 1 μM	43,26 ± 9,68 (3)	7,29 ± 0,91 (3)

Cell cultures were incubated with solvent or drugs for 30 min and then [^{3}H]-Glutamate (10 nM) plus unlabelled glutamate (10-1000 μM) for further 10 min. Effects of drugs on glutamate uptake were evaluated using [^{3}H]-glutamate uptake as an indicator of the total glutamate taken up by the cells. Values are means ± S.E.M. for (*n*) experiments.

The next step was designed to test whether P2Y receptors agonists which are active at $P2Y_1$, $P2Y_{12}$ and $2Y_{13}$ receptor subtypes would influence the uptake of glutamate from astrocytes; therefore we tested the effect of ADPβS and ATPγS.

ADPβS is agonist of $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptor subtypes whether ATPγS activates almost all P2Y receptor subtypes, except the $P2Y_6$. As shown in **Figure 4**, both agonists influenced glutamate uptake from astrocytes.

Values for Km and Vmax obtained from control treated cultures are shown in **Table 3**.

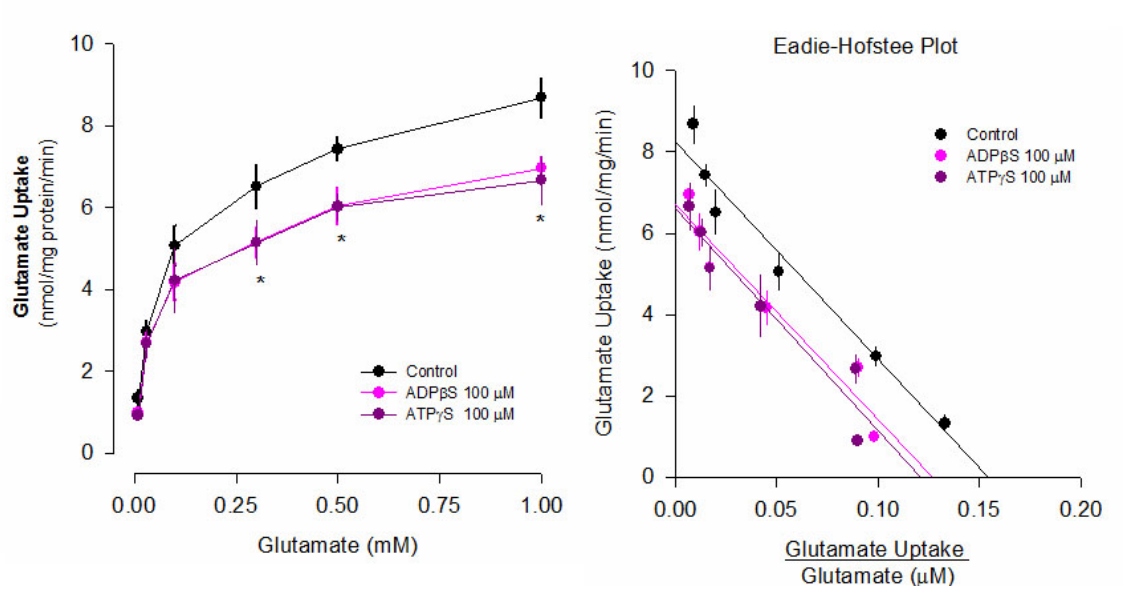

Figure 4. Glutamate uptake from 20 DIV astrocyte cultures: effect of ADPβS and ATPγS. Cell cultures were incubated with solvent or drugs for 30 min and then with [^{3}H]-Glutamate (10 nM) plus unlabelled glutamate (10-1000 µM) for further 10 min. Effects of drugs on glutamate uptake were evaluated using [^{3}H]- glutamate uptake as an indicator of the total glutamate taken up by the cells. Values are means ± S.E.M. for (*n*) experiments. Significant differences from solvent: * $P < 0.05$.

The Km values of control cultures and cultures incubated with ADPβS and ATPγS were very similar indicating that they caused no change on the affinity of glutamate transporters; but they decreased maximal capacity of glutamate transporters as indicated by the values of Vmax in **Table 3**. The effect of these agonists on glutamate uptake were very similar in proliferating and synchronized cultures of astrocytes, as shown in **Figure 5** and **Table 4**.

Table 3. Glutamate uptake from 20 DIV astrocyte cultures.

Drugs present	Km (μM)	Vmax (nmol/mg protein/min)
Solvent	49,40 ± 7,28 (4)	7,89 ± 0,35 (4)
ADPβS 100 μM	52,72 ± 3,37 (4)	6,71 ± 0,36 (4)*
ATPγS 100 μM	52,55 ± 0,35 (3)	6,55 ± 0,62 (3)*

Cell cultures were incubated with solvent or drugs for 30 min and then [^{3}H]-Glutamate (10 nM) plus unlabelled glutamate (10-1000 μM) for further 10 min. Effects of drugs on glutamate uptake were evaluated using [^{3}H]-glutamate uptake as an indicator of the total glutamate taken up by the cells. Values are means ± S.E.M. for (*n*) experiments.

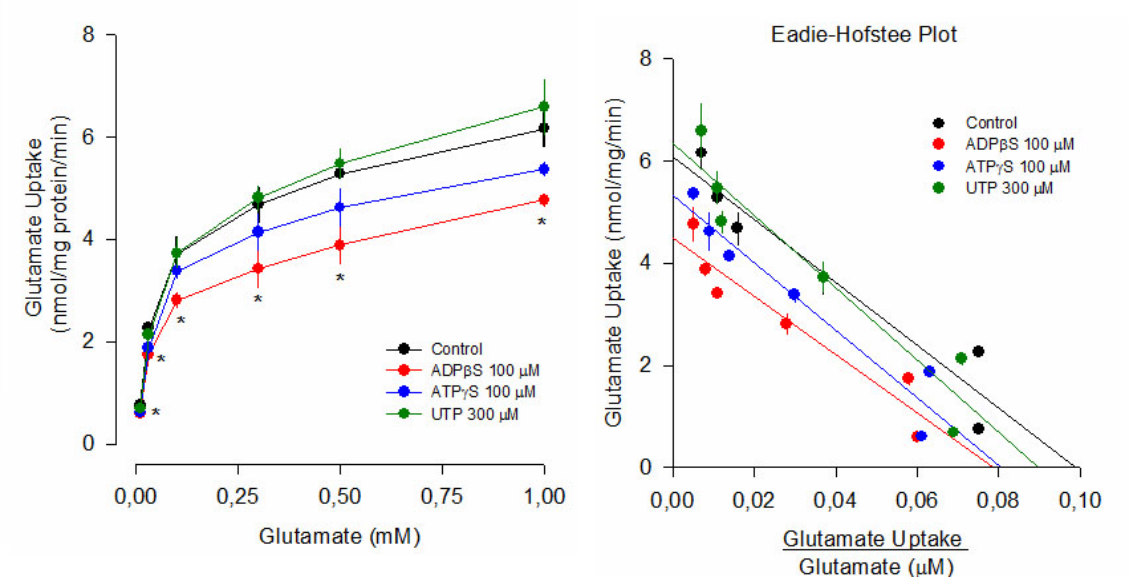

Figure 5. Glutamate uptake from 20 DIV astrocyte cultures. Comparison of the effect of P2 agonists synchronised and non-synchronized cell cultures. Cell cultures were incubated with solvent or drugs for 30 min and then with [^{3}H]-Glutamate (10 nM) plus unlabelled glutamate (10-1000 μM) for further 10 min. Effects of drugs on glutamate uptake were evaluated using [^{3}H]- glutamate uptake as an indicator of the total glutamate taken up by the cells. Values are means ± S.E.M. for (*n*) experiments. Significant differences from solvent: * $P < 0.05$.

In spite of the glutamate uptake in astrocytic cultures being lower in synchronized cultures than in proliferating cultures ADPβS inhibited glutamate uptake, whether as previously observed with proliferating cultures, UTP also had no effect on glutamate uptake (see Table 4).

Table 4. Glutamate uptake from synchronized 20 DIV astrocyte cultures.

Drugs present	Km (μM)	Vmax (nmol/mg protein/min)
Solvent	62,11 ± 4,38 (4)	6,13 ± 0,21 (4)
ADPβS 100 μM	57,64 ± 5,34 (4)	4,48 ± 0,17 (4)*
ATPγS 100 μM	65,61 ± 4,58 (4)	5,25 ± 0,27 (4)
UTP 300 μM	69,08 ± 2,57 (4)	6,25 ± 0,44 (4)

Synchronized cell cultures were incubated with solvent or drugs for 30 min and then [^{3}H]-Glutamate (10 nM) plus unlabeled glutamate (10-1000 μM) for further 10 min. Effects of drugs on glutamate uptake were evaluated using [^{3}H]- glutamate uptake as an indicator of the total glutamate taken up by the cells. Values are means ± S.E.M. for (*n*) experiments. Significant differences from solvent: * $P < 0.05$.

None of P2Y receptor-agonists tested changed the value of Km, therefore it can be concluded that they have no influence on the affinity of glutamate transporters. The most meaningful effect was mediated by ADPβS, which decreased the maximal capacity of glutamate transporters in all culture conditions tested.

6. DISCUSSION

The present work was designed to study the influence of agonists of P2Y receptors on glutamate uptake in primary astrocyte cell cultures. Astrocytes play a key role in glutamate uptake. This process is essential to protect neurones against excessive excitatory stimulation. Indeed, high concentration of glutamate elicits neuronal damage and related excitotoxic events are suggested to participate in the progression of several insults of the CNS.

Release of ATP from neuronal and glial cells trigger intercellular calcium waves in astrocytes that spread and constitute a signalling pathway and serve important physiological functions, namely in the modulation of synaptic transmission and control of vascular perfusion. Calcium waves involve gap junction channels for propagation from cell to cell. Binding of ATP to P2 receptors results further ATP release and increases the propagation of the waves. Several mechanisms for ATP release have been proposed, including vesicular release and channel-mediated release through a variety of membrane channels (Bao et al., 2007).

Purinergic receptors are widely distributed throughout the central nervous system. ATP, an endogenous ligand of these receptors, may directly mediate synaptic transmission as a fast neurotransmitter, or it may modulate synaptic efficacy as a neuromodulator. Both potentiating and suppressing effects of glutamate on synaptic transmission have been reported (Zhang et al., 2003).

Therefore aim of this study was to investigate the role of P2Y receptors on the glutamate uptake, to investigate the influence of agonists of P2Y receptors on glutamate uptake and to identify the subtype of P2Y receptors involved. We have used 20 DIV cell cultures that were tested in different phases of cell cycle: during the proliferative phase and in resting cells by foetal calf serum deprivation during the 48 hours immediately before the experiments. Cultures with non-proliferation astrocytes were called synchronized cultures and all cells in these cultures were in G_0 phase of the cell cycle. In these cultures there was a different expression of glutamate transporters and P2Y. The results revealed significant differences in glutamate uptake in non- synchronized and synchronized cultures (Figure 1).

Effect of PKC on glutamate uptake

PMA (phorbol- 12- myristate- 13- acetate) is activator of protein kinase C (PKC). Presently, 11 isoforms of PKC have been identified in mammalian tissues, and they are divided into three subfamilies. The individual members of the PKC family show different tissue distribution, mode of activation, kinetic properties and substrate specifities suggesting

different and specialized physiological processes (Hyun Lee et al., 2003). In our experiment we used two concentrations of PMA, 1μM and 10μM. We observed increasing effect on glutamate uptake in both concentrations; more significant increasing was for higher concentration of PMA.
Then we confirmed the inhibitory effect of DL-TBOA, the most potent competitive nontransportable blocker of glutamate uptake.

The role of P2Y pyrimidine preferring receptors in glutamate uptake

UTP is full agonist of $P2Y_2$ and $P2Y_4$ receptors and weak agonist of $P2Y_6$ receptor. All of these receptors are coupled via G_q- proteins to stimulation of phospholipase C followed by increases in inositol phosphates and mobilisation of Ca^{2+} from intracellular stores (Kugelgen, 2006). In spite of the fact, that UTP is agonist of these receptors, in our experiment had UTP no effect on glutamate uptake. Therefore, we could exclude an involvement of these P2Y receptor subtypes on modulation of glutamate uptake from glial cells.

The role of P2Y adenine nucleoside preferring receptors in glutamate uptake

ADP is the endogenous agonist at the $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors and it interacts at these subtypes with generally greater affinity than does ATP. At $P2Y_1$ receptors, derivatives of ADP tend to be full agonists, whereas ATP appears to be partial agonist. At $P2Y_{12}$ receptors, ADP derivatives activate and 5´- triphosphate derivatives antagonize. At $P2Y_{13}$ receptors, both ADP and ATP might act as full agonists. ADPβS is potent agonist of $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors (Abbracchio, 2006). $P2Y_1$ receptor couple via G_q- proteins (see above), $P2Y_{12}$ and $P2Y_{13}$ receptors couple via G_i- proteins to inhibition of adenylate cyclase followed by a decrease in intracellular cAMP levels (Kugelgen, 2006). $P2Y_1$ receptor is coupled via G_q-proteins to stimulate protein kinase C, it means, that we could expect increasing effect on glutamate uptake. But Figure.4. clearly determined decreasing effect of ADPβS on glutamate uptake, thus we could exclude $P2Y_1$ receptor from this way of affection of glutamate uptake. Decrease of glutamate uptake was likely caused by effect on $P2Y_{12}$ and $P2Y_{13}$ receptors, which are coupled via G_i- proteins and where we could expect decreasing effect.

As mentioned above, we registered significant differences in glutamate uptake in synchronized and non- synchronized cultures. When we compared the values of Vmax of glutamate uptake influenced by ADPβS and ATPγS, we could confirm, that in non-synchronised cultures was maximal capacity of glutamate transporters higher than in synchronized cultures. It means that the effect of those drugs on glutamate uptake was higher in non- synchronized cultures.

7. CONCLUSIONS

Glial cells, their division into astrocytes, oligodendrocytes, Schwann cells and microglia, the communication between neurones and glial cells, the ways of it's interference, the role of glutamate as the main excitatory neurotransmitter in CNS, glutamate receptors and transporters, the role of ATP in communication between nerve cells and finally P2 receptors, their division into P2X and P2Y receptors with a view to P2Y receptors, their function in CNS, subtypes of those receptors and the influence of agonists and antagonists of those receptors, were described in introduction, the theoretical part of this diploma thesis.

The main aim of this work was to clarify the role of P2Y receptors in glutamate transmission and define the subtypes of P2Y receptors involved including the influence of agonists and antagonists.

We worked with 20 days old astrocytic cells cultures prepared from newborn rat's brains. We added define amount of glutamate and agonists of P2Y receptors, than we measured how the agonist influenced the glutamate uptake by astrocytes.

In some experiments we worked with synchronized cultures, it means with cultures deprived of FCS for 48 hours before the uptake experiments. All cells in those cultures were in the same phase of cell division (G_0). We were interested whether the glutamate uptake in those synchronized cultures differs from glutamate uptake in nonsynchronized cultures.

We tested the influence of agonists (UTP, PMA, DL- TBOA, 3- Phe- UDP, ATPγS a ADPβS) in nonsynchronized cultures.

We added UTP in two concentrations (100μM a 300 μM), but we didn't prove any effect on glutamate uptake.

During the next experiments we tested the influence of activator of protein kinase C (PMA) that we used in two concentrations (1 μM a 10 μM). We expected a positive effect on the glutamate uptake that we confirmed later for both concentration. In the case of DL- TBOA we proved the inhibitory effect on glutamate uptake.

Between next tested agonists belonged ADPβS a ATPγS. In nonsynchronized cultures we reached the conclusion that both drugs decreased Vmax and thereby glutamate uptake almost identically. In order to better characterise glutamate uptake in synchronized cultures we conducted another experiment in that we proved insignificant effect of UTP on glutamate uptake, ATPγS showed some effect, but we couldn't confirm this effect in serie of another experiments. Only testing of ADPβS brought demonstrable results. ADPβS significantly decreased glutamate uptake in nonsynchronized and also i synchronized cell cultures.

8. ZÁVĚR

V teoretické části této diplomové práce byly podrobně popsány gliové buňky, jejich rozdělení na astrocyty, oligodendrocyty, Schwannovy buňky a mikroglia, byla popsána komunikace mezi neurony a gliovými buňkami a způsoby jejího ovlivnění, role glutamátu jakožto nejvýznamnějšího excitačního neurotransmiteru v CNS, glutamátové receptory a transportéry, dále role ATP v komunikaci mezi nervovými buňkami a konečně P2 receptory, jejich rozdělení na P2X a P2Y receptory se zaměřením na P2Y receptory, jejich funkci v CNS, jednotlivé podtyby těchto receptorů a ovlivnění za pomoci agonistů a antagonistů.

Účelem této práce bylo objasnit úlohu P2Y receptorů v přenosu glutamátu a definovat podtypy P2Y receptorů zahrnuté v tomto přenosu, včetně vlivu agonistů těchto receptorů.

Pracovali jsme s 20 dní starými buněčnými astrocytárními kulturami připravenými z mozků čerstvě narozených krys, ke kterým jsme přidávali definované množství glutamátu a agonisty P2Y receptoru, abychom posléze změřili, jak tento agonista ovlivnil vychytávání glutamátu astrocyty.

Některé experimenty jsme prováděli na synchronizovaných kulturách,tj. kulturách z jejichž živného media bylo 48 hodin před experimentem odstraněno sérum. V těchto kulturách se nacházely všechny buňky ve stejné fázi buněčného dělení (G_0). Zajímalo nás, zda se bude vychytávání glutamátu v těchto synchronizovaných kulturách lišit od vychytávání glutamátu v nesynchronizovaných kulturách.

Nejprve jsme testovali vliv agonistů (UTP, PMA, DL- TBOA, 3- Phe- UDP, ATPγS a ADPβS) na nesynchronizovaných kulturách.

UTP jsme přidávali ve 2 koncentracích (100μM a 300 μM), ale vliv na vychytávání glutamátu jsme neprokázali.

V dalších experimentech jsme testovali vliv aktivátoru protein kinásy C (PMA), kterou jsme použili ve 2 koncentracích (1μM a 10 μM). Očekávali jsme pozitivní vliv na vychytávání glutamátu, který jsme posléze potvrdili v obou koncentracích. V případě DL- TBOA, inhibitoru vychytávání glutamátu, jsme si ověřili tento jeho efekt.

Mezi další testované agonisty patří ADPβS a ATPγS. V nesynchronizovaných kulturách došlo u obou látek k téměř identickému snížení Vmax a tím ke sníženému vychytávání glutamátu. Abychom mohli lépe charakterizovat vychytávání glutamátu v synchronizovaných kulturách, provedli jsme další experiment, ve kterém se prokázal zanedbatelný vliv UTP na vychytávání glutamátu, ATPγS sice vykazoval jistý efekt, který jsme ale nemohli sérií dalších experimentů potvrdit. Jediné prokazatelné výsledky přineslo testování ADPβS, který prokazatelně snížil

vychytávání glutamátu nejenom v nesynchronizovaných buněčných kulturách, ale i v kulturách synchronizovaných.

9. REFERENCES

Abbracchio M.P., Burnstock G., Boeynaems J-M., Barnard E.A., Boyer J.L., Kennedy C., Knight G.E., Fumagalli M., Gachet C., Jacobson K., Weisman G.A. (2006): International union of pharmacology LVIII: Update on the P2Y G protein coupled nucleotide receptors: From molecular mechanisms and patophysiology to therapy, Pharmacol Rev 58, 281- 341

Araque A., Parpura V., Sanzgiri R. P., Haydon P. G. (1999): Tripartite synapses: the unknowledged partner, Trends in neurosciences 22, 208- 215

Bao L., Samuels S., Locovei S., Macagno E.R., Muller K. J., Dahl G. (2007): Innexins form two types of channels, FEBS Letters 581, 5703- 5708

Beart P. M., O'Shea (2007): Transporters for L- glutamate: An update of their molecular pharmacology and pathological involvement, British Jounal of Pharmacology 150, 5-7

Bender A. S., Young L. P., Norenberg M. D. (1997): Effect of lactic acid on L- glutamate uptake in cultured astrocytes: mechanistic consideration, Brain research 750, 59- 66

Bezzi P., Domercq M., Vesce S., Volterra A. (2001): Neuron- astrocyte cross- talk during synaptic transmission: physiological and neuropathological implications, Progress in brain research 132, 255-65

Burnstock G. (2006): Patophysiology and therapeutic potential of purinergic signaling, Pharmacol Rev 58, 58-86

Carmignoto G. (2000): Reciprocal communication systems between astrocytes and neurones, Progress in Neurobiology 62, 561- 581

Chen B.-C., Lin W. - W. (1999): PKCβI mediates the inhibition of P2Y receptor- induced inositol phosphate formation in endothelial cells, British Jounal of Pharmacology 127, 1908- 1914

Dev K. K., Nishimune A., Henley J.M, Nakanishi S. (1999): The protein kinase Cα binding protein PICK1 interacts with short but not long form alternative splice variants of AMPA receptor subunits, Neuropharmacology 38, 635- 644

Dissing-Olesen L, Ladeby L, Nielsen HH, Toft-Hansen H, Dalmau I, Finsen B. (2007): Axonal lesion-induced microglial proliferation and microglial cluster formation in the mouse, Neuroscience 149, 112- 122.

Eguchi Y., Ogiue- Ikeda M., Ueno S. (2003): Control of orientation of rat Schwann cells using an 8- T static magnetic field, Neuroscience letters 351, 130- 132

Fields R. D., Stevens B. (2000): ATP: an extracellular signalling molecule between neuron and glia, Trends neurosci 23, 625- 633

Fisahn A., Heinemann S.F., McBain C.J.(2005): The kainate receptor subunit GluR6 mediates metabotropic regulation of the slow and medium AHP currents in mouse hippocampal neurones, J Physiol 562, 199-203

Gehrmann J., Matsumoto Y., Kreutzberg G.W. (1995): Microglia: intrinsic immuneffector cell of the brain, Brain Research Reviews 20, 269- 287.

Gever J.R., Cockayne A., Dillon M.P., Burnstock G., Ford A.P.D.W. (2006): Pharmacology of P2X channels, Pflugers Arch- Eur J Physiol 452, 513- 537

Grassi S., Frondaroli A., Pettorossi V.E. (2002): Different metabotropic glutamate receptors play opposite roles in synaptic plasticity of the rat medial vestibular nuclei, JPhysiol 543, 795-806

Halassa M., Fellin T., Haydon P.G. (2007): The tripartide synapse: roles for gliotransmission in health and disease, Trends in Molecular Medicine 13, 54- 63

Heinrich A., Kittel A., Csolle C., Vizi E.S., Sperlagh B. (2008): Modulation of neurotransmitter release by P2X and P2Y receptors in the rat spinal cord, Neuropharmacology 54, 375- 386

Hegarty D.M., Mitchell J.L., Swanson K.C., Aicher S.A. (2007): Kainate receptors are primarily postsynaptic to SP- containing axon in the trigeminal dorsal horn, Brain Research 1184, 149-159

Hertz L., Zielke H. R. (2004): Astrocytic control of glutamatergic activity: astrocytes as stars of the show, Trends in neurosciences 27, 12

Hertz, L. (2006): Glutamate, a neurotransmitter – And so much more, A synopsis of Wierzba III, Neurochemistry international 48, 416- 425

Hoogland G., Bos I.W.M., Kupper F., Willigen G.van, Spierenburg H.A., Nieuwenhuizen O. van, Graan P.N.E. de (2005): Thrombin- stimulated glutamate uptake is predominantly mediated by the glial glutamate transporter EAAT2, Neurochemistry international 47, 499- 506

Hussel S., Boehm S. (2006): Functions of neuronal P2Y receptors, Pflugers Arch- Eur J Physiol 452, 538- 551

Hyun Lee, Bo-Hwa Choi, Byung-Chang Suh, Sun-Kyong Lee and Kyong-Tai Kim (2003):Attenuation of signal flow from P2Y6 receptor by protein kinase C- a in SK- N-BE (2)C human neuroblastoma cells, Journal of Neurochemistry 85, 1043- 1053

Illes P., Ribeiro A. (2004): Molecular physiology of P2 receptors in the central nervous system, European Journal of Pharmacology 483, 5- 17

James G., Butt A.M. (2002): P2Y and P2X purinoreceptor mediated Ca2+ signalling in glial cell pathology in the central nervous system, European Journal of Pharmacology 447, 247- 260

Keyser J., Mostert J. P., Koch M. W. (2007): Dysfunctional astrocytes as key players in the patogenesis of central nervous system disorders, Journal of neurobiological Sciences, article in press

Khanna J.M., Morato G.S., Kalant H. (2002): Effects of NMDA antagonists, NMDA agonists, and serotonin depletion on acute tolerance to ethanol, Pharmacol Biochem Behav 72, 291-8

Kreutzberg GW (1995): The first line of defense in brain pathologies, Drug research 45, 357-360

Kugelgen I. von (2006): Pharmacological profiles of cloned mammalian P2Y receptor subtypes, Pharmacology & Therapeutics 110, 415- 432

Liu L., Wong T.P., Pozza M. F., Lingenhoehl K., Wang Y., Sheng M., Auberson I.P., Wang Y.P.(2004): Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity, Science 304, 1021-4

Miller B.A., Jeannine M.C., Tovar C.A., Ferguson A.R. (2007): Developmental stage of oligodendrocytes determines their response to activated microglia in vitro, Journal of neuroinflammation 4, 28

Muir D., Varon S., Manthorpe M. (1990): Schwann cell proliferation in vitro is under negative autocrine control, Journal of cell biology 111, 2663- 2671

Nakatsuka G., Gu J. G. (2001): ATP P2Y receptor- mediated enhacement of glutamate release and evoked EPSCs in dorsal horn neurons of the rat spinal cord, Jounal of neuroscience 21, 6522- 31

Ndubaku U., Bellard M. E. de (2007): Glial cells: Old cells with new twist, Acta Histochemica, in press

Park C. J., Park S. A., Yoon T. G., Lee S. J., Yum K. W., Kim H. J. (2005): Bupivacaine induces apoptosis via ROS in the Schwann cell line, J Dent Res 84, 852- 857

Parpura V., Scemes E., Spray D. C. (2004): Mechanisms of glutamate release from astrocytes: gap junction “hemichannels”, purinergic receptors and exocytotic release, Neurochemistry international 45, 259- 264

Pertusa M., García- Matas S., Rodríguez- Farré E., Sanfeliu C., Cristófol R. (2007): Astrocytes aged in vitro show a decreased neuroprotective capacity, Journal of neurochemistry 101, 794- 805

Popik P., Layer R.T., Skolnick P. (1994): The putative anti- addictive drug ibogaine is a competitive inhibitor of [3H]MK-801 binding to NMDA receptor komplex, Psychopharmacology 114, 672-4

Queiroz G., Meyer D. K., Meyer A., Starke K., Kugelgeln I. (1999): A study of the mechanism of the release of ATP from rat cortical astroglial cells evoked by activation of glutamate receptors, Neuroscience 91, 1171-1181

Rodrigues R. J., Almeida T., Richardson P. J., Oliveira C. R., Cunha R. A. (2005): Dual presynaptic control by ATP release via facilitatory P2X1, P2X2/3, and P2X3 and inhibitory P2Y1, P2Y2, and/ or P2Y4 receptors in the rat hippocampus, Journal of Neuroscience 25, 6286- 95

Rossi D., Oshima T., Attwell D.(2000): Glutamate release in severe brain ischaemia is mainly by reversed uptake, Nature 403, 316- 321

Sager C., Tapken D., Hollmann M. (2008): Functional modulation of AMPA receptors by TARPs, Neuroscience, in press

Shigeri Y, Seal R. P., Shiamoto K. (2004): Molecular pharmacology of glutamate, EAATs and VGLUTs, Brain research reviews 45, 250- 265

Shimamoto K., Lebrun B., Yasuda – Kamatani Y., Sakaitani M., Shigeri Y., Yumoto N., Nakajima T. (1998): DL- threo- beta- benzyloxyaspartate, a potent blocker of excitatory amino acid transporters, Mol Pharmacol 53, 195- 201

Shoepp D.D. (1993): The biochemical pharmacology of metabotropic glutamate receptors, Biochem Soc Trans 21, 97-102

Sperlágh B., Kofalvi A., Deuchars J., Atkinson L., Milligan C.J., Buckley N.J., Visi E. S. (2002): Involvement of $P2X_7$ receptors in the regulation of neurotransmitter release in the rat hippocampus, Journal of Neurochemistry 81, 1196- 211

Stephenson F.A. (2006): Structure and traffickin of NMDA and GABAA receptors, Biochem Soc Trans 34, 877-81

Takano T., J. Kang, J. K. Jaiswal, S. M. Simon, J. H.-C. Lin, Y. Yu, Y. Li, J. Yang, G. Dienel, H. R. Zielke, M. Nedergaard (2005): Receptor-mediated glutamate release from volume sensitive channels in astrocytes. Proc. Natl. Acad. Sci. USA 102, 16466- 16471

Vermeiren C., Najimi M., Maloteaux J.- M., Hermans E. (2005): Molecular and functional characterisation of glutamate transporters in rat cortical astrocytes exposed to a defined combination of growth factors during in vitro differentiation , Neurochemistry international 46, 137- 147

Waagepetersen H.S., Qu H., Sonnewald U., Shimamoto K., Schousboe A. (2005): Role of glutamine and neuronal glutamate uptake in glutamate homeostasis and synthesis during

vesicular release in cultured glutamatergic neurons, Neurochemistry Intenational 47, 92-102

Woodhoo A., Sahni V., Gilson J., Setzu A., Franklin R.J.M., Blakemore W.F., Mirsky R., Jessen K.R. (2007): Schwann cell precursor: a favourable cell for myelin repair in the central nervous system, Brain 130, 2175- 2185

Ye Z.- C., Sontheimer H. (1999): Glioma cells release excitotoxic concentrations of glutamate, Cancer research 59, 4383- 4391

Zarate C.A. Jr., Manji H.K. (2008): The role of AMPA receptor modulation in the treatment of neuropsychiatric diseases, Experimental Neurology, article in press

Zhang J- M., Wang H-K., Ye C- Q., Ge W., Jiang Z- L., Wu C- P., Poo M- M., Duan S. (2003): ATP released by astrocytes mediates glutamatergic activity- dependent heterosynaptic suppression, Neuron 40, 971- 982

Zimmermann H. (2008): ATP and acetylcholine, equal brethren, Neurochemistry international 52, 634- 648